HANDBOOK OF Cell Signaling

Volume 1

Editors-in-Chief

RALPH A. BRADSHAW

EDWARD A. DENNIS



Handbook of Cell Signaling

Volume 1

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Contents

VOLUME 1

Contributors xlv
Preface lxvii

CHAPTER 1
Cell Signaling: Yesterday, Today, and Tomorrow 1
Ralph A. Bradshaw and Edward A. Dennis

Origins of Cell Signaling
Enter Polypeptide Growth Factors
Cell Signaling at the Molecular Level
Lipid Signaling
Cell Signaling Tomorrow
References

PART I
INITIATION: EXTRACELLULAR AND MEMBRANE EVENTS
James Wells, Editor

Section A: Molecular Recognition
Ian Wilson, Editor

CHAPTER 2
Structural and Energetic Basis of Molecular Recognition 11
Emil Alexov and Barry Honig

Introduction
Principles of Binding
Nonspecific Association with Membrane Surfaces
Protein–Protein Interactions
Prospects
References

CHAPTER 3
Computational Genomics: Prediction of Protein Functional Linkages and Networks 15
Todd O. Yeates and Michael J. Thompson

Introduction
Approaches to Analyzing Protein Functions on a Genome-Wide Scale
Current Issues and Future Prospects for Computing Functional Interactions
References

CHAPTER 4
Molecular Sociology 21
Irene M. A. Nooren and Janet M. Thornton

Transmembrane Signaling Paradigms
Structural Basis of Protein–Protein Recognition
Conclusion
References

CHAPTER 5
Free Energy Landscapes in Protein–Protein Interactions 27
Jacob Piehler and Gideon Schreiber

Introduction
Thermodynamics of Protein–Protein Interactions
Interaction Kinetics
The Transition State
Association of a Protein Complex
Dissociation of a Protein Complex
Summary
References

CHAPTER 6
Antibody–Antigen Recognition and Conformational Changes 33
Robyn L. Stanfield and Ian A. Wilson

Introduction
Antibody Architecture
Conformational Changes
Conclusion
References

CHAPTER 7
Binding Energetics in Antigen–Antibody Interfaces 39
Roy A. Mariuzza

Introduction
Thermodynamic Mapping of Antigen–Antibody Interfaces
Conclusions
References

CHAPTER 8
Immunoglobulin–Fc Receptor Interactions 45
Brian J. Sutton, Rebecca L. Beavil, and Andrew J. Beavil

Introduction
IgG–Receptor Interactions
IgE–Receptor Interactions
Summary
References

CHAPTER 9
Plasticity of Fc Recognition 51
Warren L. DeLano

Introduction
Structures of the Natural Fc Binding Domains
The Consensus Binding Site on Fc
Evolution of an Fc Binding Peptide
Factors Promoting Plasticity
Conserved and Functionally Important Molecular Interactions
Conclusion
References

CHAPTER 10
Ig-Superfold and Its Variable Uses in Molecular Recognition 57
Nathan R. Zaccai and E. Yvonne Jones

Introduction
The Immunoglobulin Superfamily
Ig-Superfold-Mediated Recognition
References

CHAPTER 11
T-Cell Receptor/pMHC Complexes 63
Markus G. Rudolph and Ian A. Wilson

TCR Generation and Architecture
Peptide Binding to MHC Class I and II
TCR/pMHC Interaction
Conclusions and Future Perspectives
References

CHAPTER 12
Mechanistic Features of Cell-Surface Adhesion Receptors 74
Steven C. Almo, Anne R. Bresnick, and Xuewu Zhang

Mechanosensory Mechanisms
Cell–Cell Adhesions/Adherens Junctions
T-Cell Costimulation
Axon Guidance and Neural Development
Conclusions
References

CHAPTER 13
The Immunological Synapse 79
Michael L. Dustin

Introduction
Migration and the Immunological Synapse
The Cytoskeleton and the Immunological Synapse
The Role of Self MHCp in T-Cell Sensitivity to Foreign MHCp
Integration of Adaptive and Innate Responses
Summary
References

CHAPTER 14
NK Receptors 83
Roland K. Strong

Introduction
Immunoreceptors
Natural Killer Cells
Ig-Type NK Receptors: KIR
C-Type Lectin-Like NK Receptors: Ly49A
C-Type Lectin-Like NK Receptors: NKG2D
References

CHAPTER 15
Carbohydrate Recognition and Signaling 87
James M. Rini and Hakon Leffler

Introduction
Biological Roles of Carbohydrate Recognition
Carbohydrate Structure and Diversity
Lectins and Carbohydrate Recognition
Carbohydrate-Mediated Signaling
Conclusions
References

CHAPTER 16
Rhinovirus–Receptor Interactions **95**
Elizabeth Hewat

References

CHAPTER 17
HIV-1 Receptor Interactions **99**
Peter D. Kwong

Molecular Interactions
Atomic Details
Recognition in the Context of a Humoral Immune Response
References

CHAPTER 18
Influenza Virus Neuraminidase Inhibitors **105**
Garry L. Taylor

Introduction
Flu Virus: Role of NA
Structure of NA
Active Site
Inhibitor Development
Conclusion
References

CHAPTER 19
Signal Transduction and Integral Membrane Proteins **115**
Geoffrey Chang and Christopher B. Roth

Introduction
Electrophysiology: Rapid Signal Transduction
Mechanosensation: How Do We Feel?
Active Transporters: Rapid Response and Energy Management
Receptors: Gate Keepers for Cell Signaling
References

CHAPTER 20
Structural Basis of Signaling Events Involving Fibrinogen and Fibrin **119**
Russell F. Doolittle

References

CHAPTER 21
Structural Basis of Integrin Signaling **123**
Robert C. Liddington

Introduction
Structure
Quaternary Changes
Tertiary Changes
Tail Interactions
Concluding Remarks
References

CHAPTER 22
Structures of Heterotrimeric G Proteins and Their Complexes **127**
Stephen R. Sprang

Introduction
Gα Subunits
Ga-Effector Interactions
GTP Hydrolysis by Gα and Its Regulation by RGS Proteins
Gβγ Dimers
GPR/GoLoco Motifs
Gα-GPCR Interactions
References

Section B: Vertical Receptors
Henry Bourne, Editor

CHAPTER 23
Structure and Function of G-Protein-Coupled Receptors: Lessons from the Crystal Structure of Rhodopsin **139**
Thomas P. Sakmar

Introduction
Introduction to Rhodopsin: a Prototypical G-Protein-Coupled Receptor
Molecular Structure of Rhodopsin
Molecular Mechanism of Receptor Activation
References

CHAPTER 24
Human Olfactory Receptors **145**
Orna Man, Tsviya Olender, and Doran Lancet

References

CHAPTER 25
Chemokines and Chemokine Receptors: Structure and Function **149**
Carol J. Raport and Patrick W. Gray

Introduction
Chemokine Structure and Function
Chemokine Receptors
References

CHAPTER 26
The Binding Pocket of G-Protein-Coupled Receptors for Biogenic Amines, Retinal, and Other Ligands **155**
Lei Shi and Jonathan A. Javitch

Introduction

The Binding Pocket of GPCRs
A Role of the Second Extracellular Loop in Ligand Binding
References

CHAPTER 27
Glycoprotein Hormone Receptors: A Unique Paradigm for Ligand Binding and GPCR Activation 161
Gilbert Vassart, Marco Bonomi, Sylvie Claeysen, Cedric Govaerts, Su-Chin Ho, Leonardo Pardo, Guillaume Smits, Virginie Vlaeminck, and Sabine Costagliola

Introduction
Molecular Pathophysiology
Structure Function Relationships of the Glycoprotein Hormone Receptors
Conclusions and Perspectives
References

CHAPTER 28
Protease-Activated Receptors 167
Shaun R. Coughlin

Introduction
Mechanisms of Activation
Protease-Activated Receptor Family
Roles of PARs *In Vivo*
References

CHAPTER 29
Constitutive and Regulated Signaling in Virus-Encoded 7TM Receptors 173
Thue Schwartz

Virus-Encoded Proteins Are Developed through Targeted Evolution *In Vivo*
The Redundant Chemokine System Is an Optimal Target for Viral Exploitation
Multiple Virus-Encoded 7TM Receptors
Constitutive Signaling through Altered Pathways
Viral Receptors Recognize Multiple Ligands with Variable Function
Attempts to Identify the Function of Virus-Encoded Receptors *In Vivo*
References

CHAPTER 30
Frizzleds as G-Protein-Coupled Receptors for Wnt Ligands 177
Sarah H. Louie, Craig C. Malbon, Randall T. Moon

Introduction
Wnt Signaling
Evidence for Frizzleds as G-Protein- Coupled Receptors
Perspective
References

CHAPTER 31
Agonist-Induced Desensitization and Endocytosis of G-Protein-Coupled Receptors 181
Mark von Zastrow

Introduction
General Processes of GPCR Regulation
Mechanisms of GPCR Desensitization and Endocytosis
Functional Consequences of GPCR Endocytosis
References

CHAPTER 32
Functional Role(s) of Dimeric Complexes Formed from G-Protein-Coupled Receptors 187
Marta Margeta-Mitrovic and Lily Yuh Jan

References

CHAPTER 33
The Role of Chemokine Receptors in HIV Infection of Host Cells 191
Jacqueline D. Reeves and Robert W. Doms

Introduction
HIV Entry
Coreceptor Use *In Vivo*
Env Domains Involved in Coreceptor Interactions
Coreceptor Domains Involved in HIV Infection
Receptor Presentation and Processing
Role of Signaling in HIV Infection
Summary
References

CHAPTER 34
Chemotaxis Receptor in Bacteria: Transmembrane Signaling, Sensitivity, Adaptation, and Receptor Clustering 197
Weiru Wang and Sung-Hou Kim

Signaling at Periplasmic Ligand Binding Domain
Signaling at the Cytoplasmic Domain
Adaptation
Clustering of the Chemoreceptor and Sensitivity
Future Studies
References

CHAPTER 35
Overview: Function and Three-Dimensional Structures of Ion Channels 203
Daniel L. Minor, Jr.

Introduction
Studies of Full-Length Ion Channels
General Pore Features Revealed by Bacterial Channels

Pore Helices: Electrostatic Aids to Permeation
Open Channels
Eukaryotic Ion Channels at High Resolution: Divide and Conquer
Ion Channel Accessory Subunits: Soluble and Transmembrane
The Future: Ion Channels as Electrosomes
References

CHAPTER 36
How Do Voltage-Gated Channels Sense the Membrane Potential? 209
Chris S. Gandhi and Ehud Y. Isacoff

Introduction
The Voltage-Sensing Gating Particle
S4 Is the Primary Voltage Sensor
Physical Models of Activation: Turning a Screw through a Bolt
Coupling Gating to S4 Voltage-Sensing Motions
References

CHAPTER 37
Ion Permeation: Mechanisms of Ion Selectivity and Block 215
Bertil Hille

Aqueous Pore
Ion Selectivity
Block
References

CHAPTER 38
Agonist Binding Domains of Glutamate Receptors: Structure and Function 219
Mark L. Mayer

References

CHAPTER 39
Nicotinic Acetylcholine Receptors 223
Arthur Karlin

Function
Structure
References

CHAPTER 40
Small Conductance Ca^{2+}-Activated K^+ Channels: Mechanism of Ca^{2+} Gating 227
John P. Adelman

Introduction
Clones Encoding SK Channels
Biophysical and Pharmacological Profiles
Mechanisms of Ca^{2+}-gating
Pantophobiac After All
References

CHAPTER 41
Regulation of Ion Channels by Direct Binding of Cyclic Nucleotides 233
Edgar C. Young and Steven A. Siegelbaum

Introduction
The Cyclic Nucleotide-Gated Channels
Other Channels Directly Regulated by Cyclic Nucleotides
References

Section C: Horizontal Receptors
Robert Stroud, Editor

CHAPTER 42
Overview of Cytokine Receptors 239
Robert M. Stroud

CHAPTER 43
Growth Hormone and IL-4 Families of Hormones and Receptors: The Structural Basis for Receptor Activation and Regulation 241
Anthony A. Kossiakoff

Introduction
The Growth Hormone Family of Hormones and Receptors
Structural Basis for Receptor Homodimerization
Hormone Specificity and Cross-Reactivity Determine Physiological Roles
Hormone-Receptor Binding Sites
Receptor–Receptor Interactions
Hormone–Receptor Binding Energetics
Biological Implications of Transient Receptor Dimerization
A High-Affinity Variant of hGH (hGH_v) Reveals an Altered Mode for Receptor Homodimerization
Site1 and Site2 Are Structurally and Functionally Coupled
IL-4 Hormone-Induced Receptor Activation
IL-4–α-Chain Receptor Interface
Binding of the γ-Chain Receptor
Comparisons of IL-4 with GH(PRL)
Concluding Remarks
References

CHAPTER 44
Erythropoietin Receptor as a Paradigm for Cytokine Signaling 251
Deborah J. Stauber, Minmin Yu, and Ian A. Wilson

Introduction
Biochemical Studies Supporting Preformed Dimers

Other Cytokine Receptor Superfamily Members
Conclusions
References

CHAPTER 45
A New Paradigm of Cytokine Action Revealed by Viral IL-6 Complexed to gp130: Implications for GCSF Interaction with GCSFR 259
Dar-chone Chow, Lena Brevnova, Xiao-lin He, and K. Christopher Garcia

Introduction
Receptor/Ligand Interactions
The gp130 System
Viral Interleukin-6
GCSF and GCSFR
Structure of the Viral IL-6–gp130 Complex
Site 1
The Site 2 Interface
The Site 3 Interface
Implications of the vIL-6–gp130 Tetramer Structure for the Active GCSF–GCSFR Extracellular Signaling Complex
References

CHAPTER 46
The Fibroblast Growth Factor (FGF) Signaling Complex 265
Fen Wang and Wallace L. McKeehan

Introduction
FGF Polypeptides
FGFR Tyrosine Kinases
Heparan Sulfate
Oligomeric FGF–FGFR–HS Signaling Complex
Intracellular Signal Transduction by the FGFR Complex
References

CHAPTER 47
Structure of IFN-γ and Its Receptors 271
Mark R. Walter

References

CHAPTER 48
Structure and Function of Tumor Necrosis Factor at the Cell Surface 275
Stephen R. Sprang

Introduction
Structure of Tumor Necrosis Factor
TNF Receptors
Extracellular (Ligand Binding) Domains of TNF Family Receptors
Ligand–Receptor Complexes
Consequences of Ligand–Receptor Complex Formation
Receptor Preassociation
Conclusion
References

CHAPTER 49
The Mechanism of NGF Suggested by the NGF–TrkA-D5 Complex 281
Abraham M. de Vos and Christian Wiesmann

Introduction
Neurotrophins
Trks
NGF–TrkA-D5 Complex
$p75^{NTR}$
References

CHAPTER 50
The Mechanism of VEGFR Activation Suggested by the Complex of VEGF–flt1-D2 285
Christian Wiesmann and Abraham M. de Vos

Introduction
Heparin-Binding Domain of VEGF
Receptor-Binding Domain VEGF
VEGF Receptors
VEGF–flt1-D2 Complex
References

CHAPTER 51
Receptor–Ligand Recognition in the TGFβ Family as Suggested by the Crystal Structures of BMP-2–BR-IA_{ec} and TGFβ3–TR-II_{ec} 289
Matthias K. Dreyer

Introduction
Ligand and Receptor Structures
Receptor–Ligand Complexes
BMP-2–BR-IA_{ec} Complex
Complex Formation with TGFβ Is Different than for BMP-2
References

CHAPTER 52
Insulin Receptor Complex and Signaling by Insulin 293
Lindsay G. Sparrow and S. Lance Macaulay

Introduction
Insulin Receptor Domain Structure
Binding Determinants of the IR
Insulin Signaling to Glucose Transport
References

CHAPTER 53
Structure and Mechanism of the Insulin Receptor Tyrosine Kinase **299**
Steven R. Hubbard

Introduction
Structural/Mechanistic Studies
Prospects
References

CHAPTER 54
What Does the Structure of Apo2L/TRAIL Bound to DR5 Tell Us About Death Receptors? **305**
Sarah G. Hymowitz and Abraham M. de Vos

Introduction
Novel Features in the Structure of Apo2L/TRAIL
Apo2L/TRAIL:DR5 Structures
Ligand-Independent Receptor Assembly
Intracellular Consequences of Ligand Binding
Conclusion
References

Section D: Membrane Proximal Events
Tom Alber, Editor

CHAPTER 55
TNF Receptor Associated Factors **311**
Jee Y. Chung, Young Chul Park, Hong Ye, and Hao Wu

References

CHAPTER 56
Assembly of Signaling Complexes for TNF Receptor Family Molecules **315**
Gail A. Bishop and Bruce S. Hostager

Introduction
Receptor Aggregation
Raft Recruitment
Ubiquitination
Receptor Interactions
Conclusions
References

CHAPTER 57
Mechanisms of CD40 Signaling in the Immune System **319**
Aymen Al-Shamkhani, Martin J. Glennie, and Mark S. Cragg

Introduction
Signaling Pathways Triggered by CD40 Engagement
CD40 Signaling Is Mediated by TRAF-Dependent and TRAF-Independent Pathways
References

CHAPTER 58
Role of Lipid Domains in EGF Receptor Signaling **323**
Linda J. Pike

Introduction
Localization of the EGF Receptor to Lipid Rafts
Rafts and EGF-Receptor-Mediated Signaling
The EGF Receptor and Caveolin
Summary
References

CHAPTER 59
Structure and Function of B-Cell Antigen Receptor Complexes **327**
Michael Reth and Michael Huber

Introduction
The Structure of the B Cell Antigen Receptor
Initiation of BCR Signaling Is Controlled by Redox Regulation
References

CHAPTER 60
Lipid-Mediated Localization of Signaling Proteins **331**
Maurine E. Linder

Introduction
Protein Lipidation
Summary
References

CHAPTER 61
G-Protein Organization and Signaling **335**
Maria R. Mazzoni and Heidi E. Hamm

Introduction
G-Protein Molecular Organization
Structural Features of G Protein Activation
Structural Determinants of Receptor–G-Protein Specificity
Gα Interactions with Effector Molecules
Gβγ Interactions with Effector Molecules
Conclusions
References

CHAPTER 62
JAK–STAT Signaling **343**
Rashna Bhandari and John Kuriyan

Introduction
Cytokine Signaling Proteins
JAK Structure and Localization

STAT Structure and Function
Inhibition of Cytokine Signaling
Summary
References

CHAPTER 63
Organization of Photoreceptor Signaling Complexes **349**
Susan Tsunoda

INAD Organizes Signaling Complexes
INAD-Signaling Complexes in Phototransduction
Assembly, Targeting, and Anchoring of Signaling Complexes
Signaling Complexes in Vertebrate Photoreceptors
References

CHAPTER 64
Protein Localization in Negative Signaling **355**
Jackson G. Egen and James P. Allison

Introduction
The Role of CD28 and CTLA-4 in T-Cell Activation
Expression and Localization of CTLA-4 and CD28: Consequences for Receptor Function
Mechanisms of CTLA-4-Mediated Negative Signaling
Conclusions
References

CHAPTER 65
Transmembrane Receptor Oligomerization **361**
Darren Tyson and Ralph A. Bradshaw

Introduction
Tyrosine Kinase-Containing Receptors
Cytokine Receptors
Guanylyl Cyclase-Containing Receptors
Serine/Threonine Kinase-Containing Receptors
Tumor Necrosis Factor Receptors
Heptahelical Receptors (G-Protein-Coupled Receptors)
Concluding Remarks
References

PART II
TRANSMISSION: EFFECTORS AND CYTOSOLIC EVENTS
Tony Hunter, Editor

PART II
Introduction **369**
Tony Hunter, Editor

Section A: Protein Phosphorylation
Tony Pawson

CHAPTER 66
Eukaryotic Kinomes: Genomic Cataloguing of Protein Kinases and Their Evolution **373**
Tony Hunter and Gerard Manning

Introduction
The Yeasts: *Saccharomyces cerevisiae and Schizosaccharomyces pombe*
Nematodes: *Caenorhabditis elegans*
Insects: *Drosophila melanogaster*
Vertebrates: *Homo sapiens*
Comparative Kinomics
Coda
References

CHAPTER 67
Modular Protein Interaction Domains in Cellular Communication **379**
Tony Pawson and Piers Nash

Introduction
Phosphotyrosine-Dependent Protein–Protein Interactions
Interaction Domains: A Common Theme in Signaling
Adaptors, Pathways, and Networks
Evolution of a Phospho-Dependent Docking Protein
Multisite Phosphorylation, Ubiquitination, and Switch-Like Responses
Summary
References

CHAPTER 68
Structures of Serine/Threonine and Tyrosine Kinases **387**
Matthew A. Young and John Kuriyan

Introduction
Structures of Protein Kinases
Structures of Inactive Protein Kinases
Summary
References

CHAPTER 69
Protein Tyrosine Kinase Receptor Signaling Overview **391**
Carl-Henrik Heldin

Introduction
PTK Subfamilies
Mechanism of Activation
Control of PTK Receptor Activity
Cross-Talk Between Signaling Pathways

PTK Receptors and Disease
References

CHAPTER 70
Signaling by the Platelet-Derived Growth Factor Receptor Family **397**
M. V. Kovalenko and Andrius Kazlauskas

Introduction
Platelet-Derived Growth Factors, Their Receptors, and Assembly of the PDGF Receptor Signaling Complex
Some Aspects of Regulation of the PDGF Receptor-Initiated Signaling
References

CHAPTER 71
EGF Receptor Family **405**
Mina D. Marmor and Yosef Yarden

Introduction
Domain Structure of ErbBs
Subcellular Localization of ErbB Proteins
ErbB-Induced Signaling Pathways
Negative Regulatory Pathways
Specificity of Signaling Through the ErbB Network
ErbB Proteins and Pathological Conditions
References

CHAPTER 72
IRS-Protein Scaffolds and Insulin/IGF Action **409**
Morris F. White

IRS-Proteins: The Beginnings
IRS-Proteins and Insulin Signaling
IRS-Protein Structure and Function
IRS-Protein Signaling in Growth, Nutrition, and Longevity
Interleukin-4 and IRS2 Signaling
Heterologous Regulation of IRS-Protein Signals
IRS2 and Pancreatic β-Cells
Summary
References

CHAPTER 73
Eph Receptors **421**
Rüdiger Klein

Introduction
Ephs and Ephrins
Eph Receptor Signaling Via Cytoplasmic Protein Tyrosine Kinases
Eph Receptor Signaling Via Rho Family GTPases
Effects on Cell Proliferation
Eph Receptor Signaling through PDZ-Domain-Containing Proteins
Eph Receptors and Cell Adhesion
Ephrin Reverse Signaling
EphrinB Reverse Signaling Via Phosphotyrosine
EphrinB Reverse Signaling Via PDZ Domain Interactions
Summary
References

CHAPTER 74
Cytokine Receptor Superfamily Signaling **427**
James N. Ihle

Cytokine Receptor Superfamily Signaling
References

CHAPTER 75
Negative Regulation of the JAK/STAT Signaling Pathway **431**
Joanne L. Eyles and Douglas J. Hilton

Introduction
The Phosphatases
STAT Phosphatases
PIAS (Protein Inhibitors of Activated STATS)
SOCS (Suppressors of Cytokine Signaling) Family
Concluding Comments
References

CHAPTER 76
Activation of Oncogenic Protein Kinases **441**
G. Steven Martin

Introduction
Physiological Regulation of Protein Kinases
Activation of Protein Kinases by Retroviruses
Activation of Protein Kinases in Human Cancer
Oncogenic Protein Kinases as Targets for Therapy
References

CHAPTER 77
Protein Kinase Inhibitors **451**
Alexander Levitzki

Signal Transduction Therapy
Protein Tyrosine Kinase Inhibitors
SER/THR Kinase Inhibitors
References

CHAPTER 78
Integrin Signaling: Cell Migration, Proliferation, and Survival **463**
J. Thomas Parsons, Jill K. Slack-Davis, and Karen H. Martin

Introduction
Integrins Nucleate the Formation of Multi-Protein Complexes

Cell Migration: A Paradigm for Studying Integrin Signaling
Integrin Regulation of Cell Proliferation and Survival: Links to Cancer
Concluding Remarks
References

CHAPTER 79
Downstream Signaling Pathways: Modular Interactions 471
Bruce J. Mayer

Introduction
General Properties of Interaction Modules
Roles in Signaling
Prospects
References

CHAPTER 80
Non-Receptor Protein Tyrosine Kinases in T-Cell Antigen Receptor Function 475
Kiminori Hasegawa, Shin W. Kang, Chris Chiu and Andrew C. Chan

Introduction
T-Cell Antigen Receptor Structure
Src PTKs
Csk (c-Src PTK)
ZAP-70/Syk PTKs
Tec PTKs
Summary
References

CHAPTER 81
Cbl: A Physiological PTK Regulator 483
Wallace Y. Langdon

Introduction
Domains of Cbl Proteins
Sli-1: A Negative Regulator of RPTKs
PTK Downregulation by Polyubiquitylation
Cbl-Deficient Mice
Future Directions
References

CHAPTER 82
TGFβ Signal Transduction 487
Jeffrey L. Wrana

Introduction
The Smad Pathway
Smads and the Ubiquitin–Proteasome System
Smad-Independent Signaling Pathways
Other Receptor Interaction Proteins
References

CHAPTER 83
MAP Kinases 493
James R. Woodgett

Introduction
The ERK Module
Stress-Activated MAPKs, Part 1: SAPK/JNKs
Stress-Activated MAPKs, Part 2: p38 MAPKs
MAPKKs
MAPKKKs
MAPKKKKs
Summary
References

CHAPTER 84
Cytoskeletal Regulation: Small G-Protein–Kinase Interactions 499
Ed Manser

Introduction
P21-Activated Kinases
Myotonic Dystrophy Kinase-Related Cdc42-Binding Kinase
Rho-Associated Kinase (ROK)
References

CHAPTER 85
Recognition of Phospho-Serine/Threonine Phosphorylated Proteins 505
Stephen J. Smerdon and Michael B. Yaffe

Introduction
14-3-3 Proteins
FHA Domains
WW Domains
Leucine-Rich Repeats and WD40 Domains
Concluding Remarks
References

CHAPTER 86
Role of PDK1 in Activating AGC Protein Kinase 513
Dario R. Alessi

Introduction
Mechanisms of Activation of PKB
PKB Is Activated by PDK1
Activation of Other Kinases by PDK1
Phenotype of PDK1 PKB- and S6K-Deficient Mice and Model Organisms
Hydrophobic Motif of AGC Kinases
Mechanisms of Regulation of PDK1 Activity
Structure of the PDK1 Catalytic Domain
Concluding Remarks
References

CHAPTER 87
Regulation of Cell Growth and Proliferation in Metazoans by mTOR and the p70 S6 Kinase 523
Joseph Avruch

Introduction
Functions of TOR
Signaling from TOR
Regulation of mTOR Activity
References

CHAPTER 88
AMP-Activated Protein Kinase 535
D. Grahame Hardie

Introduction
Structure of the AMPK Complex
Regulation of the AMPK Complex
Regulation in Intact Cells and Physiological Targets
Medical Implications of the AMPK System
References

CHAPTER 89
Principles of Kinase Regulation 539
Bostjan Kobe and Bruce E. Kemp

Introduction
Protein Kinase Structure
General Principles of Control
Regulatory Sites in Protein Kinase Domains
Conclusions
References

CHAPTER 90
Calcium/Calmodulin-Dependent Protein Kinase II 543
Mary B. Kennedy

Introduction
Structure of CaMKII
Regulation by Autophosphorylation
Regulatory Roles of CaMKII in Neurons
References

CHAPTER 91
Glycogen Synthase Kinase 3 547
Philip Cohen and Sheelagh Frame

Introduction
The Substrate Specificity of GSK3
The Regulation of GSK3 Activity by Insulin and Growth Factors
GSK3 as a Drug Target
The Role of GSK3 in Embryonic Development
GSK3 and Cancer
References

CHAPTER 92
Protein Kinase C: Relaying Signals from Lipid Hydrolysis to Protein Phosphorylation 551
Alexandra C. Newton

Introduction
Protein Kinase C Family
Regulation of Protein Kinase C
Function of Protein Kinase C
Summary
References

CHAPTER 93
The PIKK Family of Protein Kinases 557
Graeme C. M. Smith and Stephen P. Jackson

Introduction
Overview of PIKK Family Members
Overall Architecture of PIKK Family Proteins
MTOR: A Key Regulator of Cell Growth
DNA-Pkes: At the Heart of the DNA Nonhomologous End-Joining Machinery
ATM and ATR: Signalers of Genome Damage
SMG-1: A Regulator of Nonsense-Mediated mRNA Decay
TRRAP: A Crucial Transcriptional Co-Activator
PIKK Family Members as Guardians of Nucleic Acid Structure, Function, and Integrity?
References

CHAPTER 94
Histidine Kinases 563
Fabiola Janiak-Spens and Ann H. West

References

CHAPTER 95
Atypical Protein Kinases: The EF2/MHCK/ChaK Kinase Family 567
Angus C. Nairn

Introduction
Identification of an Atypical Family of Protein Kinases: EF2 Kinase, Myosin Heavy Chain Kinase and ChaK
The Structure of the Atypical Kinase Domain Reveals Similarity to Classical Protein Kinases and to Metabolic Enzymes with ATP-Grasp Domains
Substrate Specificity of Atypical Kinases
Regulation of Atypical Kinases
Functions of the Atypical Family of Protein Kinases
References

CHAPTER 96
Casein Kinase I and Regulation of the Circadian Clock **575**
Saul Kivimäe, Michael W. Young, and Lino Saez

Introduction
double-time: A Casein Kinase I Homolog in *Drosophila*
Casein Kinase I in the Mammalian Clock
Casein Kinase I in the *Neurospora* Clock
Similarities and Differences of CKI Function in Different Clock Systems
References

CHAPTER 97
The Leucine-Rich Repeat Receptor Protein Kinases of *Arabidopsis thaliana:* A Paradigm for Plant LRR Receptors **579**
John C. Walker and Kevin A. Lease

Introduction
LRR Receptor Protein Kinases: The Genomic Point of View
LRR Receptor Protein Kinases: The Functional View
Summary
References

CHAPTER 98
Engineering Protein Kinases with Specificity for Unnatural Nucleotides and Inhibitors **583**
Chao Zhang and Kevan M. Shokat

References

Section B: Protein Dephosphorylation
Jack E. Dixon, Editor

CHAPTER 99
Overview of Protein Dephosphorylation **591**
Jack E. Dixon

CHAPTER 100
Protein Serine/Threonine Phosphatases and the PPP Family **593**
Patricia T. W. Cohen
Current Classification of Protein Serine/Threonine Phosphatases
Background
Evolution and Conserved Features of the PPP Family
Catalytic Activities of the PPP Family Members
Eukaryotic PPP Subfamilies
Domain and Subunit Structure of PPP Family Members
Medical Importance of the PPP Family
References

CHAPTER 101
The Structure and Topology of Protein Serine/Threonine Phosphatases **601**
David Barford

Introduction
Protein Serine/Threonine Phosphatases of the PPP Family
Protein Serine/Threonine Phosphatases of the PPM Family
Conclusions
References

CHAPTER 102
Naturally Occurring Inhibitors of Protein Serine/Threonine Phosphatases **607**
Carol MacKintosh and Julie Diplexcito

Introduction
Effects of Inhibitors in Cell-Based Experiments
The Toxins Bind to the Active Sites of Protein Phosphatases
Chemical Synthesis of Protein Phosphatase Inhibitors
Microcystin Affinity Chromatography and Affinity Tagging
Avoiding the Menace of Toxins in the Real World Outside the Laboratory
References

CHAPTER 103
Protein Phosphatase 1 Binding Proteins **613**
Anna A. Depaoli-Roach

Introduction
Protein Phosphatase 1 (PP1)
PP1 Regulatory or Targeting Subunits
Conclusions
References

CHAPTER 104
Role of PP2A in Cancer and Signal Transduction **621**
Gernot Walter

Introduction
Structure of PP2A
Subunit Interaction
Association of PP2A with Cellular Proteins
Alteration or Inhibition of PP2A Is Essential in Human Cancer Development
Mutation of Aα and Aβ Isoforms in Human Cancer
Differences between Aα and Aβ Subunits
PP2A and Wnt Signaling
PP2A and MAP Kinase Pathway
Summary
References

CHAPTER 105
Serine/Threonine Phosphatase Inhibitor Proteins **627**
Shirish Shenolikar

Introduction
Protein Phosphatase 1 (PP1) Inhibitors
I-1, DARPP-32, and Other Phosphorylation-Dependent Phosphatase Inhibitors
Latent Phosphatase Complexes Activated by Inhibitor Phosphorylation
Inhibitors of Type-2 Serine/Threonine Phosphatases
Conclusions
References

CHAPTER 106
Calcineurin **631**
Claude B. Klee and Seun-Ah Yang

Introduction
Enzymatic Properties
Structure
Regulation
Distribution and Isoforms
Functions
Muscle Differentiation
Conclusion
References

CHAPTER 107
Protein Serine/Threonine-Phosphatase 2C (PP2C) **637**
Hisashi Tatabe and Kazuhiro Shiozaki

Introduction
Regulation of the Stress-Activated MAP Kinase Cascades
Control of the CFTR Chloride Channel by PP2C
Plant Hormone Abscisic Acid Signaling
Fem-2: A Sex-Determining PP2C in Nematode
Stress-Responsive PP2Cs in *Bacillus subtilis*
References

CHAPTER 108
Overview of Protein Tyrosine Phosphatases **641**
Nicholas K. Tonks

Background
Structural Diversity within the PTP Family
The Classical PTPs
The Dual Specificity Phosphates (DSPs)
Regulation of PTP Function
Oxidation of PTPs in Tyrosine Phosphorylation-Dependent Signaling
Substrate Specificity of PTPs
PTPs and Human Disease
Perspectives
References

CHAPTER 109
Protein Tyrosine Phosphatase Structure and Mechanisms **653**
Youngjoo Kim and John M. Denu

Introduction
Introduction to the Protein Tyrosine Phosphatase Family
Structure
Mechanism
Regulation
References

CHAPTER 110
Bioinformatics: Protein Tyrosine Phosphatases **659**
Niels Peter H. Møller, Peter Gildsig Jansen, Lars F. Iversen, and Jannik N. Andersen

Introduction to Bioinformatics
Amino Acid Homology Among PTP Domains and Structure-Function Studies
Identification of the Genomic Complement of PTPs
Functional Aspects of PTPs in Health and Disease: Bioinformatics
References

CHAPTER 111
PTP Substrate Trapping **671**
Andrew J. Flint

Introduction
Original C→S and D→A Substrate-Trapping Mutants
Second-Generation Trapping Mutants
Accessory or Noncatalytic Site Contributions to Substrate Recognition
New Twists on Trapping
Other Applications of Substrate Trapping Mutants
References

CHAPTER 112
Inhibitors of Protein Tyrosine Phosphatases **677**
Zhong-Yin Zhang

Introduction
Covalent PTP Modifiers
Oxyanions as PTP Inhibitors
PTyr Surrogates as PTP Inhibitors
Bidentate PTP Inhibitors
Other PTP Inhibitors
Concluding Remarks
References

CHAPTER 113
Regulating Receptor PTP Activity **685**
Erica Dutil Sonnenburg, Tony Hunter, and Joseph P. Noel

Introduction
Regulation by Dimerization
Regulation by Phosphorylation
Regulation by D2 Domain
References

CHAPTER 114
CD45 **689**
Zheng Xu, Michelle L. Hermiston, and Arthur Weiss

Introduction
Structure
Function
Regulation
References

CHAPTER 115
Properties of the Cdc25 Family of Cell-Cycle Regulatory Phosphatases **693**
William G. Dunphy

Introduction
Physiological Functions of Cdc25
Regulation of Cdc25
Concluding Remarks
References

CHAPTER 116
Cell-Cycle Functions and Regulation of Cdc14 Phosphatases **697**
Harry Charbonneau

Introduction
The Cdc14 Phosphatase Subgroup of PTPs
Budding Yeast Cdc14 is Essential for Exit from Mitosis
Fission Yeast Cdc14 Coordinates Cytokinesis with Mitosis
Potential Cell-Cycle Functions of Human Cdc14A and B
References

CHAPTER 117
MAP Kinase Phosphatases **703**
Marco Muda and Steve Arkinstall

Introduction
MAPK Phosphatases in Yeast
A MAPK Phosphatase in *C. elegans*
MAPK Phosphatases in *Drosophila melanogaster*
MAPK Phosphatases in Mammals
Summary
References

CHAPTER 118
SH2-Domain-Containing Protein–Tyrosine Phosphatases **707**
Benjamin G. Neel, Haihua Gu, and Lily Pao

History and Nomenclature
Structure, Expression, and Regulation
Biological Functions of Shps
Shp Signaling and Substrates
Determinants of Shp Specificity
Shps and Human Disease
Summary and Future Disease
References

CHAPTER 119
Insulin Receptor PTP: PTP1B **729**
Alan Cheng and Michel L. Tremblay

Introduction
PTP1B as a *Bona Fide* IR Phosphatase
PTP1B Gene Polymorphisms and Insulin Resistance
Insulin-Mediated Modulation of PTP1B
Genetic Evidence for Other PTP1B Substrates
Concluding Remarks
References

CHAPTER 120
Low-Molecular-Weight Protein Tyrosine Phosphatases **733**
Robert L. Van Etten

Introduction
Structures of LMW PTPases
Catalytic Mechanism
Inhibitors and Activators
Substrate Specificity, Regulation, and Biological Role
References

CHAPTER 121
STYX/Dead-Phosphatases **741**
Matthew J. Wishart

Introduction
Gathering Styx: Structure Implies Function
The Gratefully Undead: STYX/Dead-Phosphatases Mediate Phosphorylation Signaling
Conclusions
References

VOLUME 2

Contributors xlv

PART II
TRANSMISSION: EFFECTORS AND CYTOSOLIC EVENTS (CONTINUED FROM VOLUME 1)

Section C: Calcium Mobilization
Michael J. Berridge, Editor

CHAPTER 122
Phospholipase C **5**
Hong-Jun Liao and Graham Carpenter

Introduction
PLC Anatomy
PLC Activation Mechanisms
PLC Physiology
References

CHAPTER 123
Inositol 1,4,5-trisphosphate 3-kinase and 5-phosphatase **11**
Valérie Dewaste and Christophe Erneux

Introduction
Type I $InsP_3$5-phosphatase
$InsP_3$ 3-kinase
References

CHAPTER 124
Cyclic ADP-ribose and NAADP **15**
Antony Galione and Grant C. Churchill

Introduction
References

CHAPTER 125
Sphingosine 1-phosphate **19**
Kenneth W. Young and Stefan R. Nahorski

Introduction
Sphingolipid Metabolism
Activation of SPHK
Intracellular Target for SPP-mediated Ca^{2+} Release
Concluding Remarks
References

CHAPTER 126
Voltage-gated Ca^{2+} Channels **23**
William A. Catterall

Introduction
Physiological Roles of Voltage-gated Ca^{2+} Channels
Ca^{2+} Current Types Defined by Physiological and Pharmacological Properties
Molecular Properties of Ca^{2+} Channels
Molecular Basis for Ca^{2+} Channel Function
Ca^{2+} Channel Regulation
Conclusion
References

CHAPTER 127
Store-operated Ca^{2+} Channels **31**
James W. Putney, Jr.

Capacitative Calcium Entry
Store-operated Channels
Mechanism of Activation of Store-Operated Channels
Summary
References

CHAPTER 128
Arachidonic Acid-regulation Ca^{2+} Channel **35**
Trevor J. Shuttleworth

Introduction
Identification and Characterization of ARC Channels
Specific Activation of ARC Channels by Low Agonist Concentrations
Roles of ARC Channels and SOC/CRAC Channels in $[Ca^{2+}]_i$ Signals: "Reciprocal Regulation"
Conclusions and Implications
References

CHAPTER 129
IP_3 Receptors **41**
Colin W. Taylor

Introduction
References

CHAPTER 130
Ryanodine Receptors **45**
David H. MacLennan and Guo Guang Du

Function and Structure
Activation of Ryanodine Receptor Ca^{2+} Release Channels
Molecular Biology of Ryanodine Receptors
References

CHAPTER 131
Intracellular Calcium Signaling **51**
Martin D. Bootman, H. Llewelyn Roderick, Rodney O'Connor, and Michael J. Berridge

The "Calcium Signaling Toolkit" and Calcium Homeostasis
Multiple Channels and Messengers Underlie Ca^{2+} Increases
Temporal Regulation of Ca^{2+} Signals
Spatial Regulation of Ca^{2+} Signals
Modulation of Ca^{2+} Signal Amplitude
Ca^{2+} as a Signal within Organelles and in the Extracellular Space
References

CHAPTER 132
Calcium Pumps **57**
Ernesto Carafoli

Introduction
Reaction Cycle of the SERCA and PMCA Pumps
The SERCA Pump
The PMCA Pump
Genetic Diseases Evolving Defects of Calcium Pumps
References

CHAPTER 133
Sodium/Calcium Exchange **63**
Mordecai P. Blaustein

Introduction
Two Families of PM Na^+/Ca^{2+} Exchanges
Modes of Operation of the Na^+/Ca^{2+} Exchangers
Regulation of NCX
Inhibition of NCX
Localization of the NCX
Physiological Roles of the NCX
References

CHAPTER 134
Ca^{2+} Buffers **67**
Beat Schwaller

Introduction
Relevant Parameters for Ca^{2+} Buffers
Ca^{2+} Buffers as One Component Contributing to Intracellular Ca^{2+} Homeostasis
Biological Effects of Ca^{2+} Buffers
References

CHAPTER 135
Mitochondria and Calcium Signaling, Point and Counterpoint **73**
Michael R. Duchen

Introduction
Fundamentals
Machinery of Mitochondrial Ca^{2+} Movement
The Set Point
Quantitative Issues, Microdomains, and the Regulation of $[Ca^{2+}]_c$ Signals
Impact of Ca^{2+} Uptake on Mitochondrial Function
Mitochondrial Ca^{2+}, Disease, and Death
CODA
References

CHAPTER 136
EF-Hand Proteins and Calcium Sensing: The Neuronal Calcium Sensors **79**
Jamie L. Weiss and Robert D. Burgoyne

Introduction
Class A. Neuronal Calcium Sensor 1 (Frequenin)
Class B. Neurocalcins (VILIPs) and Hippocalcin
Class C. Recoverins
Class D. Guanylate Cyclase Activating Proteins
Class E. K^+ Channel Interacting Proteins
Future Perspectives for the NCS Protein Family
References

CHAPTER 137
Calmodulin-Mediated Signaling **83**
Anthony R. Means

Introduction
References

CHAPTER 138
The Family of S100 Cell Signaling Proteins **87**
Claus W. Heizmann, Beat W. Schäfer, and Günter Fritz

Introduction
Protein Structures and Metal-Dependent Interactions with Target Proteins
Genomic Organization, Chromosomal Localization, and Nomenclature
Translocation, Secretion, and Biological Functions
Associations with Human Diseases
Conclusion and Perspectives
References

CHAPTER 139
C_2-Domains in Ca^{2+}-Signaling **95**
Thomas C. Südhof and Josep Rizo

Structures of C_2-Domains
Ca^{2+}-Binding Mode of C_2-Domains
Phospholipid Binding Mechanism of C_2-Domains
Other Ligands of C_2-Domains
Functions of C_2-Domains
References

CHAPTER 140
Annexins and Calcium Signaling **101**
Stephen E. Moss

Introduction
Annexins as Ca^{2+} Channel Regulators
Conclusions
References

CHAPTER 141
Calpain **105**
Alan Wells and Anna Huttenlocher

Introduction
Calpain Family
Modes of Regulation
Calpain as a Signaling Intermediate: Potential Targets
Functional Roles
Future Considerations
References

CHAPTER 142
Regulation of Intracellular Calcium through Hydrogen Peroxide **113**
Sue Goo Rhee

Introduction
Sources and Chemical Properties of ROS
Activation of Ryanodine and IP_3 Receptor Ca^{2+} Release Channels by H_2O_2
Enhancement of $[Ca^{2+}]_i$ through H_2O_2-mediated Inactivation of Protein Tyrosine Phosphatase and PTEN
References

Section D: Lipid-Derived Second Messengers
Lewis Cantley, Editor

CHAPTER 143
Historical Overview: Protein Kinase C, Phorbol Ester, and Lipid Mediators **119**
Yasutomi Nishizuka and Ushio Kikkawa

Retrospectives of Phospholipid Research
Protein Kinase C and Diacylglycerol
Phorbol Ester and Cell Signaling
Structural Heterogeneity and Mode of Activation
Translocation and Multiple Lipid Mediators
Conclusion
References

CHAPTER 144
Type I Phosphatidylinositol 4-phosphate 5-kinases (PI4P 5-kinases) **123**
K. A. Hinchliffe and R. F. Irvine

Introduction
Basic Properties
Regulation
Function
References

CHAPTER 145
Type 2 PIP4-Kinases **129**
Lucia Rameh

Introduction
History
Structure
Type 2 PIP4-Kinase Isoforms
Regulation
Putative Models for the Function of the Type 2 PIP-Kinases
Conclusion
References

CHAPTER 146
Phosphoinositide 3-Kinases **135**
David A. Fruman

Introduction
The Enzymes
The Products
Lipid-Binding Domains
Effectors and Responses
Phosphatases
Genetics
Summary
References

CHAPTER 147
PTEN/MTM Phosphatidylinositol Phosphatases **143**
Knut Martin Torgersen, Soo-A Kim, and Jack E. Dixon

PTEN
Myotubularin: a Novel Family of Phosphatidylinositol Phosphatases
References

CHAPTER 148
SHIP Inositol Phosphate Phosphatases **147**
Larry R. Rohrschneider

Introduction
SHIP1 Structure, Expression, and Function
SHIP2 Structure, Expression, and Function
References

CHAPTER 149
Structural Principles of Lipid Second Messenger Recognition **153**
Roger L. Williams

Introduction
Phospholipid Second Messenger Recognition by Active Sites of Enzymes
Phosphoinositide-binding Domains
Non-phosphoinositide Lipid Messenger Recognition
Future Directions
References

CHAPTER 150
Pleckstrin Homology (PH) Domains **161**
Mark A. Lemmon

Identification and Definition of PH Domains
The Structure of PH Domains
PH Domains as Phosphoinositide-Binding Modules
Binding of PH Domains to Non-phosphoinositide Ligands
Possible Roles of Non-phosphoinositide PH Ligands
Conclusions
References

CHAPTER 151
PX Domains 171
Hui Liu and Michael B. Yaffe

History and Overview of PX Domains
Lipid-Binding Specificity and the Structure of PX Domain
Function of PX Domain-containing Proteins
References

CHAPTER 152
FYVE Domains in Membrane Trafficking and Cell Signaling 177
Christopher Stefan, Anjon Audhya, and Scott Emr

Introduction
Role for PtdIns(3)P in Membrane Trafficking and Identification of the FYVE Domain
Structural Basis for the FYVE Domain
Conservation of the FYVE Domain and Localization of PtdIns(3)P
FYVE Domains in Membrane Trafficking
FYVE Domains Involved in PtdIns(3)P Metabolism
FYVE Domains in Signaling
FYVE-like Domains
Conclusions
References

CHAPTER 153
Protein Kinase C: Relaying Signals from Lipid Hydrolysis to Protein Phosphorylation 187
Alexandra C. Newton

Introduction
Protein Kinase C Family
Regulation of Protein Kinase C
Function of Protein Kinase C
Summary
References

CHAPTER 154
Role of PDK1 in Activating AGC Protein Kinase 193
Dario R. Alessi

Introduction
Mechanism of Activation of PKB
PKB Is Activated by PDK1
Activation of Other Kinases by PDK1
Phenotype of PDK1 PKB- and S6K-Deficient Mice and Model Organisms
Hydrophobic Motif of AGC Kinases
Mechanism of Regulation of PDK1 Activity
Structure of the PDK1 Catalytic Domain
Concluding Remarks
References

CHAPTER 155
Modulation of Monomeric G Proteins by Phosphoinositides 203
Sonja Krugmann, Len Stephens, and Phillip T. Hawkins

Introduction
Rho Family Small GTPases
Arf Family GTPases
Modulation of Ras Family GTPases by PI3K
Conclusion
References

CHAPTER 156
Phosphoinositides and Actin Cytoskeletal Rearrangement 209
Paul A. Janmey, Robert Bucki, and Helen L. Yin

Historical Perspective
Stimulating Cellular Actin Polymerization
Actin-Membrane Linkers Localized or Activated by PIP2
Relation of Actin Assembly to Phsphoinositide-containing Lipid Rafts
Different Mechanisms of PPI-Actin Binding Protein Regulation
Effects on Lipid Membrane Structure
References

CHAPTER 157
The Role of PI3 Kinase in Directional Sensing during Chemotaxis in *Dictyostelium*, a Model for Chemotaxis of Neutrophils and Macrophages 217
Richard A. Firtel and Ruedi Meili

Introduction
Directional Movement
Localization of Cytoskeletal and Signaling Components
The Signaling Pathways Controlling Directional Movement
PI3K Effectors and their Roles in Controlling Chemotaxis
The Tumor Suppressor PTEN Regulates the Chemoattractant PI3K Pathways
Conclusions
References

CHAPTER 158
Phosphatidylinositol Transfer Proteins 225
Shamshad Cockcroft

Introduction
The Classical PITPs: α and β
RdgB Family of PITP Proteins
References

CHAPTER 159
Inositol Polyphosphate Regulation of Nuclear Function 229
John D. York

Introduction
Inositol Signaling and the Molecular Revolution
Links of Inositol Signaling to Nuclear Function
The Inositol Polyphosphate Kinase (IPK) Family
References

CHAPTER 160
Ins(1,3,4,5,6)P_5: A Signal Transduction Hub 233
Stephen B. Shears

Introduction
References

CHAPTER 161
Phospholipase D 237
Paul C. Sternweis

Introduction
Structural Domains and Requirements for Activity
Catalysis: Mechansim and Measurement
Modification of Mammalian PLDs
Regulatory Inputs for Mammalian PLD
Regulatory Pathways
Physiological Function of PA
Localization of PLD
Future Directions
References

CHAPTER 162
Diacylglycerol Kinases 243
M. K. Topham and S. M. Prescott

Introduction
The DGK Family
Regulation of DGKs
Paradigms of DGK Function
Conclusions
References

CHAPTER 163
Sphingosine-1-Phosphate Receptors 247
Michael Maceyka and Sarah Spiegel

Introduction
The S1PRs
S1P Signaling via S1PRs
Transactivation of S1PRs
Downstream Signaling from S1PRs
References

CHAPTER 164
SPC/LPC Receptors 253
Linnea M. Baudhuin, Yijin Xiao, and Yan Xu

Introduction
Physiological and Pathological Functions of LPC and SPC
Identification of Receptors for SPC and LPC
Perspectives
References

CHAPTER 165
The Role of Ceramide in Cell Regulation 257
Yusuf A. Hannun and L. Ashley Cowart

Ceramide-Mediated CellR
Biochemical Pathways of Ceramide Generation
Ceramide Targets
Conclusions
References

CHAPTER 166
Phospholipase A_2 Signaling and Arachidonic Acid Release 261
Jesús Balsinde and Edward A. Dennis

Introduction
PLA_2 Groups
Cellular Function
Summary
References

CHAPTER 167
Prostaglandin Mediators 265
Emer M. Smyth and Garret A. Fitzgerald

Introduction
The Cyclooxygenase Pathway
Prostanoid Receptors
Thromboxane A_2 (TxA_2)
Prostacyclin (PGI_2)
Prostaglandin D_2 (PGD_2)
Prostaglandin E_2 (PGE_2)
Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)
Concluding Remarks
References

CHAPTER 168
Leukotriene Mediators 275
Jesper Z. Haeggström and Anders Wetterholm

Introduction
Five-Lipoxygenase
Leukotriene A_4 Hydrolase
References

CHAPTER 169
Lipoxins and Aspirin-Triggered 15-epi-Lipoxins: Mediators in Anti-inflammation and Resolution **281**
Charles N. Serhan

Lipoxin Signals in the Resolution of Inflammation
Novel Anti-Inflammatory Signals and Pathways
Concluding Remarks
References

CHAPTER 170
Cholesterol Signaling **287**
Peter A. Edwards, Heidi R. Kast-Woelbern, and Matthew A. Kennedy

Introduction
Cholesterol Precursors
Cholesterol
Cholesterol Derivatives: Ligands for Nuclear Receptors
References

Section E: Protein Proximity Interactions
John D. Scott, Editor

CHAPTER 171
Protein Proximity Interactions **293**
John D. Scott

Introduction
Techniques for the Analysis of Protein-Protein Interactions
Subcellular Structures and Multiprotein Complexes
Kinase and Phosphatase Targeting Proteins

CHAPTER 172
Protein Interaction Mapping by Coprecipitation and Mass Spectrometric Identification **295**
Shao-En Ong and Matthias Mann

Introduction
General Considerations of the Coprecipitation Experiment
GST-Tagged Proteins
Antibodies
Epitope Tags
Mass Spectrometric Approaches
Outlook
References

CHAPTER 173
Proteomics, Fluorescence, and Binding Affinity **301**
Paul R. Graves and Timothy A. J. Haystead

Introduction
Isolation of Specific Proteomes
Affinity Chromatography for the Isolation of Protein Complexes
Specificity of Protein-Protein or Protein-Ligand Interactions
References

CHAPTER 174
FRET Analysis of Signaling Events in Cells **305**
Peter J. Verveer and Philippe I. H. Bastiaens

Introduction
Fluorescent Probes for FRET
FRET Detection Techniques
Conclusions and Prospects
References

CHAPTER 175
Peptide Recognition Module Networks: Combining Phage Display with Two-Hybrid Analysis to Define Protein-Protein Interactions **311**
Gary D. Bader, Amy Hin Yan Tong, Gianni Cesareni, Christopher W. Hogue, Stanley Fields, and Charles Boone

Introduction
References

CHAPTER 176
The Focal Adhesion: A Network of Molecular Interactions **317**
Benjamin Geiger, Eli Zamir, Yariv Kafri, and Kenneth M. Yamada

Introduction
Connectivity-Based Ordering of FA Components
Molecular Switches in FA
Future Challenges
References

CHAPTER 177
WASp/Scar/WAVE **323**
Charles L. Saxe

Introduction
WASp
Scar/WAVE
References

CHAPTER 178
Synaptic NMDA-Receptor Signaling Complex **329**
Mary B. Kennedy

Introduction
Structure of the NMDA Receptor Signaling Complex
Orchestration of Responses to Ca^{2+} Entering through the NMDA Receptor
References

CHAPTER 179
Toll Family Receptors 333
Hana Bilak, Servane Tauszig-Delamasure, and Jean-Luc Imler

Introduction
Structure Function of Toll Receptors
Signaling by Toll Family Receptors
References

CHAPTER 180
Signaling and the Immunological Synapse 339
Andrey S. Shaw

Introduction
Brief Introduction to T Cell Biology
Initiation of TCR Signaling
Definition of the Immunological Synapse
Immunological Synapses and T-Cell Development
Synapses and Different Kinds of T Cells
Natural Killer Cell Synapses
The Function of the Immunological Synapse
Immunological Synapses and TCR Downregulation
Conclusion
References

CHAPTER 181
The Ubiquitin-Proteasome System 347
Mark Hochstrasser

Introduction
Overview of the Ubiquitin-Proteasome System
Components of the Ubiquitin Ligation and Deubiquitination Pathways
The 20S and 26S Proteasomes
Degradation Signals or Degrons
Examples of Regulation of Protein Ubiquitination
References

CHAPTER 182
Caspases: Cell Signaling by Proteolysis 351
Guy S. Salvesen

Protease Signaling
Apoptosis and Limited Proteolysis
Caspase Activation
Regulation by Inhibitors
IAP Antagonists
References

CHAPTER 183
MAP Kinase in Yeast 357
Elaine A. Elion

Introduction
Yeast Cells Use Multiple MAPKs to Respond to a Wide Variety of Stimuli
Functionally Defining *S. cerevisiae* MAPK Cascades
Major Regulatory Mechanisms that Control Specificity in *S. cerevisai* MAPK Cascades
References

CHAPTER 184
$X^{c,v}$ Mammalian MAP Kinases 365
Roger J. Davis

Introduction
The ERK Group of MAP Kinases
The p38 Group of MAP Kinases
The JNK Group of MAP Kinases
MAP Kinase-Related Protein Kinases
MAP Kinase Docking Interactions
Scaffold Proteins
References

CHAPTER 185
Subcellular Targeting of PKA through AKAPs: Conserved Anchoring and Unique Targeting Domains 377
Mark L. Dell'Acqua

Introduction
Structurally Conserved PKA Anchoring Determinants
Unique Subcellular Targeting Domains
Probing Cellular Functions of AKAP-PKA Anchoring
Conclusions and Future Directions
References

CHAPTER 186
AKAP Signaling Complexes: The Combinatorial Assembly of Signal Transduction Units 383
John D. Scott and Lorene K. Langeberg

Introduction
G-Protein Signaling Through AKAP Signaling Complexes
Kinase/Phosphatase Signaling Complexes
CAMP Signaling Units
Conclusions and Perspectives
References

CHAPTER 187
Protein Kinase C–Protein Interaction 389
Peter J. Parker, Joanne Durgan, Xavier Iturrioz, and Sipeki Szabolcs

Introduction
Priming
Activation
Substrates and Pathways
PKC Inactivation
Perspectives
References

CHAPTER 188
Dendrite Protein Phosphatase Complexes 397
Roger J. Colbran

Introduction
The Importance of Dendritic Localization
Protein Phosphatase 1
Calcineurin (Protein Phosphatase 2B)
Dendritic Phosphatase Substrates
Role of Phosphatases in Synaptic Plasticity
Summary
References

CHAPTER 189
Protein Phosphatase 2A 405
Adam M. Silverstein, Anthony J. Davis, Vincent A. Bielinski, Edward D. Esplin, Nadir A. Mahmood, and Marc C. Mumby

Introduction
PP2A Regulatory Subunits Mediate Proximity Interactions
PP2A-Interacting Proteins
References

Section F: Cyclic Nucleotides
Jackie Corbin, Editor

CHAPTER 190
Adenylyl Cyclases 419
Matt Whorton and Roger K. Sunahara

Introduction
Structure-Function
Regulation
Physiology
Summary
References

CHAPTER 191
Guanylyl Cyclases 427
Ted D. Chrisman and David L. Garbers

Historic Perspectives
Guanylyl Cyclases
Guanylyl Cyclase Ligands
cGMP Effectors
Guanylyl Cyclases and Cell Growth Regulation
References

CHAPTER 192
Phosphodiesterase Families 431
Jennifer L. Glick and Joseph A. Beavo

Introduction
The Gene Families
Implications of Multiple Gene Families/Splice Variants
PDE Inhibitors as Therapeutic Agents
Where Do We Go from Here?
References

CHAPTER 193
The cAMP-Specific Phosphodiesterases: A Class of Diverse Enzymes That Defines the Properties and Compartmentalization of the cAMP Signal 437
Marco Conti

Structure of the cAMP-PDEs: Catalytic and Regulatory Domains
Subcellular Targeting of the cAMP-PDEs and cAMP Signal Compartmentalization
Regulation of cAMP-PDEs
References

CHAPTER 194
cAMP/cGMP Dual-Specificity Phosphodiesterases 441
Marie C. Weston, Lena Stenson-Holst, Eva Degerman, and Vincent C. Manganiello

Introduction
PDE1 (Ca^{2+}/Calmodulin-dependenet PDE)
PDE2 (cGMP-stimulated PDE)
PDE3 (cGMP-inhibited cAMP PDE)
PDE10
PDE11
Conclusions
References

CHAPTER 195
Phosphodiesterase-5 447
Sharron H. Francis and Jackie D. Corbin

Introduction
Gene Organization and Regulation of Expression
General Structure
Concluding Remarks
References

CHAPTER 196
Structure, Function, and Regulation of Photoreceptor Phosphodiesterase (PDE6) 453
Rick H. Cote

Introduction
Structure and Subcellular Localization of Rod PDE6
Regulation of Rod PDE6 Catalysis by γ
Catalytic Properties of Nonactivated and Activated PDE6P
Roles of the GAF Domains in PDE6 Regulation
Conclusion
References

CHAPTER 197
Spatial and Temporal Relationships of Cyclic Nucleotides in Intact Cells 459
Manuela Zaccolo, Marco Mongillo, and Tullio Pozzan

The Complexity of Cyclic Nucleotides Signaling
Methodological Advances
Functional Compartments of cAMP in Heart Cells
Spatio-temporal Aspects of Cyclic Nucleotides Signaling in Neurons
Conclusions
References

CHAPTER 198
Regulation of Cyclic Nucleotide Levels by Sequestration 465
Jackie D. Corbin, Jun Kotera, Venkatesh K. Gopal, Rick H. Cote, and Sharron H. Francis

Introduction
Sequestration of cGMP in Rod Photoreceptor Cells by PDE6
Sequestration of cGMP by PDE5
References

CHAPTER 199
cAMP-Dependent Protein Kinase 471
Susan S. Taylor and Elzbieta Radzio-Andzelm

Introduction
Catalytic Subunit
Protein Kinase Inhibitor
Regulatory Subunits
References

CHAPTER 200
Cyclic GMP-Dependent Protein Kinase 479
Thomas M. Lincoln

Introduction
Biochemical and Molecular Biology of PKG Isoforms
Physiologic Roles of PKG
Concluding Remarks
References

CHAPTER 201
Inhibitors of Cyclic Nucleotide-Dependent Protein Kinases 487
Wolfgang R. G. Dostmann

Introduction
Cyclic Nucleotide Binding Site-Targeted Inhibitors
ATP Binding Site-Targeted Inhibitors
Peptide Binding in Site-Targeted Inhibitors
Conclusions
References

CHAPTER 202
Peptide Substrates of Cyclic Nucleotide-Dependent Protein Kinases 495
Ross I. Brinkworth, Bostjan Kobe, and Bruce E. Kemp

Introduction
Peptide Substrate Recognition
Comparison of Kinase Substrate Acceptor Loci
Optimum Recognition Sequences
Comparison of PKA and PKG Specificity
Conclusions
References

CHAPTER 203
Physiological Substrates of PKA and PKG 501
Kjetil Tasken, Anja Ruppelt, John Shabb, and Cathrine R. Carlson

Introduction
Abundance of PKA and PKG Phosphorylation Sites in the Human Proteome
Physiological Substrates
Concluding Remarks
References

CHAPTER 204
Effects of cGMP-Dependent Protein Kinase Knockouts 511
Franz Hofmann, Robert Feil, Thomas Kleppisch, and Claudia Werner

Cyclic GMP-Dependent Protein Kinases: Genes and Knockouts
Outlook
References

CHAPTER 205
Cyclic Nucleotide-Regulated Cation Channels 515
Martin Biel and Andrea Gerstner

Introduction
General Features of Cyclic Nucleotide-Regulated Cation Channels
CNG Channels
HCN Channels
References

CHAPTER 206
Epacs, cAMP-Binding Guanine Nucleotide Exchange Factors for Rap1 and Rap2 521
Holger Rehman, Johan de Rooij, and Johannes L. Bos

Introduction
The Epac Family

The cAMP-Binding Domain of Epac Closely Resembles Those of PKA and Channels
Epac Is Conserved Through Evolution
Properties of Epac
Expression and Subcellular Localization of Epacs
Cellular Function of Epacs
References

CHAPTER 207
Cyclic Nucleotide-Binding Phosphodiesterase and Cyclase GAF Domains 525
Sergio E. Martinez, Xiao-Bo Tang, Stewart Turley, Wim G. J. Hol, and Joseph A. Beavo

Introduction
Atomic Structure
References

CHAPTER 208
cAMP Signaling in Bacteria 531
J. M. Passner

Introduction and Significance
Background and History
Transcriptional Regulation by CAP
CAP Permits Differential Gene Regulation at Different cAMP Concentrations
A Second cAMP-Binding Site in a CAP Monomer
Perspectives and Conclusions
References

CHAPTER 209
Cyclic Nucleotide Signaling in Paramecium 535
Jürgen U. Linder and Joachim E. Schultz

Introduction
cAMP Formation and Adenylyl Cyclase
Guanylyl Cyclase and cGMP Formation
Downstream of Cyclic Nucleotide Formation
References

CHAPTER 210
Cyclic Nucleotide Signaling in Trypanosomatids 539
Roya Zoraghi and Thomas Seebeck

Introduction
Cyclic Nucleotide Signaling, Cell Proliferation, and Differentiation
Individual Components of the Cyclic Nucleotide Signaling Pathways
Cyclic Nucleotides and Host Parasite Intervention
Concluding Remarks
References

CHAPTER 211
Cyclic Nucleotide Specificity and Cross-Activation of Cyclic Nucleotide Receptors 545
Clay E. S. Comstock and John B. Shabb

cAMP Cross-Activation of PKG
cGMP Cross-Activation of PKA
Molecular Basis for cAMP/cGMP Selectivity of PKA and PKG
Other Cyclic Nucleotide Receptors
References

CHAPTER 212
Cyclic Nucleotide Analogs as Tools to Investigate Cyclic Nucleotide Signaling 549
Anne Elisabeth Christensen and Stein Ove Døskeland

Introduction
Use of cNMP Analogs: Guidelines and Examples
Chemistry and Properties of Cyclic Nucleotide Analogs
Future Developments
References

Section G: G Proteins
Heidi Hamm, Editor

CHAPTER 213
Signal Transduction by G Proteins — Basic Principles, Molecular Diversity, and Structural Basis of Their Actions 557
Lutz Birnbaumer

Introduction
Ras, the Prototypic Regulatory GTPases
Heterotrimeric G Proteins
Mechanism of G-Protein Activation by Receptors and Modulation of Activity
References

CHAPTER 214
Genetic Analysis of Heterotrimeric G-Protein Function 571
Juergen A. Knoblich

Introduction
Signaling by Heterotrimeric G Proteins in Yeast
Heterotrimeric G-Protein Function in *Drosophila*
Conclusions
References

CHAPTER 215
Heterotrimeric G Protein Signaling at Atomic Resolution 575
David G. Lambright

Introduction
Architecture and Switching Mechanism of the G_α Subunits

Insight into the GTP Hydrolytic Mechanism from an Unexpected Transition State Mimic
$G_{\beta\gamma}$ with and without G_{α}
Phosducin and $G_{\alpha\gamma}$
$G_{s\alpha}$ and Adenylyl Cyclase
Filling in the GAP
Visual Fidelity
What Structures May Follow
References

CHAPTER 216
***In Vivo* Functions of Heterotrimeric G Proteins** **581**
Stefan Offermanns

Introduction
Development
Central Nervous System
Immune System
Heart
Sensory Systems
Hemostasis
Conclusions
References

CHAPTER 217
Regulation of G Proteins by Covalent Modification **585**
Jessica E. Smotrys and Maurine E. Linder

Introduction
N-Terminal Acylation of G_{α}
C-Terminal Modification of G_{γ}
Conclusions
References

CHAPTER 218
G-Protein-Coupled Receptors, Cell Transformation, and Signal Fidelity **589**
Hans Rosenfeldt, Maria Julia Marinissen, and J. Silvio Gutkind

Introduction
Heptahelical Receptors and Tumorigenesis
G-Protein Signaling in Cancer
A Matrix of MAPK Cassettes Links GPCRs to Biological Outcomes
G-Protein-Independent Signaling
GPCR Effectors Are Organized by Scaffolding Molecules
Conclusion: GPCR Biology Requires Both Signal Integration and Separation
References

CHAPTER 219
Signaling through G_z **601**
Jingwei Meng and Patrick J. Casey

General Properties
Receptors That Couple to G_z
Regulators of G_z Signaling: RGS Proteins
Effectors of G_z Signaling
$G\alpha_z$ Knockout Mice
Summary
References

CHAPTER 220
Effectors of $G_{\alpha 0}$ Signaling **605**
Prahlad T. Ram, J. Dedrick Jordan, and Ravi Iyengar

Introduction
Conclusions
References

CHAPTER 221
Phosphorylation of G Proteins **609**
Louis M. Luttrell and Deirdre K. Luttrell

Introduction
Serine Phosphorylation
Tyrosine Phosphorylation
Conclusions
References

CHAPTER 222
Mono-ADP-Ribosylation of Heterotrimeric G Proteins **613**
Maria Di Girolamo and Daniela Corda

Introduction
The Mono-ADP-Ribosylation Reaction
Bacterial Toxin-Induced ADP-Ribosylation
Endogenous Mono-ADP-Ribosylation
References

CHAPTER 223
Using Receptor-G-Protein Chimeras to Screen for Drugs **619**
Graeme Milligan, Richard J. Ward, Gui-Jie Feng, Juan J. Carrillo, and Alison J. McLean

Receptor-G-Protein Chimeras: An Introduction
Defining the Signal
Guanine Nucleotide Exchange Assays
Constitutive Activity and Inverse Agonism
Conclusions
References

CHAPTER 224
Specificity of G Protein $\beta\gamma$ Dimer Signaling **623**
Janet D. Robishaw, William F. Schwindinger, and Carl A. Hansen

Introduction
Diversity of the β and γ Gene Families
Assembly of the $\beta\gamma$ Dimer
Specificy of G Protein $\beta\gamma$ Dimer Signaling

Conclusion
References

CHAPTER 225
The RGS Protein Superfamily **631**
David P. Siderovski and T. Kendall Harden

Introduction
The Signature RGS-Box as a Gα GAP
Gα GAP and Other Signaling Regulatory Activities of RGS Family Members
References

CHAPTER 226
Mechanism of βγ Effector Interaction **639**
Tohru Kozasa

Introduction
Effectors Interacting with βγ Subunits
Specificity of the Interaction between βγ Subunit and Effectors
References

CHAPTER 227
βγ Signaling in Chemotaxis **645**
Carol L. Manahan and Peter N. Devreotes

Introduction
Evidence that G Proteins Are Involved in Chemotaxis
PI3Ks — Role in Chemotaxis?
Lipid Phosphatases, PTEN and SHIP
References

CHAPTER 228
Reversible Palmitoylation in G-Protein Signaling **651**
Philip Wedegaertner

Introduction
Sites of Palmitoylation in G_α and RGS Proteins
Activation-Regulated Palmitoylation of Gα
Mechanisms of Reversible Palmitoylation
Functions of Reversible Palmitoylation
Conclusion
References

CHAPTER 229
G Proteins Mediating Taste Transduction **657**
Sami Damak and Robert F. Margolskee

Introduction
α-Gustducin
α-Transducin
Other G Protein α Subunits
βγ Subunits
G-Protein-Coupled Receptors
Second Messenger Pathways
Conclusion
References

CHAPTER 230
Regulation of Synaptic Fusion by Heterotrimeric G Proteins **663**
Simon Alford and Trillium Blackmer

Introduction
The Vesicle Fusion Machinery
G Protein-Coupled Receptor Mediated Modulation at the Presynaptic Terminal
Possible Mechanisms of Presynaptic Inhibition by G Proteins
Presynaptic Ca^{2+} Stores and Modulation of Neurotransmitter Release
G Proteins and Phosphorylation
References

CHAPTER 231
G Protein Regulation of Channels **667**
Wiser Ofer and Lily Yeh Jan

Interaction with K^+ Channels
The Gβγ Interacting Domain of GIRK
Coupling of GIRK Activation to Specific Receptors
Calcium Channel Interaction with G Proteins
G Protein Interacting Domains
The Gβγ Interacting Domain of HVA $Ca^{2\pm}$ Channels
Modulation of Gβγ Inhibition
Voltage-Independent G-Protein-Mediated Inhibition of Calcium Channels
References

CHAPTER 232
Ras and Cancer **671**
Frank McCormick

Introduction: Ras Activation in Cancer
Pathways Downstream of Ras
Mouse Models of Cancer
Prospects for Cancer Therapy Based on Ras
References

CHAPTER 233
The Influence of Cellular Location on Ras Function **675**
Janice E. Buss, Michelle A. Booden, and John T. Stickney

Cytosolic Ras Is not Functional
After Modifications by Endomembrane Enzymes, Ras Proteins Move Toward the Cell Surface
Destination-Cell Surface: Ras Proteins Distribute Among Several Plasma Membrane Domains
Ras Proteins Finally Become Active at the Plasma Membrane
Endocytosis — A New Stage for Ras Signaling
Drugs that Affect Ras Membrane Binding
References

CHAPTER 234
Role of R-Ras in Cell Growth 681
Gretchen A. Murphy, Adrienne D. Cox, and Channing J. Der

Introduction
General Properties of R-Ras Proteins: Variations on Ras
R-Ras
TC21/R-Ras-2
M-Ras/R-Ras-3
Conclusions
References

CHAPTER 235
Molecular and Structural Organization of Rab GTPase Trafficking Networks 689
Christelle Alory and William E. Balch

Introduction
Rab Proteins are Recycling GTPases
Rab Proteins: An Evolutionary Conserved Family
Structural Organization of the Rab Proteins
Posttranslational Modification and Localization
Effector Molecules: REP/CHM, GEF, Effectors (Motors/Tethers/Fusogens), GAP, and GDI
Rab Dysfunction and Disease
Perpective
References

CHAPTER 236
Cellular Roles of the Ran GTPase 695
Jomon Joseph and Mary Dasso

Introduction
Introduction to the Ran Pathway
Structural Analysis of Ran Pathway Components
Ran's Role in Nuclear Transport
Ran's Function in Mitotic Progression
Ran's Function in Spindle Assembly
Ran's Role in Postmitotic Nuclear Assembly
Conclusions
References

CHAPTER 237
Rho Proteins and Their Effects on the Actin Cytoskeleton 701
Anja Schmidt and Alan Hall

Introduction
Effects of Rho GTPases on the Actin Cytoskeleton
Signaling from Rho GTPases to the Actin Cytoskeleton
Conclusions
References

CHAPTER 238
Regulation of the NADPH Oxidase by Rac GTPase 705
Becky A. Diebold and Gary M. Bokoch

Components and Regulation of the NADPH Oxidase
The Role of Rac in NADPH Oxidase Regulation
Current Models of Rac Function in NADPH Oxidase Regulation
Rac GTPase—A More General Role in Regulating Oxidant-Bases Signaling?
References

CHAPTER 239
The Role of Rac and Rho in Cell Cycle Progression 711
Laura J. Taylor and Dafna Bar-Sagi

Introduction
Regulation of G1 Progression
The Function of Rac and Rho in Cell Cycle Progression and Transformation
Cell Cycle Targets of Rac and Rho
Future Perspectives
References

CHAPTER 240
Cdc42 and Its Cellular Functions 715
Wannian Yang and Richard A. Cerione

Introduction
Biological Effects of Cdc42
Cell Adhesion and Migration
Cell Polarity
Molecular Mechanisms Underlying the Biological Activities of Cdc42
Conclusions
References

CHAPTER 241
Tissue Transglutaminase: A Unique GTP-Binding/GTPase 721
Richard A. Cerione

Introduction
TGase as a GTP-Binding/GTPase
New Links to Biological Function
Future Directions
References

CHAPTER 242
The Role of ARF in Vesicular Membrane Traffic 727
Melissa M. McKay and Richard A. Kahn

The ARF Family of Regulatory GTPases
ARF as a Regulator of Membrane Traffic
References

CHAPTER 243
Yeast Small G Protein Function: Molecular Basis of Cell Polarity in Yeast **733**
Hay-Oak Park and Keith G. Kozminski

Introduction
Conclusion
References

CHAPTER 244
Farnesyltransferase Inhibitors **737**
James J. Fiordalisi and Adrienne D. Cox

Introduction
Farnesylation and Protein Function
Ras—The Prototype of Farnesylated Proteins
Indentification and Development of FTIs
FTI Activity in Cell Culture and Animal Models
Alternative Prenylation in the Presence of FTIs
FTIs as Pharmacological Tools to Study Signaling and Biology
Targets of FTIs
Inhibition of Signaling by FTIs
Summary of Prospects
References

CHAPTER 245
Structure of Rho Family Targets **745**
Helen R. Mott and Darerca Owen

CRIB Proteins
Non-CRIB Rac Effectors
Rho Effectors
Concluding Remarks
References

CHAPTER 246
Structural Features of RhoGEFs **751**
Jason T. Snyder, Kent L. Rossman, David K. Worthylake, and John Sondek

Introduction
Structural Accomplishments
DH Domain Features
DH-Associated PH Domains
PH Domain Configurations
Mechanisms of Nucleotide Exchange
Molecular Recognition of Rho GTPase Substrates
External Regulation of the DH and PH Domains
References

CHAPTER 247
Structural Considerations of Small GTP-Binding Proteins **757**
Alfred Wittinghofer

Introduction
The G Domain Functional Unit
Guanine Nucleotide Exchange Factors
Effector B Via Switches and Others
Conclusions
References

CHAPTER 248
Conventional and Unconventional Aspects of Dynamin GTPases **763**
Sandra L. Schmid

Introduction
Common and Unique Features of Dynamin as a GTPase
Dynamin's Function in Endocytic Vesicle Formation
Dynamin's Siblings: The Dynamin Subfamily of GTPases
Dynamin as a Signaling Molecule
Conclusion and Perspectives
References

CHAPTER 249
Mx Proteins: High Molecular Weight GTPases with Antiviral Activity **771**
George Kochs, Othmar G. Engelhardt, and Otto Haller

Antiviral Activity of Mx GTPases
Mx Proteins Belong to the Superfamily of High Molecular Weight GTPases
Cellular Interaction Partners of Mx GTPases
References

Section H: Developmental Signaling
Geraldine Weinmaster, Editor

CHAPTER 250
Toll-Dorsal Signaling in Dorsal-Ventral Patterning and Innate Immunity **779**
Ananya Bhattacharya and Ruth Steward

The Toll-Dorsal Pathway
Maturation of the Toll Ligand
Toll Signaling Establishes the Embryonic Dorsal Gradient
Dorsal Regulates the Function of Zygotic Genes
The Intracellular Pathway Is Conserved in the *Drosophila* Immune Response
Nuclear Import of Rel Proteins
References

CHAPTER 251
Developmental Signaling: JNK Pathway in *Drosophila* Morphogenesis **783**
Beth E. Stronach and Norbert Perrimon

Introduction
The Paradigm of JNK Signaling: Dorsal Closure
Thorax Closure
Follicle Cell Morphogenesis
A New Paradigm: Planar Cell Polarity
Cellular Stress Response and Wound Healing

Perspectives
References

CHAPTER 252
Wnt Signaling in Development **789**
Christian Wehrle, Heiko Lickert, and Rolf Kemler

Introduction
Wnt Signaling in Invertebrate Development
Wnt Signaling in Vertebrate Development
Wnt/β-Catenin Target Genes
References

CHAPTER 253
Hedgehog Signaling and Embryonic Development **793**
Mark Merchant, Weilan Ye, and Frederic de Sauvage

The Hedgehog Proteins: Generation and Distribution
Transmitting the Hh Signal
Hh in Development and Disease
References

CHAPTER 254
Control of Left-Right (L/R) Determination in Vertebrates by the Hedgehog Signaling Pathway **799**
Javier Capdevila and Juan Carlos Izpisúa Belmonte

Introduction
The Discovery of the First Molecular Asymmetries in Vertebrate Embryos and the Role of SHH
The Role of a Composite HH Signal during L/R Determination in the Mouse
References

CHAPTER 255
EGF-Receptor Signaling in *Caenorhabditis elegans* Vulval Development **805**
Nadeem Moghal and Paul W. Sternberg

The Core LET-23 Signaling Pathway
Tissue Specificity
Positive and Negative Regulators
Prospects
References

CHAPTER 256
Induction and Lateral Specification Mediated by LIN-12/Notch Proteins **809**
Sophie Jarriault and Iva Greenwald

The LIN-12/Notch Pathway
Cell-Cell Interactions Mediated by the LIN-12/Notch Pathway
The Role of LIN-12/Notch Proteins: Suppression of Differentiation versus Specification of Binary Cell Fate Decisions
References

CHAPTER 257
Notch Signaling in Vertebrate Development **813**
Chris Kintner

Introduction
Components Mediating Vertebrate Notch Signaling
Notch Signaling in Vertebrate Development
Summary
References

CHAPTER 258
Reiterative and Concurrent Use of EGFR and Notch Signaling during *Drosophila* Eye Development **827**
Raghavendra Nagaraj and Utpal Banerjee

Introduction
Establishment of the Eye Primordium
Proliferation and D/V Patterning
Morphogenetic Furrow and R8 Specification
R-Cell Specification
Sequential Linkage between Notch and EGFR Pathways
Parallel Linkage between EGFR and Notch
Pigment Cell Differentiation and Apoptosis
Conclusion
References

CHAPTER 259
BMPs in Development **833**
Karen M. Lyons and Emmanuele Delot

Introduction
Gradients of BMP Activity
Establishing BMP Ligand Gradients
Extracellular Modifiers of BMP Activity
Interpreting the Gradient-Role of BMP Receptors
Differential Gene Activity in Response to BMP Signal Transduction
Intracellular Negative Regulation of BMP Signaling
Lessons from Loss-of-Function Studies in Mammals
Conclusions
References

CHAPTER 260
Neurotrophin Signaling in Development **839**
Albert H. Kim and Moses V. Chao

Introduction
The Neurotrophin Ligands
Neurotrophin Receptors
Signaling Specificity during Development
The Importance of Tetrograde Transport
Interacting Proteins
References

CHAPTER 261
PDGF Receptor Signaling in Mouse Development **845**
Richard A. Klinghoffer

Introduction
PDGTβR Signaling *In Vivo*
PDGFαR Signaling *In Vivo*
Specificity of PDGFR Signaling *In Vivo*
References

CHAPTER 262
VEGF and the Angiopoietins Activate Numerous Signaling Pathways that Govern Angiogenesis **849**
Christopher Daly and Jocelyn Holash

Introduction
Endothelial Cell Proliferation
VEGF Promotes Vascular Permeability
Ang-1 Inhibits Vascular Permeabilitly
Vessel Destabilization and EC Migration
Regulation of EC Survival during Angiogenesis
Conclusion
References

CHAPTER 263
Vascular Endothelial Growth Factors and their Receptors in Vasculogenesis, Angiogenesis, and Lymphangiogenesis **855**
Marja K. Lohela and Kari Alitalo

Vasculogenesis, Angiogenesis, and Lymphangiogenesis
The Vascular Endothelial Growth Factors and their Receptors
VEGF and VEGFR-1 and -2 are Essential for Vasculogenesis and Angiogenesis
Lymphangiogenesis is Regulated by VEGFR-3 and its Ligands VEGF-C and -D
Concluding Remarks
References

CHAPTER 264
Signaling from FGF Receptors in Development and Disease **861**
Monica Kong and Daniel J. Donoghue

Introduction
Expression of FGFR during Development
Role of FGFR in Development
Syndromes Associated with FGFRs
Signaling Pathways Mediated by FGFRs
Summary
References

CHAPTER 265
The Role of Receptor Protein Tyrosine Phosphatases in Axonal Pathfinding **867**
Andrew W. Stoker

Introduction
RPTPs and the Visual System
Neuromuscular System
Further Axon Growth and Guidance Roles
Axonal Signaling by RPTPs
References

CHAPTER 266
Attractive and Repulsive Signaling in Nerve Growth Cone Navigation **871**
Guo-li Ming and Mu-ming Poo

Introduction
Netrin Signaling
Semaphorin Signaling
Slit Signaling
Ephrin Signaling
Nogo and Myelin-Associated Clycoprotein Signaling
Critical Roles of Modulatory Signals
Concluding Remarks
References

CHAPTER 267
Semaphorins and their Receptors in Vertebrates and Invertebrates **877**
Eric F. Schmidt, Hideaki Togashi, and Stephen M. Strittmatter

The Semaphorin Family
Receptors for Semaphorins
Intracellular Signaling Pathways
Summary and Future Directions
References

CHAPTER 268
Signaling Pathways that Regulate Neuronal Specification in the Spinal Cord **883**
Ann E. Leonard and Samuel L. Pfaff

Patterning along the Dorsoventral Axis
Dorsal Spinal Cord Development
Ventral Spinal Cord Development
Rostrocaudal Specification
References

CHAPTER 269
Cadherins: Interactions and Regulation of Adhesivity **889**
Barbara Ranscht

Introduction
The Members of the Family
Multiple Modes for Regulating Cadherin Adhesive Activity
Conclusions and Perspectives
References

VOLUME 3

Contributors xlv

PART III
NUCLEAR AND CYTOPLASMIC EVENTS: TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL REGULATION
Michael Karin, Editor

PART III
Introduction
Michael Karin

Section A: Nuclear Receptors
Michael G. Rosenfeld, Editor

CHAPTER 270
History of Nuclear Receptors 7
Elwood V. Jensen

Introduction
Discovery of Receptors and Shift in Research Direction
Receptor Forms and Physiological Action
Subsequent Discoveries Relevant to Receptor Structure and Function
References

CHAPTER 271
Regulation of Basal Transcription by RNA Polymerase II 11
Sohail Malik and Robert G. Roeder

Introduction
The Preinitiation Complex
Global Mechanisms of PIC Function
Gene-Specific Regulation of PIC Function by Transcriptional Activators
Conclusion
References

CHAPTER 272
Structural Mechanisms of Ligand-Mediated Signaling by Nuclear Receptors 21
H. Eric Xu and Millard H. Lambert

Introduction
Overall Structure of the LBD
Ligand-Binding Pockets
Ligand-Mediated Activation: Mouse Trap versus Charged Clamp
Ligand-Mediated Repression
Dimerization
Summary
References

CHAPTER 273
Nuclear Receptor Coactivators 25
Riki Kurokawa and Christopher K. Glass

Introduction
Mechanism of Coactivator Recruitment
General Classes of Coactivator Complexes
Coactivators as Targets of Signal Transduction Pathways
Conclusion
References

CHAPTER 274
Corepressors in Mediating Repression by Nuclear Receptors 29
Kristen Jepsen and Michael G. Rosenfeld

Introduction
N-CoR and SMRT in Repression by Nuclear Receptors
Purification of Corepressor Complexes
Other Nuclear Receptor and Transcription Factor Partners of N-CoR/SMRT
Multiple Mechanisms of N-CoR/SMRT Regulation
Roles in Development and Disease
Other Mediators of Nuclear Receptor Repression
Conclusion
References

CHAPTER 275
Steroid Hormone Receptor Signaling 35
Vincent Giguère

Introduction
Activation by the Hormone
Hormone-Independent Activation
Cross-Talk with Other Transcription Factors
Nongenomic Action of Steroid Hormones
Estrogen Related Receptors
Selective Steroid Hormone Receptor Modulators
References

CHAPTER 276
PPARγ Signaling in Adipose Tissue Development 39
Robert Walczak and Peter Tontonoz

Introduction
PPARγ: A Dominant Regulator of Adipose Tissue Development
Analysis of PPARγ Function in Animal Models
Transcriptional Networks in Adipose Tissue Development
Negative Regulation of Adipocyte Differentiation
PPARγ, TNF-α Signaling Antagonism and Insulin Resistance
PPARγ and Cell Cycle Regulation
References

CHAPTER 277
Orphan Nuclear Receptors 47
Barry Marc Forman

Classical Receptors versus Orphan Receptors
Orphan Receptors and Metabolite-Derived Signals
Orphan Receptors and Xenobiotic Signals
Future Directions
References

CHAPTER 278
Identification of Ligands for Orphan Nuclear Receptors 53
Steven A. Kliewer and Timothy M. Willson

Introduction
PPARs: Fatty Acid Sensors
LXRs: Cholesterol Sensors
FXR: Bile Acid Sensor
PXR and CAR: Xenobiotic Sensors
Ligands for Other Orphan Nuclear Receptors
Conclusion
References

CHAPTER 279
Orphan Receptor COUP-TFII and Vascular Development 57
Fabrice G. Petit, Sophia Y. Tsai, and Ming-Jer Tsai

Introduction
Vascular Development
PPARγ: Inhibitor of Angiogenesis
COUP-TFII: Positive Effector in Angiogenesis
Conclusion
References

CHAPTER 280
Crosss-Talk between Nuclear Receptors and Other Transcription Factors 61
Peter Herrlich

Introduction
Proliferation and Proinflammatory Pathways
Nuclear Receptors
Induced Expression of Inhibitory Molecules
Immediate Hormone Responses
Direct Modulation of Transcription Factors
Conclusion
References

CHAPTER 281
***Drosophila* Nuclear Receptors** 69
Kirst King-Jones and Carl S. Thummel

Introduction
Nuclear Receptors and Embryonic Pattern Formation
Ecdysone Regulatory Hierarchies
The Neuronal Connection
References

Section B: Transcription Factors
Marc Montiminy, Editor

CHAPTER 282
JAK-STAT Signaling 77
Christian W. Schindler

Introduction
The JAK-STAT Paradigm
The JAK Family
The STAT Family
A Promising Future
References

CHAPTER 283
FOXO Transcription Factors: Key Targets of the PI3K-Akt Pathway That Regulate Cell Proliferation, Survival, and Organismal Aging 83
Anne Brunet, Hien Tran, and Michael E. Greenberg

Introduction
Identification of the FOXO Subfamily of Transcription Factors
Regulation of FOXO Transcription Factors by the PI3K-Akt Pathway
Other Regulatory Phosphorylation Sites in FOXOs
Mechanism of the Exclusion of FOXOs from the Nucleus in Response to Growth Factor Stimulation
Transcriptional Activator Properties of FOXOs
FOXOs and the Regulation of Apoptosis
FOXOs Are Key Regulators of Several Phases of the Cell Cycle
FOXOs in Cancer Development: Potential Tumor Suppressors
Role of FOXOs in the Response to Stress and Organismal Aging
FOXOs and the Regulation of Metabolism in Relation to Organismal Aging
Conclusion
References

CHAPTER 284
Multiple Signaling Routes to Histone Phosphorylation 91
Claudia Crosio and Paolo Sassone-Corsi

Introduction
Histone Phosphorylation and Gene Activation
Histone Phosphorylation and DNA Repair
Histone Phosphorylation and Apoptosis
Histone Phosphorylation and Mitosis

Conclusions
References

CHAPTER 285
Multigene Family of Transcription Factor AP-1 **99**
Peter Angel

Introduction
General Structure of AP-1 Subunits
Transcriptional and Posttranslational Control of AP-1 Activity
Function of Mammalian AP-1 Subunits: Lessons from Loss-of-Function Approaches in Mice
References

CHAPTER 286
NFκB: A Key Integrator of Cell Signaling **107**
John K. Westwick, Klaus Schwamborn, and Frank Mercurio

References

CHAPTER 287
Transcriptional Regulation via the cAMP Responsive Activator CREB **115**
Marc Montminy and Keyong Du

The Transcriptional Response to cAMP
Mechanism of Transcriptional Activation via CREB
Signal Discrimination via CREB
Secondary Phosphorylation of CREB: Ser142
Methylation of the KIX Domain
Cooperative Binding with MLL
References

CHAPTER 288
The NFAT Family: Structure, Regulation, and Biological Functions **119**
Fernando Macian and Anjana Rao

Introduction
Structure and DNA-Binding
Regulation
Transcriptional Functions
Biological Programs Regulated by NFAT
Perspectives
References

CHAPTER 289
Transcriptional Control through Regulated Nuclear Transport **125**
Steffan N. Ho

Introduction
Regulated Nuclear Transport: Overview
Coordinate Regulation of Nuclear Import and Export: Calcium-Dependent Nuclear Localization of NFATc Transcription Factors
Regulated Nuclear Transport of Non-DNA-Binding Transcriptional Regulatory Proteins
Conclusion
References

CHAPTER 290
Proteasome/Ubiquitination **129**
Daniel Kornitzer and Aaron Ciechanover

Protein Degradation and the Ubiquitin/Proteasome System
Regulation of Ubiquitination by Substrate Modification
Regulation of Ubiquitin Ligase Activity
Protein Processing by the Ubiquitin System
Modulation of Kinase Activity by Ubiquitination
Conclusion
References

CHAPTER 291
Fluorescence Resonance Energy Transfer Microscopy and Nuclear Signaling **135**
Ty C. Voss and Richard N. Day

Introduction
References

CHAPTER 292
The Mammalian Circadian Timing System **139**
Ueli Schibler, Steven A. Brown, and Jürgen A. Ripperger

Introduction
The Molecular Oscillator
Photic Entrainment of the Central Pacemaker
Outputs of the SCN Pacemaker
Outputs via Subsidiary Clocks
Conclusions and Perspectives
References

CHAPTER 293
Protein Arginine Methylation **145**
Michael David

Introduction
Arginine Methylation and Arginine-Methyltransferases
Function of Arginine Methylation
Role of Arginine Methylation in Signal Transduction
References

CHAPTER 294
Transcriptional Activity of Notch and CSL Proteins **149**
Elise Lamar and Chris Kintner

Introduction
Components of the Notch Transcriptional Complex

Notch Transcriptional Activity *In Vivo*
Conclusion
References

CHAPTER 295
The β-Catenin: LEF/TCF Signaling Complex: Bigger and Busier than Before 161
Reiko Landry and Katherine A. Jones

Introduction
Regulated Proteolytic Turnover of β-Cat
Regulation of the Wnt-Assembled Enhancer Complex in the Nucleus
Enter Pygopus and Legless (hBcl9)
Perspectives
References

CHAPTER 296
Cubitus Interruptus 167
Sarah M. Smolik and Robert A. Holmgren

Introduction
Protein Structure and Expression Patterns of Ci
Regulation of Ci by Hedgehog
Regulation of Ci by PKA
Ci Transcriptional Regulation
References

CHAPTER 297
The Smads 171
Malcolm Whitman

Introduction
Families: R-Smads, Co-Smads, and I-Smads
Smad Oligomerization and Regulation by Receptors
Transcriptional Regulation by Smads
Down-Regulation and Cross-Regulation of Smads
Function *In Vivo*: Gain of Function Loss of Function
References

Section C: Damage/Stress Responses
Albert J. Fornace, Jr., Editor

CHAPTER 298
Complexity of Stress Signaling and Responses 179
Sally A. Amundson and Albert J. Fornace, Jr.

Introduction: A Variety of Stresses
Origin of Signals
Signal Transduction
Functional Genomics and Proteomics Approaches
References

CHAPTER 299
Signal Transduction in the *Escherichia coli* SOS Response 185
Penny J. Beuning and Graham C. Walker

SOS Response
LexA Cleavage and Other Self-Cleavage Reactions Regulating the SOS Response
Structures of Y-Family Polymerases
Conclusions
References

CHAPTER 300
Oxidative Stress and Free Radical Signal Transduction 191
Bruce Demple

Introduction: Redox Biology
Oxidative Stress Responses in Bacteria: Well-Defined Models of Redox Signal Transduction
Responses to Superoxide Stress and Nitric Oxide: SoxR Protein
Response to H_2O_2 and Nitrosothiols: OxyR Protein
Parallels in Redox and Free-Radical Sensing
Themes in Redox Sensing
References

CHAPTER 301
Budding Yeast DNA Damage Checkpoint: A Signal Transduction-Mediated Surveillance System 197
Marco Muzi-Falconi, Michel Giannattasio, Giordano Liberi, Achille Pelliccioli, Paolo Plevani, and Marco Foiani

Introduction
Sensing
Downstream Events
References

CHAPTER 302
Finding Genes That Affect Signaling and Toleration of DNA Damage, Especially DNA Double-Strand Breaks 203
Craig B. Bennet and Michael A. Resnick

Introduction
Nature of DSB and Repair and Genetic Consequences
Checkpoint Activation and Adaptation as Signaling Responses to DSBs
DNA Damage Signaling Networks
Identifying Checkpoint Defects by Screening Radiation-Sensitive Mutants
Checkpoint Mutants Revealed through Screening DNA Replication Mutants
Screening for Checkpoint Defects
Screen for Altered Checkpoint and Adaptation Responses to a Single DSB
Other Screens for DNA Damage Checkpoint Pathway Genes
Implications of DNA Damage Checkpoint Signaling
References

CHAPTER 303
Radiation Responses in *Drosophila* **213**
Naoko Sogame and John M. Abrams

Introduction
Sensors and Transmitters
Effectors
Conclusions: What Can We Learn from the *Drosophila* Model?
References

CHAPTER 304
Double-Strand Break Recognition and Its Repair by Nonhomologous End Joining **219**
Jane M. Bradbury and Stephen P. Jackson

Introduction
Repair of DSBs: Homologous Recombination and NHEJ
Recognition of DNA DSBs
Signal Transduction
DNA Repair
Other Sensors and Transducers of DNA Damage
New Factors in NHEJ
Future Prospects
References

CHAPTER 305
Role of ATM in Radiation Signal Transduction **225**
Martin F. Lavin, Shaun Scott, Philip Chen, Sergei Kozlov, Nuri Gueven, and Geoff Birrell

Introduction
Sensing Radiation Damage in DNA
ATM Signaling: Recognition of Breaks in DNA
Checkpoint Activation
Role of ATM in More General Signaling
Perspective
References

CHAPTER 306
Signaling to the p53 Tumor Suppressor through Pathways Activated by Genotoxic and Nongenotoxic Stresses **237**
Carl W. Anderson and Ettore Appella

Introduction
p53 Protein Structure
Posttransitional Modifications to p53
Regulation of p53 Activity
Activation of p53 by Genotoxic Stresses
Activation of p53 by Nongenotoxic Stresses
Conclusions
References

CHAPTER 307
Abl in Cell Signaling **249**
Jean Y. J. Wang

Introduction
Functional Domains of Abl
Proteins that Interact with Abl
Abl in Signal Transduction
Future Prospects
References

CHAPTER 308
Radiation-Induced Cytoplasmic Signaling **257**
Christine Blattner and Peter Herrlich

Introduction
Cytoplasmic Signaling Network
Redox Sensitivity and Metal Toxicity: Toxic Agents Activate Signaling Pathways
Activation of Signaling Components
Primary Radiation Targets: DNA Damage versus Cytoplasmic Signaling
Other Signaling-Initiating Principles
Conclusions
References

CHAPTER 309
Endoplasmic Reticulum Stress Responses **263**
David Ron

Introduction
ER Stress Defined
The UPR in Yeast
The UPR Is Metazoans
Conclusions
References

CHAPTER 310
The Heat-Shock Response: Sensing the Stress of Misfolded Proteins **269**
Richard I. Morimoto and Ellen A. A. Nollen

Introduction
Transcriptional Regulation of the Heat-Shock Response
Molecular Chaperones: Folding, Misfolding, and the Assembly of Regulatory Complexes
Neurodegenerative Diseases: When Aggregation-Prone Proteins Go Awry
References

CHAPTER 311
Hypoxia-Mediated Signaling Pathways **277**
Albert C. Koong and Amato J. Giaccia

Introduction
HIF-1 Signaling

Unfolded Protein Response
Conclusions
References

CHAPTER 312
Regulation of mRNA Turnover by Cellular Stress **283**
Myriam Gorospe

Introduction
mRNA Stability
Stress-Activated Signaling Molecules that Regulate mRNA Turnover
Conclusions
References

Section D: Post-Translational Control
Nahum Sonenberg, Editor

CHAPTER 313
RNA Localization and Signal Transduction **293**
Vaughan Latham and Robert H. Singer

Introduction
Growth Factors Induce mRNA Localization
Signaling from the Extracellular Matrix Induces mRNA Localization
mRNAs Localized via the Cytoskeleton
mRNA Granule Movement in Neurons
Regulation of mRNA Localizing Proteins
GTPase Signals Regulating Actomyosin Interactions Are Involved in mRNA Localization
Conclusion
References

CHAPTER 314
Translational Control by Amino Acids and Energy **299**
Tobia Schmelze, José L. Crespo, and Michael N. Hall

Introduction
GCN System
TOR Signaling Pathway
References

CHAPTER 315
Translational Control and Insulin Signaling **305**
Thomas Radimerski and George Thomas

References

CHAPTER 316
Unfolded Protein Response: An Intracellular Signaling Pathway Activated by the Accumulation of Unfolded Proteins in the Lumen of the Endoplasmic Reticulum **311**
Randal J. Kaufman

Introduction
UPR in *Saccharomyces cerevisiae*
UPR Transcriptional Activation in Metazoan Species
Physiological Role for the UPR in Mammals
Future Directions
References

CHAPTER 317
Regulation of mRNA Turnover **319**
Perry J. Blackshear and Wi S. Lai

Introduction
Current Models of mRNA Stability in Vertebrate Cells
Presence of Instability Elements in Vertebrate mRNAs
Effects of ARE Binding Proteins on mRNA Turnover
Regulation of TTP Activity in Cells
Conclusion
References

CHAPTER 318
CPEB-Mediated Translation in Early Vertebrate Development **323**
Joel D. Richter

Introduction
Mechanism of Translational Control
CPEB and Early Development
Conclusions
References

CHAPTER 319
Translational Control in Invertebrate Development **327**
Paul Lasko

Introduction
Translational Control Targets Oskar to the Pole Plasm
Translational Control Targets Nanos to the Pole Plasm
Translational Control in the *Drosophila* Nervous System
Role for Translational Control in Regulation Growth
Translational Repression through MicroRNAs
References

CHAPTER 320
Role of Alternative Splicing During the Cell Cycle and Programmed Cell Death 331
Chanseok Shin and James L. Manley

Introduction
Apoptosis and Splicing
Cell Cycle and Splicing Regulation
References

CHAPTER 321
NF90 Family of Double-Stranded RNA-Binding Proteins: Regulators of Viral and Cellular Function 335
Trevor W. Reichman and Michael B. Mathews

Summary
Introduction
Members of the NF90 Protein Family
Domain Structure of NF90 Family Proteins
Proteins that Interact with NF90
Nucleic Acid Binding Properties of NF90
Functions of NF90 Homologs
Cellular Regulation of NF90 and NF45
Conclusions
References

CHAPTER 322
Signaling Pathways that Mediate Translational Control of Ribosome Recruitment of mRNA 343
Nahum Sonenberg and Emmanuel Petroulakis

Introduction
eIF4F Complex Formation
Repressors of Cap-Dependent Translation
Modulation of 4E-BP Phosphorylation FRAP/mTOR
Phosphorylation of eIF4G and eIF4B
Control of Cell Growth and Proliferation by eIF4E: Link to Cancer
Conclusions
References

PART IV
EVENTS IN INTRACELLULAR COMPARTMENTS
Marilyn Farquhar, Editor

CHAPTER 323
SREBPs: Gene Regulation through Controlled Protein Trafficking 353
Peter J. Espenshade, Joseph L. Goldstein, and Michael S. Brown

Introduction
SREBPs: Membrane-Bound Transcription Factors
SCAP: Sterol Sensor and Escorter of SREBP from ER to Golgi
Sterols Control Sorting of SCAP/SREBP into ER Vesicles
ER Retention of SCAP/SREBP
Conclusions
References

CHAPTER 324
Endoplasmic Reticulum Stress Responses 359
David Ron

Introduction
Conclusion
References

CHAPTER 325
Signaling Pathways from Mitochondria to the Nucleus 365
Zhengchang Liu and Ronald A. Butow

Introduction
Milestones in Mitochondrial Research
Mitochondrial Signaling
Aging and Retrograde Regulation
Conclusions
References

CHAPTER 326
Signaling During Exocytosis 375
Lee E. Eiden

Introduction
Functional, Morphological, and Historical Aspects of Exocytosis and Stimulus-Secretion Coupling
Secretion Begins with Secretagogues
Secretagogues Act at Target Cell Receptors
Calcium and Cyclic AMP: The Two Main Second Messengers for Secretion
Calcium and the Regulation of Exocytosis
Exocytosis and SNAREs
Calcium and cAMP Sensors for Exocytosis
Role of Signal Summation in Regulated Exocytosis
Role of PKC and Other PMA Targets in Regulated Secretion
Negative Regulation of Secretion
Upstream Regulation of Secretion
Far Upstream Regulation of Secretion
Conclusions and Future Outlook for Signaling in Exocytosis
References

CHAPTER 327
Nonclassical Pathways of Protein Export **393**
Igor Prudovsky, Anna Mandinova, Cinzia Bagala, Raffaella Soldi, Stephen Bellum, Chiara Battelli, Irene Graziani, and Thomas Maciag

Introduction
Fibroblast Growth Factor Export Pathways
The Export of FGF-1 as a Multiprotein Complex
Interleukin-1 Export Pathways
Acidic Phospholipids and the Molten Globule Hypothesis
The Potential Pathophysiological Implication of Nonclassical Release
References

CHAPTER 328
Regulation of Cell Cycle Progression **401**
Clare H. McGowan

Introduction
Being There: Cyclins Define Cell Cycle Phase
Signals to Slow Processes: Regulation of Cdks by Inhibitory Proteins
Cdks Are Positively and Negatively Regulated by Phosphorylation
Degradation: The Importance of Being Absent
Location, Location, Location
Checkpoint Signaling
References

CHAPTER 329
Endocytosis and Cytoskeleton **411**
Pier Paolo Di Fiore and Giorgio Scita

Introduction
Actin Dynamics and Endocytosis
Role of Microtubule Cytoskeleton in Receptor Endocytosis
Physical and Functional Interactions of Dynamin and Dynamin-Interacting Proteins with the Actin Cytoskeleton
Integration of Signals in Endocytosis and Actin Dynamics by Small GTPases
Conclusions
References

CHAPTER 330
Molecular Basis for Nucleocytoplasmic Transport **419**
Gino Cingolani and Larry Gerace

Introduction
Transport Signals
Transport Receptors
The Small GTPase Ran
Nuclear Pore Complex
Mechanism of Transport
Future Directions
References

CHAPTER 331
Apoptosis Signaling: A Means to an End **431**
Lisa J. Pagliari, Michael J. Pinkoski, and Douglas R. Green

Introduction
The End of the Road
Caspase-8 Activation via Death Receptors
Mitochondria and the Activation of Caspase-9
Mitochondrial Outer Membrane Permeabilization
The Bcl-2 Family
Cell Cycle versus Apoptosis
Conclusions
References

CHAPTER 332
Signaling Down the Endocytic Pathway **441**
Jeffrey L. Benovic and James H. Keen

Introduction
RTK Signaling from the Cell Surface
RTK Signaling from Endocytic Compartments
GPCR Signaling Paradigms and Desensitization
Control of RTK and GPCR Trafficking Leading to Degradation
GPCR Activation of MAP Kinases
Endocytic Signaling in Developmental Systems
Signaling between Neuronal Cell Body and Terminal
References

PART V
CELL-CELL AND CELL-MATRIX INTERACTIONS
E. Brad Thompson, Editor

PART V
Introduction
Brad Thompson

CHAPTER 333
Overview of Cell-Cell and Cell-Matrix Interactions **452**
E. Brad Thompson and Ralph A. Bradshaw

References

CHAPTER 334
Angiogenesis: Cellular and Molecular Aspects of Postnatal Vessel Formation **455**
Carla Mouta, Lucy Liaw, and Thomas Maciag

Introduction
Initiators of Angiogenesis: Cellular, Metabolic, and Mechanical
Vessel-Specific Requirements in Angiogenesis
Cellular and Soluble Regulators

Coordination of Angiogenesis by Cellular and Molecular Interactions
References

CHAPTER 335
Signaling Pathways Involved in Cardiogenesis 463
Deepak Srivastava

Introduction
Cardiomyocyte and Heart Tube Formation
Cardiac Looping and Left-Right Asymmetry
Patterning of the Developing Heart Tube
Myocardial Growth
Cardiac Valve Formation
Cardiac Outflow Tract and Aortic Arch Development
Conclusions
References

CHAPTER 336
Development and Regulatory Signaling in the Pancreas 471
Murray Korc

Introduction
Ontogeny of the Pancreas
Pancreatic Islet-Acinar Interactions
Cell-Cell and Matrix Interactions in the Endocrine Pancreas
Matrix and Cell-Cell Interactions in the Exocrine Pancreas
Conclusions
References

CHAPTER 337
Tropic Effects of Gut Hormones in the Gastrointestinal Tract 477
B. Mark Evers and Robert P. Thomas

Introduction
Tropic Effects of Gut Peptides in the Stomach, Small Bowel, and Colon
GI Hormone Receptors and Signal Transduction Pathways
Signaling Pathways Mediating the Effects of Intestinal Peptides
Conclusions
References

CHAPTER 338
Integrated Response to Neurotrophic Factors 485
J. Regino Perez-Polo

Introduction
Neural Cell Death
The Neurotrophic Hypothesis
Neurotrophins
Neurotrophin Receptors
Neurotrophin Signaling Pathways
Transcriptional Regulation
AP-1
NFκB Transcription Factor
Role of NFκB
Conclusions
References

CHAPTER 339
Cell-Cell and Cell-Matrix Interactions in Bone 497
L. F. Bonewald

Introduction
Diseases of Bone
Bone Cells and Their Functions
Mechanical Strain
Hormone Responsible for Bone Development, Growth, and Maintenance
Growth and Transcription Factors Responsible for Bone Development and Growth
Fibroblast Growth Factors
Bone Extracellular Matrix
Conclusions
References

CHAPTER 340
Cell-to-Cell Interactions in Lung 509
Joseph L. Alcorn

Introduction
Lung Organogenesis and Development
Soluble Factors of Cell-to-Cell Interactions Involved in Lung Injury
Conclusion
References

CHAPTER 341
Mechanisms of Stress Response Signaling and Recovery in the Liver of Young versus Aged Mice: The p38 MAPK and SOCS Families of Regulatory Proteins 515
John Papaconstantinou

Introduction
The p38 MAPK Pathway in Stress Response Signaling
SOCS Family of Negative Regulators of Inflammatory Response
Conclusions
References

CHAPTER 342
Cell-Cell Signaling in the Testis and Ovary 531
Michael K. Skinner

Introduction
Cell-Cell Signaling in the Testis
Cell-Cell Signaling in the Ovary

Conclusions
References

CHAPTER 343
T Lymphocytes **546**
Rolf König and Wenhong Zhou

Introduction
Signaling Receptors in T Cells form Dynamic Macromolecular Signaling Complexes
Coreceptor and Costimulatory Proteins Modulate T-Cell Signaling Pathways
Intracellular Signaling Pathways Induced by Antigen Stimulation of T Cells
Conclusions
References

CHAPTER 344
Signal Transduction via the B-Cell Antigen Receptor: A Crucial Regulator of B-Cell Biology **555**
Louis B. Justement

Introduction
Initiation of Signal Transduction through the BCR
Propagation of Signal Transduction via the BCR
Conclusions
References

CHAPTER 345
Signaling Pathways in the Normal and Neoplastic Breast **565**
Danica Ramljak and Robert B. Dickson

Introduction
Signaling Molecules: A Class of Growth Factors
PI3K/Akt, MEK/Erk, and Stats: Major Proliferation/Survival Molecules Downstream of Growth Factor Receptors in Breast
Conclusions and Future Prospects
References

CHAPTER 346
Kidney **573**
Elsa Bello-Reuss and William J. Arendshorst

Overview of Kidney Functions and Cell-to-Cell Interactions
Vascular Endothelial Cells
Vascular Smooth Muscle Cells
Tubulovascular Interactions: The Juxtaglomerular Apparatus
Tubulovascular Interactions: The Juxtaglomerular Apparatus and Tubuloglomerular Feedback
Vasculotubular Communication
Tubule-Tubule Communication: Paracrine Agents Released from Epithelial Cells
Interstitial Cell-Tubule Communication
Conclusions
References

CHAPTER 347
Prostate **591**
Jean Closset and Eric Reiter

Introduction
Development of the Prostate during Fetal Life
The Adult Prostate
The Prostate during Aging
Conclusions
References

CHAPTER 348
Retrograde Signaling in the Nervous System: Dorsal Root Reflexes **607**
William D. Willis

Cell-to-Cell Signaling in the Nervous System
Retrograde Signaling
Neurogenic Inflammation
Dorsal Root Reflexes as Retrograde Signals
Conclusions
References

CHAPTER 349
Cytokines and Cytokine Receptors Regulating Cell Survival, Proliferation, and Differentiation in Hematopoiesis **615**
Fiona J. Pixley and E. Richard Stanley

General Aspects of Hematopoiesis
Signaling through Cytokine Receptors
Conclusions
References

CHAPTER 350
Regulation of Bartlett Endogenous Stem Cells in the Adult Mammalian Brain: Promoting Neuronal Repair **625**
Rodney L. Rietze and Perry F. Bartlett

Adult Neurogenesis Revealed
Isolation and Culture of Neural Stem Cells
Regulation of Stem Cell Differentiation into Neuron
References

Index

CHAPTER 1

Cell Signaling: Yesterday, Today, and Tomorrow

Ralph A. Bradshaw and Edward A. Dennis

Cell signaling, which is also often referred to as signal transduction or transmembrane signaling, is the process by which cells communicate with their environment and respond temporally to external cues that they sense there. All cells have the capacity to achieve this to some degree, albeit with a wide variation in purpose, mechanism, and response. At the same time, there is a remarkable degree of similarity over quite a range of species, particularly in the eukaryotic kingdom, and comparative physiology has been a useful tool in the development of this field. The central importance of this general phenomenon (sensing of external stimuli by cells) has been appreciated for a long time, but it has truly become a dominant part of cell and molecular biology research in the past ten years, in part because a description of the dynamic responses of cells to external stimuli is in essence a description of the life process itself. This approach lies at the core of the new field of proteomics, and its importance to human and animal health is already plainly evident. Here, we briefly consider the origins of cell signaling, broadly summarize the current state of the art, and speculate on future directions with an eye toward what questions must be answered and which ones likely will be answered in the near future.

Origins of Cell Signaling

Although cells from polycellular organisms derive substantial information from interactions with other cells and extracellular structural components, it was humoral components that first were appreciated to be intracellular messengers. This idea was certainly inherent in the "internal secretions" initially described by Claude Bernard in 1855 and thereafter, as it became understood that ductless glands, such as the spleen, thyroid, and adrenals, secreted material into the bloodstream. However, Bernard did not directly identify hormones as such. This was left to Bayliss and Starling and their description of secretin in 1902 [1].

Recognizing that it was likely representative of a larger group of chemical messengers, the term *hormone* was introduced by Starling in a Croonian Lecture presented in 1905. The word, derived from the Greek word meaning "I excite or arouse," was apparently proposed by a colleague, W. B. Hardy, and was adopted, even though it did not particularly connote the messenger role but rather emphasized the positive effects exerted on target organs via cell signaling (see Wright [2] for a general description of these events). The realization that these substances could also produce inhibitory effects, gave rise to a second designation, "chalones", introduced by Schaefer in 1913 (see Schaefer [3]), for the inhibitory elements of these glandular secretions. The word *autocoid* was similarly coined for the group as a whole (hormones and chalones). Although the designation chalone is occasionally applied to some growth factors with respect to certain of their activities (e.g., transforming growth factor β), autocoid has essentially disappeared. Thus, if the description of secretin and the introduction of the term *hormone* are taken to mark the beginnings of molecular endocrinology and the eventual development of cell signaling, then we are at or near the 100th anniversary of this field.

The origins of endocrinology, as the study of the glands that elaborate hormones and the effect of these entities on target cells, naturally gave rise to a definition of hormones as substances produced in one tissue type that traveled systemically to another tissue type to exert a characteristic response. Of course, initially these responses were couched in organ and whole animal responses, although they increasingly

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were defined in terms of metabolic and other chemical changes at the cellular level. The early days of endocrinology were marked by many important discoveries, such as the discovery of insulin [4], to name one, that solidified the definition, and a well-established list of hormones, composed primarily of three chemical classes (polypeptides, steroids, and amino acid derivatives), was eventually developed. Of course, it was appreciated even early on that the responses in the different targets were not the same, particularly with respect to time. For example, adrenalin was known to act very rapidly while growth hormone required much longer time frames to exert its full range of effects. However, in the absence of any molecular details of mechanism, the emphasis remained on the distinct nature of the cells of origin versus those responding and on the systemic nature of transport, and this remained the case well into the 1970s. An important shift in endocrinological thinking had its seeds well before that, however, even though it took about 25 years for these "new" ideas that greatly expanded endocrinology to be enunciated clearly.

Enter Polypeptide Growth Factors

Although the discovery of polypeptide growth factors as a new group of biological regulators is generally associated with nerve growth factor (NGF), it can certainly be argued that other members of this broad category were known before NGF. However, NGF was the source of the name *growth factor* and has been in many important respects a Rosetta stone for establishing many of the principles that are now known to underpin much of signal transduction. Thus, its role as the progenitor of the field and the entity that keyed the expansion of endocrinology, and with it the field of cell signaling, is quite appropriate. There are numerous accounts of the discovery of NGF [5–8] and how this led directly to identification of epidermal growth factor (EGF), another regulator that has been equally important in providing novel insights into molecular endocrinology and signal transduction. However, it was not till the sequences of NGF and EGF were determined [9,10] that the molecular phase of growth factor research truly began. Of particular importance was the suggestion that NGF and insulin were related entities [11], which suggested a similar molecular action (which, indeed, turned out to be remarkably clairvoyant) and was the first indication that the identified growth factors, which at that time were quite limited in number, were hormonal like. This hypothesis led quickly to the identification of receptors for NGF on target neurons, using the tracer binding technology of the time (see Raffioni *et al.* [12] for a summary of these contributions), which further confirmed their hormonal status. Over the next several years, similar observations were recorded for a number of other growth factors that in turn led to the redefinition of endocrine mechanisms to include paracrine and autocrine interactions (see Section V Introduction – Bradshaw/Thompson).

Cell Signaling at the Molecular Level

At the same time that the growth factor field was undergoing rapid development, major advances were also occurring in studies on hormonal mechanisms. In particular, Sutherland and colleagues [13] were redefining hormones as messengers and their ability to produce second messengers. This was, of course, based primarily on the identification of cyclic AMP (cAMP) and its production by a number of classical hormones. However, it also became clear that not all hormones produce this second messenger nor was it stimulated by any of the growth factors known at that time. This enigma remained unresolved for quite a long time until tyrosine kinases were identified, and it was shown, first with the EGF receptor [14], that these entities were responsible for the signal transduction for many of those hormones and growth factors that did not stimulate the production of cAMP. Aided by the tools of molecular biology, it was a fairly rapid transition to the cloning of the receptors (for all hormones and growth factors) and the subsequent development of the main classes of signaling mechanisms. These include, in addition to the receptor tyrosine kinases described above, the G-protein receptors (including the receptors that produce cAMP and probably constituting the largest class of cell surface receptors); cytokine receptors, which recruit soluble tyrosine kinases; serine/threonine receptors; the tumor necrosis factor (TNF) (TRAF) receptors that activate nuclear factor kappa B (NFκB), among other pathways; guanyl cyclase receptors and nuclear receptors utilized by steroids; and other signaling entities. Structural biology has not maintained the same pace and there are still both ligands and receptors for which we do not have full three-dimensional information as yet, but this gap is rapidly closing and it may be anticipated that the full catalog of these structures will soon be available. Of particular importance will be the anticipated first structure of a full-length transmembrane receptor with a regular ligand bound. That would certainly provide important insights into signal transmission that currently are lacking.

In parallel with the development of our understanding of ligand/receptor organization, structure, and general mechanism, an equally important advance has occurred in the appreciation of the intracellular events that these various receptor classes initiate. Indeed, the very substantial repertoire of molecules includes effectors (kinases of various types, phospholipases, etc.), scaffolds, adaptors, and regulatory proteins (see Section B and multiple entries therein). Many are quite common and are activated by several types of receptors in a variety of cell types while others are quite narrow in specificity and distribution. That different receptors can cross-activate other receptors and/or signaling systems adds considerably to the complexity of cellular responses and in turn leads to an even broader array of cellular and organ responses. Indeed, today's endocrinology is a far cry from the simple pathways envisioned by the early physiologists who define this field a century ago. They would be

amazed indeed to read this Handbook and see where their early seminal observations have gone.

Lipid Signaling

The elucidation of cell signaling mechanisms and the variety of molecules that are employed in these myriad of processes is particularly well exemplified by the lipid messengers. Except for the above mentioned steroid hormones, lipids have long been thought to function mainly in energy metabolism and membrane structure. This last decade has culminated in the broad recognition that membrane phospholipids provide many of the important cell signaling molecules via phospholipases and lipid kinases. Key is the role of phospholipase C in hydrolyzing phosphatidylinositol bisphosphate (PIP2) to release diglyceride that activates protein kinase C (PKC) and inositol triphosphate (IP3), which mobilizes intracellular Ca^{2+}, central to so many regulatory processes (see Section II X-Berridge). The phosphorylation of PIP2 at the 3-position to produce PIP3 promotes vesicular trafficking and other cellular processes. Phospholipase D releases phosphatidic acid, and phospholipase A2 provides arachidonic acid, which is converted into prostaglandins, leukotrienes, and lipoxins; these ligands in turn bind to unique families of receptors as does platelet activating factor (PAF). The more recent recognition of the importance of sphingolipids and ceramide in signaling and the discoveries of the unique lysophosphatidic acid and sphingosine phosphate families of receptors has sparked the search for other new receptors for lipids. It is clear that the search for new lipid second messengers and their receptors and functions will continue unabated into the future.

Cell Signaling Tomorrow

As the humane genome and importantly the genomes of several other key research paradigms reach completion in terms of sequence, interpretation, and full annotation, it will be possible to know, in a general sense, the complete complement of proteins, involved in cell signaling. Of course, this "signaling proteome" will contain a vast number of variants arising from message splicing and posttranslational modification. Appreciating how all of these variants interact as a function of time in response to stimulation will be a mammoth if not an infinite task. But, this level of knowledge will not be required to make considerable advances over what we know at present. Indeed, we can expect that "expression proteomics," which some really define as "systems biology," will provide much insight in the coming years, particularly through the clever applications of advances in separations methodology, mass spectrometry, and hybridization assays. Both protein and nucleic acid arrays have already demonstrated their worth, and much more information will be obtained from these powerful techniques. Of utmost importance will be the application of quantification to all types of measurements so that these data can eventually be accurately modeled to produce a true picture of signal fluxes through cells as they undergo their transcriptional, phenotypic, and ultimately cell and organ responses. Although one cannot accurately predict over the next ten years what discoveries will be made, other than that there will be many and some of them will be quite unexpected, it seems certain that cell signaling will remain one of the primary areas of expanding biological research. It also seems safe to predict that many singularly important findings in terms of human and animal health will be made and that society, at all levels, will be the better for these efforts.

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PART I

Initiation: Extracellular and Membrane Events

James A. Wells, Editor

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PART I

Introduction

James A. Wells

Cells within multicellular organisms communicate via extracellular mediators: either through diffusible molecules or by direct cell–cell contact. These extracellular signals are interpreted for the most part by specific membrane receptors that in turn trigger intracellular machinery that directs the cellular response. The past decade has seen an explosion in our understanding of the molecular basis for membrane receptor signaling. Part I in this Handbook surveys the diversity of mechanisms for membrane receptor signaling.

The first section in Part I, edited by Dr. Ian Wilson, deals with molecular recognition properties at extracellular protein–protein interfaces. The first set of chapters shows how molecular recognition can be studied, ranging from theoretical and database analyses to analysis of structures, mutational studies, and thermodynamics. Next, a series of detailed structural studies are presented to reveal specific examples of the molecular recognition within immune complexes. These binding surfaces do not appear to be rigid locks and keys; they are adaptive and capable of flexing to bind, or evolving to bind, one or more ligands. This section concludes with a variety of examples of recognition complexes that bind viruses, fibrin, and integrins that mediate cell–cell adhesion. These fundamental binding events involve protein–protein interactions that are found in virtually all steps of cell signaling.

Sections B and C, edited by Drs. Henry Bourne and Robert Stroud, respectively, deal with the mechanisms of receptor binding and activation. The activated form of all known receptors or receptor complexes contain at least two transmembrane helices, because for signals to be transmitted requires a change in disposition of at least two helical segments. Membrane receptors can be classified broadly into two groups depending on whether they are fully assembled prior to ligand binding (such as ion channels and most G-coupled receptors) or whether they assemble after the ligand binding (such as most growth factor receptors). The former group can be referred to as *vertical* because ligand binding immediately transduces a signal vertically through the membrane via a conformational change in the receptor that allows it to associate with proteins on the inner membrane leaflet. The latter receptor class can be referred to as *horizontal* because ligand binding first facilitates lateral association (or change in association) of receptor subunits. This causes a change in the juxtaposition of the intracellular domains of the receptor and induces further association with intracellular signaling molecules. These receptor classes have very different evolutionary origins and cellular roles.

The vertical receptors, covered in Section B, contain multiple membrane-crossing segments and are found in all organisms from bacteria to eukaryotes. They tend to be sensors for small molecules (even photons) and peptides, and in higher eukaryotes they can also bind large proteins such as glycopolypeptide hormones and chemokines. Some of these may also undergo lateral association to form higher order complexes with possible roles in signaling. The vertical receptors often promote immediate and reversible changes in pH, membrane polarity, calcium flux etc. that control cellular metabolism or cell migration.

The horizontal receptors, surveyed in Section C, contain a single helical membrane-crossing segment and are found in multicellular eukaryotes. These tend to be sensors for protein signaling ligands such as growth factors and cytokines. Ligand binding promotes the lateral association of accessory receptor subunits or, in some cases, a change in the mode of arrangement of receptor subunits already associated. The horizontal receptors function to cause slower and irreversible changes in the cells such as proliferation, differentiation, or apoptosis.

The final section in Part I, edited by Dr. Tom Alber, reviews what is known about the cellular machinery proximal to the inside of the membrane that responds to the activated receptor complex. Many of the growth factor receptors have intracellular kinase domains that become activated

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upon ligand binding and lead to phosphorylation, which seeds growth of larger signaling complexes via adaptor proteins. The receptors for helical cytokines lack a covalently fused kinase domain but recruit specific kinases (JAKs) to the membrane after oligomerization. Still others, such as the trimeric cytokine receptors (e.g., TNF receptor), provide oligomerized scaffolds that directly recruit adaptor proteins to form the intracellular signaling complex. It is also clear that lipids can play a role in concentrating receptors and signaling molecules both by covalent modification and by forming clusters known as lipid rafts. Many of the vertical receptors, such as the G-coupled receptors and photoreceptors, have proteins on the inner leaflet of the membrane called G proteins with which they interact to send the signal on to the cytosol. Thus, ligand binding to a receptor on the outside of the cell membrane causes even larger changes in protein assemblies just inside the cell membrane.

Membrane receptors have long been known to act as the cellular gatekeepers to the outside world. Work over the past decade has begun to provide insight into the mechanisms by which extracellular signaling molecules transmit information into the cell without actually passing through the membrane. Part I of this handbook is intended to present the state-of-the-art information about initiation of cell signaling.

James A. Wells

SECTION A

Molecular Recognition

Ian Wilson, Editor

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CHAPTER 2

Structural and Energetic Basis of Molecular Recognition

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Introduction

Molecular recognition can be thought of as the process by which two or more molecules bind to one another in a specific geometry. Any binding process requires that the associating molecules prefer to interact with each other rather than the alternative, in which the individual binding interfaces interact with the solvent in which they are found. The forces that drive binding are reasonably well understood in a qualitative sense, although the accurate prediction of binding free energies or the structure of a complex given the structures of the interacting subunits remain largely unsolved problems. This chapter will briefly review the physical chemical principles of binding and summarize what has been learned so far from the analysis of the three-dimensional structures of interacting molecules and their complexes. A number of recent reviews should be consulted for more extensive discussion of the topics covered here (see, for example, references [1–4]).

Principles of Binding

What drives proteins to associate with other molecules? The hydrophobic effect clearly plays a central role, and it is possible that close packing at interfaces may allow stronger van der Waals interactions between molecules than either one undergoes with solvent molecules. Both types of forces, in general, will increase as the interfacial surface area increases, and these contributions to binding are often assumed to be proportional to the surface area of both proteins that is buried upon binding. In general, there will always be some factors that oppose binding, including the loss of translational and rotational degrees of freedom as two or more species form a complex [5] and the "strain" induced in each monomer as a result of complex formation [6]. This can involve an increase in the conformational energy of each monomer or entropic losses, such as, for example, side chains in the interface that lose some configuration freedom upon binding.

Electrostatic interactions [2] also play an important role in binding; however, the magnitude and even sign of the effect are more difficult to predict. The complication in predicting the role of electrostatic interactions is that they generally reflect a balance between two large and opposing forces. For example, the formation of an ion pair as a result of complex formation requires that both charges be removed from the solvent and be completely or partially buried at an interface. For a completely buried ion pair, the loss of solvation is believed to be a larger effect than the gain of Coulomb energy in the complex so that individual ion pairs and hydrogen bonds are believed to oppose complex formation. However, ion pairs close to the surface can remain partially hydrated while still stabilized by Coulomb interactions. In some cases, these may provide a favorable driving force for association [7].

Even when charge–charge interactions oppose binding in a thermodynamic sense, they play a crucial role in specificity, as it would be extremely unfavorable energetically to remove a charge from the solvent and not to form any compensatory interactions. The requirement that buried charges and hydrogen bonding groups be satisfied upon complex formation is fairly strictly observed in known complexes. In some cases, there appear to be interfaces where networks of hydrogen bonds and ion pairs are formed [7]. These can result in a strong enough favorable interaction to compensate for the

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loss of solvation while at the same time placing fairly stringent specificity requirements on the geometry of the complex.

Overall, the binding of proteins to other proteins, nucleic acids, and membranes can be thought of as being driving by hydrophobic interactions (including stacking when nucleic acids are involved), constrained by the need to minimize the desolvation of charged and polar groups while optimizing the favorable interaction of these groups at an interface. Within the context of these constraints, as well as that of shape complementarity, the great flexibility in the design of different interfaces allows for the wide range of regulated and highly specific interactions that characterize signaling pathways.

Nonspecific Association with Membrane Surfaces

The interaction of proteins with membrane surfaces provides an example of how different combinations of hydrophobic and electrostatic interactions are combined to achieve various specificities. Many biological membranes contain acidic phospholipids that produce a negative electrostatic potential that can be used to attract positive charges to the membrane surface [8]. A number of membrane-binding motifs are used to anchor proteins to membrane, and these generally consist of some combination of nonpolar groups and positively charged amino acids. Some proteins such as Src use unstructured regions for membrane binding and, in the case of Src, this binding involves the N-terminal peptide, which contains basic amino acids and a myristate group [8]. Binding is regulated by phosphorylation, which reduces the electrostatic attraction between the basic amino acids and the acidic phospholipids [9]. Other unstructured regions, such as those of MARCKS and caveolin, use different combinations of aromatic and basic amino acids to effect membrane binding [10].

The same principles operate for structured proteins that bind to membrane surfaces. Many proteins involved in interfacial signaling contain a lipophilic modification (e.g., myristate, farnesyl) that contributes to membrane association by partitioning hydrophobically into the membrane interior. In addition, it appears that peripheral membrane proteins often have positively charged surfaces that provide an additional attraction to the surface of acidic phospholipids. In the case of the β,γ heterodimer of G proteins, the effect appears secondary to that of nonpolar penetration [11], while for many C2 domains electrostatics appears to be the dominant interaction [12]. For example, the C2 domain from protein kinase Cβ (PKC-β) and the C2A domain from synaptotagmin I (SytI) associate peripherally with membranes containing anionic phospholipids driven primarily by electrostatic interactions. In contrast, the C2 domain from cytosolic phospholipase A2 (cPLA2) penetrates into the hydrocarbon core of membranes and prefers electrically neutral, zwitterionic phospholipids. Other C2 domains may use a combination of these effects.

Protein–Protein Interactions

Theoretical calculations of electrostatic interactions can account quantitatively for many of the observed binding properties of peripheral membrane proteins. This is not the case for protein–protein association, in part because the highly specific interactions that characterize protein interfaces place greater demands on the level of theoretical description. In addition, binding is often associated with conformational changes and, possibly, changes in ionization state, and these are extremely difficult to predict. Much of what we know of how protein–protein interfaces are designed has been obtained from the analysis of crystal structures of complexes [13–15]. A somewhat surprising finding has been that protein–protein interfaces are in general very similar in composition to the rest of the protein surface, and they tend to be much less nonpolar than the protein core. Some interfaces may be primarily nonpolar, while others appear to be characterized by a great deal of electrostatic complementarity [16]. Indeed, the two factors may well be anti-correlated, with some interfaces exploiting hydrophobic interactions and incurring a large electrostatic penalty for binding while others appear to be designed so as to optimize electrostatic interactions and to exploit hydrophobic interactions to a much lesser extent [7].

Given the knowledge of the structures of the isolated monomers it would be extremely useful to be able to predict the structure of the complex they form. This problem is known as the docking problem (see Smith and Sternberg [3] and Camacho and Vajda [4] for recent reviews), and it has been widely studied with the goal of predicting the binding modes of small molecules to proteins. The docking problem is frequently divided into two steps. One involves a geometric matching of the interacting molecules and the other involves "scoring" the model complexes generated in the first step. Scoring functions based on the principles discussed above, maximizing surface area and geometric complementarity while optimizing electrostatic interactions, appears to work quite well. Indeed, assuming that one knows the structure of the monomers as they exist in the complex, it generally appears possible to reproduce the correct complex geometry (see, for example, Norel *et al.* [17]). This suggests that the physical basis of binding is reasonably well understood. Of course, the more meaningful problem is to predict the structure of a complex based on the structures of the free monomers; however, this problem is far from being solved due to unknown conformational changes that accompany complex formation. In general, the larger these changes, the less accurate the result.

Prospects

Although the accurate prediction of binding free energies remains an unsolved problem, the current level of understanding of molecular recognition is such that computational methods can be extremely useful in the design and interpretation of experimental results. Moreover, the use of bioinformatics

tools can significantly expand the range of problems that can be addressed. For example, evolutionary information can be used to map regions on a protein surface involved in binding [18,19]. Moreover, once the binding properties of a few members of a protein family have been determined, it should be possible to understand the behavior of many other family members through a combined analysis of sequence, structure, and energetics. Comparing multiple sequence alignments, multiple structure alignments, and the physicochemical properties of protein surfaces can provide a great deal of information as to how binding affinity and specificity are coded onto the three-dimensional structures of proteins.

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CHAPTER 3

Computational Genomics: Prediction of Protein Functional Linkages and Networks

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THE RAPIDLY GROWING GENOMIC SEQUENCE DATABASES ARE CREATING NEW CHALLENGES CONCERNING HOW TO USE GENOMIC DATA TO LEARN ABOUT THE FUNCTIONS OF PROTEINS AND THEIR FUNCTIONAL RELATIONSHIPS WITH EACH OTHER IN THE CELL. A VARIETY OF EXPERIMENTAL AND COMPUTATIONAL APPROACHES ARE EMERGING FOR DECIPHERING WHICH PROTEINS ARE WORKING WITH WHICH OTHERS AS PARTS OF FUNCTIONAL NETWORKS IN THE CELL. HERE, WE REVIEW SOME OF THE VARIOUS NEW COMPUTATIONAL TECHNIQUES THAT ARE ABLE TO DRAW CONNECTIONS BETWEEN DISTINCT BUT FUNCTIONALLY LINKED PROTEINS BASED ON CERTAIN PATTERNS OF OCCURRENCE OR ARRANGEMENT ACROSS MULTIPLE FULLY SEQUENCED GENOMES.

Introduction

In the last few years, advances in genomic and proteomics technologies have produced an explosion of raw data on biological systems at the molecular level. The rapidly growing number of organisms for which the genomes have been completely sequenced serves as a dramatic example. At the time of this writing, the genome sequences of more than 100 organisms are publicly available [1], including a draft sequence of the human genome released last year [2,3]. As a result, the speed with which protein sequences are being acquired has vastly outpaced our ability to assign functions to them directly by experimental (e.g., biochemical and genetic) methods. This growing disparity between known sequences and known functions for these proteins has created a unique challenge. How can we infer the functions of proteins on the genomic scale? A variety of methods have been devised to meet this post-genomic challenge. While some genome-wide analyses are mainly experimental in nature, others are predominantly computational, and some combine aspects of both approaches. In this chapter, we touch first on experimental approaches to genome-wide analysis (covered in more detail in Chapter II.C) and then focus on computational analyses of whole genomes.

Approaches to Analyzing Protein Functions on a Genome-Wide Scale

One theme emerging from recent work is that consideration of the genomic context of a protein can provide valuable information about the function of a protein, even in the absence of experimental studies. Analyses of various kinds of patterns across the burgeoning genomic databases can provide insight into functional relationships among distinct (nonhomologous) protein sequences. Consequently, this has led to a natural shift from asking *what a particular protein*

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does to asking *what other biomolecules that protein interacts with* or, to use a broader phrasing, *is functionally linked to*. This expanded perspective of protein function within the context of pathways and networks forms the basis for many of the recent developments in genomics.

Experimental Data on a Genome-Wide Scale

Some experimental genomic approaches make direct observations of functional linkages, while others require subsequent computational analyses to make statistical inferences about the existence of such linkages. Two techniques for making direct observations of physical protein–protein interactions are the yeast two-hybrid methods [4–6] and mass-spectrometry methods [7,8]. Both approaches are being applied on a genome-wide scale to generate maps of physical protein–protein interactions. Another genome-wide experimental approach, the synthetic genetic array [9], makes observations of functional linkages among proteins at the genetic rather than physical level.

Some genome-wide studies combine experimentation and subsequent computational analysis. One example of this combined approach is the inference of functional linkages from mRNA expression data obtained from DNA microarrays. In these studies, computational analysis of the raw expression data produces functional linkages between genes for which expression patterns vary in correlated ways with respect to changes in variables such as time, growth conditions, or tissue type [10–14].

Functional Linkages from Genome Sequence Data: Nonhomology Methods

The traditional computational method for inferring the function of an uncharacterized protein relies on establishing a statistically significant similarity between the sequence of the uncharacterized protein and that of a protein whose function has already been experimentally determined. The vast majority of entries in the sequence databases have acquired their functional annotations via this technique. Here, we refer to this large family of sequence-based approaches as the *homology method* because they assign functions to proteins based on homology. While this classical approach has played a major role in shaping molecular biology, its limitation is clear. It can only infer relationships between similar sequences. The homology approach does not shed light on functional linkages between different (nonhomologous) proteins. In one situation of special interest, typically accounting for a third to half of the open reading frames in a newly sequenced genome, a protein sequence from one genome may have homologs in other genomes, but it may be that none of these proteins has ever been characterized experimentally. Sequence comparison would tell us that these proteins are all evolutionarily related to each other, but nothing more.

A series of recent computational innovations (reviewed in references [15–18]), denoted here as *nonhomology methods*, utilize patterns discovered at the higher level of genomic organization to infer functional linkages between nonhomologous proteins. Such linkages provide a rich source of functional information, even for the problematic situation of proteins without any characterized homologs. We describe three different nonhomology methods followed by an illustration of their application.

Protein Phylogenetic Profiles

Two or more proteins that act together in the cell as part of the same complex or pathway should all be present in any organism that uses that complex or pathway. Conversely, it is natural to expect them all to be absent from organisms that do not use that complex or pathway. A protein phylogenetic profile is a vector that describes the presence or absence of a particular protein across a set of genomes. Two or more different (nonhomologous) proteins that share very similar phylogenetic profiles are likely to be functionally coupled. Pellegrini *et al*. [19] developed the phylogenetic profile method to establish functional linkages among proteins on the genomic scale. Related ideas and data structures were also discussed by others [20]. Statistical treatments have improved the original calculations [17], and the profiles have been used in other applications such as predicting the subcellular localization of proteins [21].

The Rosetta Stone Method

Two proteins, A and B, that are separate entities in one organism are sometimes found fused together in a single larger protein A–B in the genome of another organism. The evolutionary fusion of these two proteins is taken as evidence that they are functionally linked. The fusion protein is dubbed a *Rosetta Stone* because it allows a functional linkage to be drawn between the two separate proteins A and B. This idea was first applied on a genome-wide scale by Marcotte *et al*. [22] and then by others [23].

Conserved Gene Clusters

Especially in prokaryotic organisms, functionally linked proteins are sometimes encoded near each other on the chromosome (e.g., as in operons). When two or more proteins tend to be encoded in proximity, especially in relatively divergent microorganisms, this argues strongly for a functional linkage between the proteins. The information embodied in conserved gene order or proximity was first applied on a genome-wide scale to establish possible functional linkages by Overbeek and coworkers [24,25].

An Example Relevant to Cell Signaling

To illustrate the ideas here, algorithms based on the three methods discussed above were applied to the genome of *Escherichia coli*, and the results for a well-known cell signaling pathway were investigated. The protein flgE was chosen as a somewhat arbitrary starting point for investigating the bacterial flagellar complex. Using the multiple methods, high confidence links were established for this protein. Subsequently, high confidence links were established to those proteins first connected to flgE. This process was repeated until links of third order from the central protein were included. The results are shown in Fig. 1. Many of the

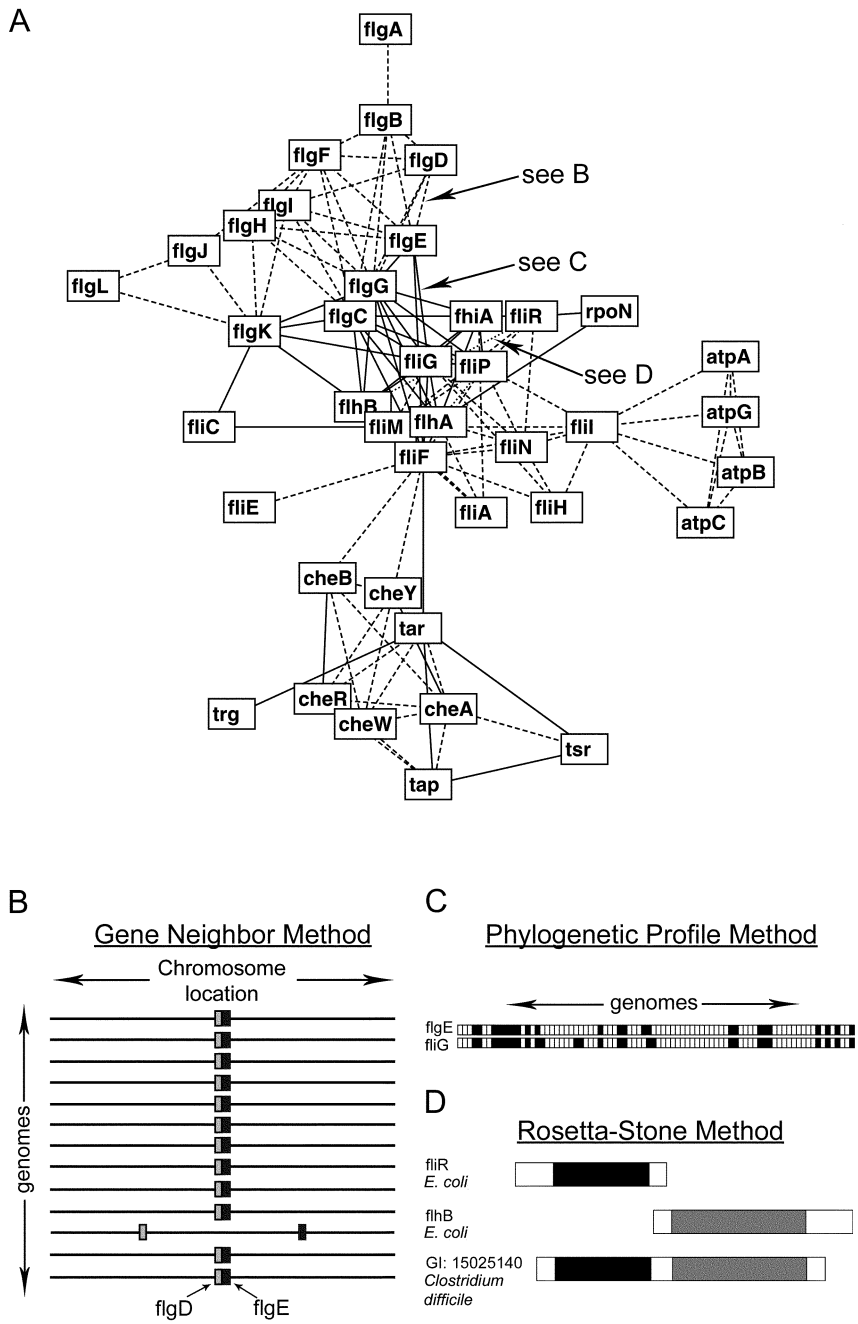


Figure 1 An illustration of functional linkages inferred by computational analysis of genomic data (nonhomology methods). The flagellar protein flgE was taken as a query protein. Computational methods were used to predict functional linkages between flgE and other proteins in the *E. coli* genome. Subsequent links (of second and third order) were generated from these to others. (A) This procedure produced the network shown, which includes many proteins known to participate in motility and chemotaxis. The computed functional links include proteins involved in various aspects of this biological system, from signal transduction to flagellar assembly and regulation. Each link is coded according to the computational method by which it was inferred. Links from the method of phylogenetic profiles are in solid lines, gene neighbor links are dashed, and Rosetta stone links are dotted. In some instances (not illustrated), multiple methods produced the same link. The three methods are shown at the bottom. Each panel illustrates the pattern in the genomic data that allowed one of the inferences at the top to be made. (B) The gene neighbor method draws a functional linkage between two proteins if they tend to be encoded in adjacent or nearby positions on the chromosomes of multiple organisms [25,26]. (C) In the method of protein phylogenetic profiles [16], the presence or absence of a protein across a set of genomes is analyzed. The two linked proteins shown have profiles for which the similarity is statistically significant. (D) In the Rosetta Stone method, the two separate proteins from one genome are functionally linked because they are found in some other genome as combined parts of a single larger protein [23,24].

proteins involved in flagellar biosynthesis and assembly (flgA, flgB, flgC, flgD, flgE, flgF, flgG, flgH, flgI, flgJ, flgK, flgL, fhiA, fliE, fliF, flip, fliR), export (flhA, flhB, fliH), and motor switching (fliG, fliM, fliN) were recovered. Links emanating from these flagellar proteins established connections to the chemosensing proteins (cheA, cheB, cheR, cheY, cheW, tap, tar, trg, tsr) whose signals ultimately drive the flagella, to the transcriptional proteins (fliA, rpoN) that regulate the production of flagellar proteins, and to the ATPase complex (fliI, atpA, atpB, atpC, atpG) that supplies energy for the flagellar motion. The illustration of the chemotaxis system in *E. coli* shows that in favorable cases these computational nonhomology methods not only can recover links among proteins involved in a complex or pathway but also can reveal higher order functional relations among the complexes and pathways.

Miscellaneous Methods

Other methods for inferring functional linkages have also been explored. For instance, mRNA expression data have been combined with promoter motif detection algorithms to identify regulatory networks [26,27]. In contrast to the analysis of large sets of experimental measurements, another computation approach seeks to distill large volumes of experimental results through the mining of the published literature [28–31]. These methods attempt to ascertain, in an automated fashion, the existence of experimentally established functional relationships among proteins from computational analysis of millions of biomedical literature abstracts. Efforts involving some amount of manual curation have also been conducted. The Database of Interacting Proteins (DIP) [32] is the result of one such effort.

Quality Control by Benchmarking

Computational methods like those discussed here provide only circumstantial evidence that various proteins are actually functionally linked in the cell. This makes quality control a particularly important problem. Two complementary approaches to this problem are the development of probabilistic models to evaluate statistical significance and the use of known functional relationships for benchmarking.

Statistical approaches for assessing inferences made by nonhomology methods have only begun to be addressed. One of the statistical difficulties that has not been explored deeply concerns how to handle correlated observations. For example, among the organisms whose genomes have been sequenced, some are much more closely related than others. This complicates the probabilistic treatment of features such as conserved relative positions (or presence versus absence) of proteins across the known genomes. Suppose for example that two (or more) proteins exhibit some genomic pattern that is evident only among very closely related organisms. Such a pattern has not survived over a long evolutionary time scale and so may not indicate a significant functional linkage between the proteins in question.

Regardless of the simplicity or sophistication of the statistical analyses performed, experience has shown that the various computational methods must be calibrated by examining how well they perform on proteins whose functions are already known. One reasonable benchmarking approach is to measure the fraction of predicted functional linkages that are corroborated by the linked proteins having similar functional categories or keywords in annotated protein databases (e.g., SWISS-PROT, MIPs, KEGG) [33–35]. A related strategy is to use the inferences from one computational method to evaluate another. The general idea of using multiple methods to generate linkages with higher confidence was first applied by Marcotte *et al*. [36]. Multiple sources of experimental measurements have also been used to similar effect [37].

Current Issues and Future Prospects for Computing Functional Interactions

Current and future investigations into the problem of computing functional interactions will address some of the following questions:

- Can probabilistic models be developed to overcome the problem of correlated data in order to give accurate significance scores for inferred linkages?
- Can the methods that work so powerfully on prokaryotic and lower eukaryotic genomes be extended fruitfully to higher organisms? How many eukaryotic genomes must be completed to make this possible?
- How can functional linkages from various methods be combined and visualized in the best way [38]?
- What are the large-scale properties of the biological networks that arise from these computations [39], and how can true pathways be extracted from them?

Solutions to these problems will bring a richer understanding of biological pathways and networks in the coming years.

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CHAPTER 4

Molecular Sociology

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Transmembrane Signaling Paradigms

The initiation of a cell signaling event relies primarily on interactions between molecules in the extracellular and cell-membrane space. Different types of molecules can serve as extracellular signals (Fig. 1a) [1]: hormones, cytokines, growth factors, and neurotransmitters secreted from distant or neighboring cells; antigens or antibodies free in solution or attached to (migrating) leucocytes or foreign (e.g., virus) cells; small soluble molecules (i.e., <1000 Da; ions, metabolites); and the extracellular matrix. Except for lipid-soluble signaling molecules that can migrate through the lipid bilayer (e.g., steroid hormones and NO gas), transmembrane proteins are involved in transferring the molecular signal into the target cell. Small soluble molecules can be transported across the plasma membrane by channel and carrier proteins or cell–cell GAP junctions, which provide an electrical and metabolic coupling with the extracellular space and neighboring cells, respectively. Larger soluble or tethered molecules, including filaments from the extracellular matrix, require a specific interaction with a transmembrane (co-) receptor for signal transduction across the membrane.

The transmembrane protein undergoes an intramolecular conformational change or change in the quaternary structure (e.g., dimerization) upon binding of the extracellular molecular signal. It is noncovalently or covalently linked either to an ion channel that allows the change of the ion traffic across the membrane or to intracellular membrane-proximal components that are activated to induce an intracellular signaling cascade. In the latter case, the receptor can contain intrinsic enzyme (e.g., phosphorylation) activity, such as the receptor tyrosine kinases; recruit relevant intracellular enzymes; or associate with G proteins, which in turn activate kinases or ion channels. While the transmembrane signaling process mediated by (ion) channels is immediate and brief, enzyme-linked receptors manifest a slow and more complex molecular mechanism but can achieve a great amplifying signaling effect. Subsequently, gene expression in the nucleus or other cell activities are affected. Recent studies have shown that endocytosis of transmembrane receptor complexes can be used to deliver the complex and affect activities at distant locations in the cell [2].

A careful regulation and coordination of the communication within the molecular signaling society is essential to the initiation of a signaling event, especially in the more complex multicellular organisms. Any molecular interaction, such as that between a receptor or receptor subunits and ligand molecules, is determined by the effective local molecular concentrations and (apparent) dissociation constants. The concentrations of the signaling and receptor molecules can be controlled by various factors at different stages along the path toward an encounter (Fig. 1b). After synthesis or secretion, enzymatic degradation or temporary storage can influence the concentration of the signaling molecule, whereas lateral capping and endocytosis can alter the density of receptor molecules at the membrane surface. A rapid turnover of signals and receptor molecules is required to respond to fast changes in the environment. Signaling molecules may have to travel far (e.g., endocrine signaling) and depend on fluid streams of the vascular system and diffusion to enable an encounter with their target. The gel-like layer of proteoglycans in the extracellular matrix can serve as a selective molecular sieve to regulate the traffic of migrating cells and signaling molecules.

The local environment at the cell membrane can play an important role in controlling the receptor–ligand interaction (Fig. 1b). By interacting with the ligands, membrane-associated

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molecules can localize and/or immobilize extracellular signals for internalization and degradation or present them for receptor binding (e.g., growth factors and cytokines localized by proteoglycans). The physicochemical and geometrical properties of the molecular interface that determine the (apparent) dissociation constant of the signal binding can be altered by interaction with the local environment. A change in the physiological condition or binding of an effector molecule from the cytosol, membrane, or extracellular space (e.g., ion, metabolite, other protein) can dramatically change the affinity of a receptor–ligand complex by altering the conformation or electrostatic potential at the signal–binding interface. Individual receptor–ligand interactions may be stabilized or activated by effector binding or multiple

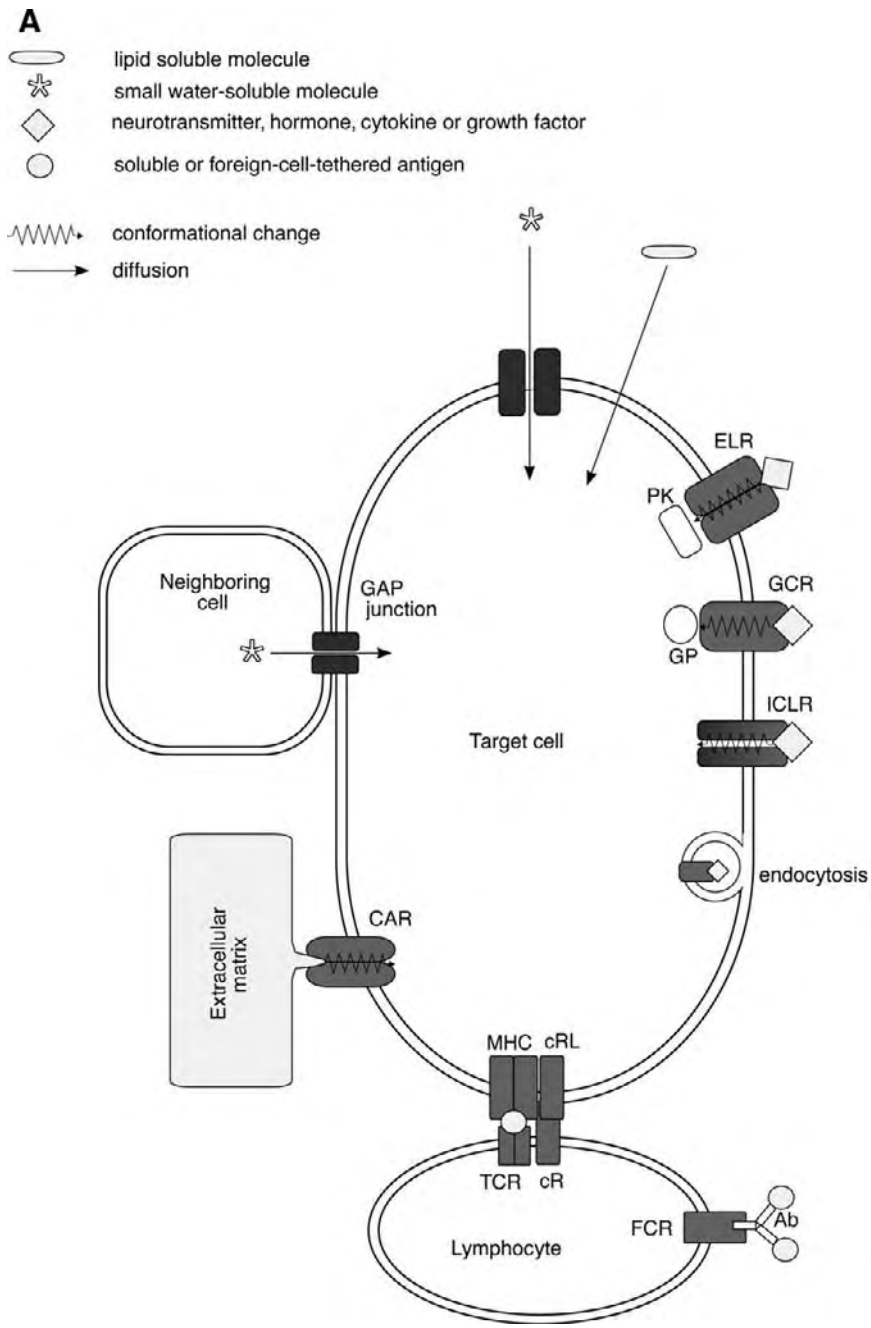


Figure 1 Transmembrane signaling paradigms: (a) molecular interactions and mechanisms, and (b) control of an interaction between a (oligomeric) receptor and (oligomeric) ligand. Extracellular signaling molecules are yellow; channels or carrier proteins and receptors are blue and red, respectively. Lipid soluble molecules comprise hydrophobic or small uncharged polar molecules; small water-soluble molecules comprise inorganic ions, sugars, amino acids, nucleotides, and vitamins. **ELR** = enzyme-linked receptor, GCR = G-coupled receptor, ICLR = ion-channel-linked receptor, PK = protein kinase, GP = G protein, Ab = antibody, MHC = major histocompatibility complex, TCR = T cell receptor, FCR = Fc receptor, cR = coreceptor, cRL = coreceptor ligand, CAR = cell adhesion receptor.

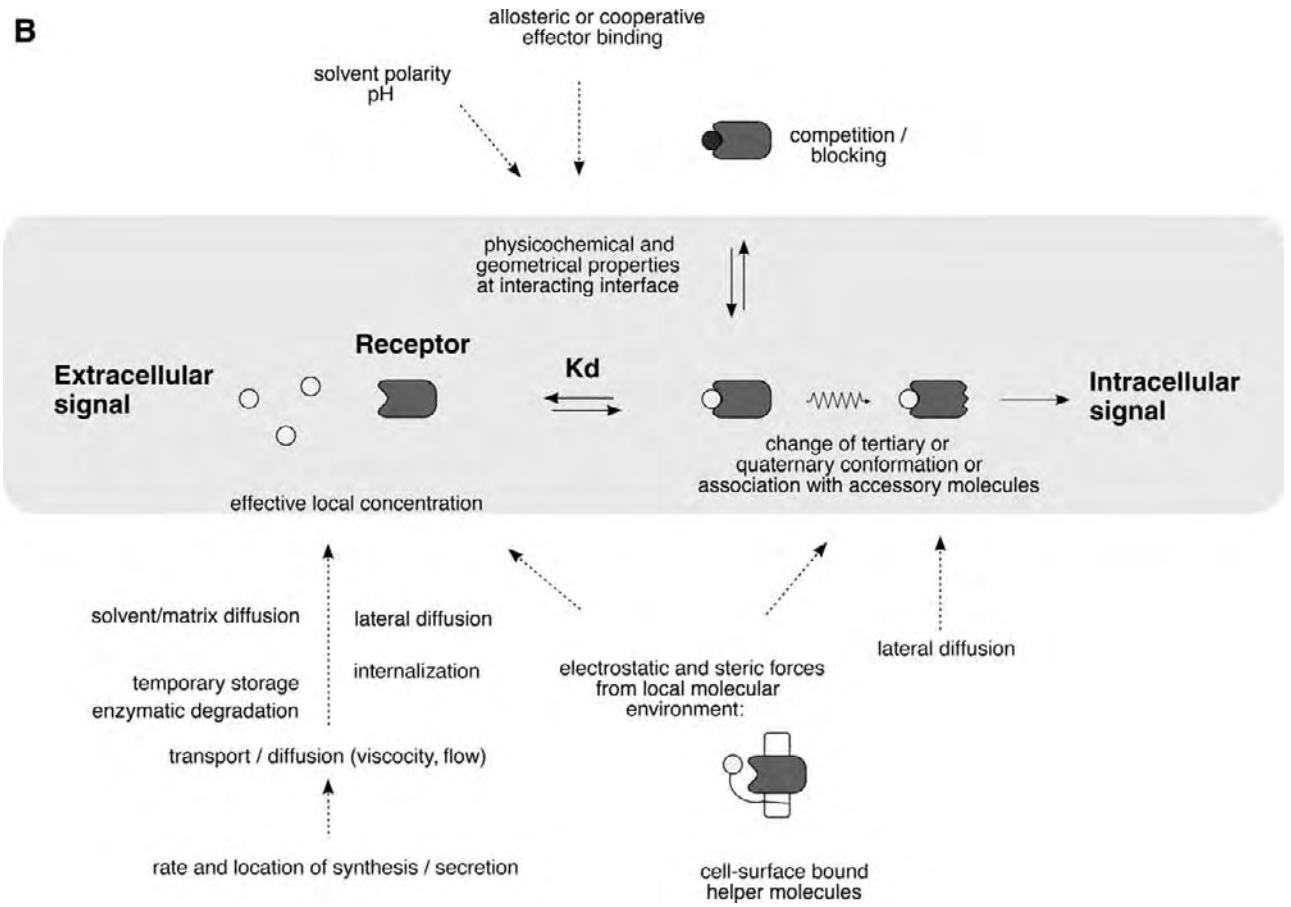


Figure 1 *(continued)*

interactions, (e.g., clustering of receptor–ligand complexes). Some complexes may be very weak on their own and require accessory proteins or coreceptors for stability or activation; for example, CD4 and CD8 coreceptors in the major histocompatibility complex (MHC) multicomponent complexes help to strengthen the adhesion between a T and antigen-presenting cell. Also, low-affinity antibody–antigen interactions may be amplified by multivalent cross-linking. Furthermore, the apparent affinity for the extracellular signaling ligand can be decreased when another molecule (i.e., antagonist) competes for the same target-binding site. These control mechanisms that regulate the signaling interaction network are similar to those that control other biomolecular interactions such as enzyme–substrate interactions. However, altering the affinity of an assembly by covalent modifications (e.g., phosphorylation) is more common in intracellular signaling, where a large repertoire of modifying enzymes is available.

Structural Basis of Protein–Protein Recognition

A specific interaction between a signaling and membrane receptor molecule is critical to obtain a well-directed signaling event. The molecular recognition process that underlies a specific interaction is provided by the complementarity of the physicochemical and geometrical properties of the two protein surfaces to obtain an energetically favorable complex. This is determined by the hydrophobic effect, close packing with favorable van der Waals interactions, and the formation of hydrogen and ionic bonds. Computational analyses of atomic structures of protein–protein complexes have identified the structural and physiochemical properties of these interfaces [3–5] (see Kleanthous [6] for reviews). Structures of various extracellular molecular signaling complexes have been elucidated so far: the extracellular domains of receptors complexed with hormones and cytokines, the major histocompatibility complex with diverse peptides (pMHC) in association with the T-cell receptor (TCR), and antibody (Fab fragments)–antigen complexes [6]. For the majority of these complexes, the individual components form homo- or hetero-oligomers by itself or upon ligand binding. Table I summarizes the receptor–ligand complexes and their oligomeric disposition [7].

In general, protein-protein interfaces exhibit a mixture of apolar and polar interactions scattered over the binding surface with the polar residues providing fine specificity. The interfaces of non-obligate complexes (i.e., between molecules that also exist on their own), such as extracellular signalling and enzyme-inhibitor complexes are generally more polar than homodimers (Fig. 2), because of the solubility

Table I Current Receptor–Protein Signaling Complexes in the Protein Data Bank

Protein1	Protein2	pdbcode(s) (resolution in Å)	Oligomeric State[1]		Receptor Activity	Ref.
Growth hormone receptor	Somatotropin (growth hormone)	3hhr (2.8)[3], 1hwg (2.5)	2:1	Receptor dimerization upon ligand binding	Non-protein kinase, associated jak kinases	12
	Gh antagonist g130r	1hwh (2.9)[3], 1a22 (2.6)	1:1			
		remodeled interface: 1axi (2.1)[3]	1:1			
Prolactin receptor	Somatotropin (growth hormone)	1bp3 (2.9)	1:1		Non-protein kinase, associated JAK kinases	13
	Placental lactogen	1f6f (2.3)	2:1			
Erythropoietin receptor	Erythropoietin	1cn4 (2.8)[3], 1eer (1.9)	2:1		Non-protein kinase, associated JAK kinases	14
Interleukin-1 receptor	Interleukin-1 receptor antagonist	1ira (2.7)	1:1		Non-protein kinase, associated accessory protein	15
Interleukin-1 receptor	Interleukin-1 beta	1itb (2.5)	1:1		Non-protein kinase, associated accessory protein	16
Interleukin-4 receptor α chain	Interleukin-4	1iar (2.3)	1:1		Non-protein kinase, associates with common γ chain, associated JAK kinase	17
Granulocyte colony-stimulating factor receptor	G-csf	1pgr (3.5)[3], 1cd9 (2.8)	2:2-I		Non-protein kinase, associated JAK kinases	18
Interleukin-6 receptor GP130 chain	Interleukin-6	1i1r (2.4)	2:2-I		Non-protein kinase, common β chain (e.g., Gp130), associated JAK kinase	19
Trka receptor	Nerve growth factor	1www (2.2)	2:2-II		Tyrosine kinase	20
Bone morphogenetic protein receptor 1a	Bone morphogenetic protein-2	1es7 (2.9)	2:2-II		Serine-threonine kinase	21
Interferon-gamma receptor α	Interferon-γ	1fg9 (2.9)[3], 1fyh (2.0)	2:2-II		Non-protein kinase, associated JAK kinases	22
Interleukin-10 receptor	Interleukin-10	1j7v (2.9)	2:2-II or 4:4[2]		Non-protein kinase, associated JAK kinases	23
Fibroblast growth factor receptor 1	Fibroblast growth factor-1	1evt (2.8)	2:2-I	Homo-and heterodimerization, interdomain ligand binding; heparin involved	Tyrosine kinase	24
Fibroblast growth factor receptor 1	Fibroblast growth factor-2	1cvs (2.8), 1fq9 (3.0; heparin bound)	2:2-I			
Fibroblast growth factor receptor 2	Fibroblast growth factor-1	1djs (2.4)	2:2-I			
Fibroblast growth factor receptor 2	Fibroblast growth factor-2	1ev2 (2.2)	2:2-I			
	Fibroblast growth factor-2 apert syndrome variant	mutant: 1iil (2.3)[3], 1ii4(2.7)[3]	2:2-I			
Death receptor 5	TRAIL	1d0g (2.4)[3], 1d4v (2.2)	3:3		Non-protein kinase, associated TRAF	25
Tumor necrosis factor receptor	Tumor necrosis factor β	1tnr (2.9)	3:3;	dimerizes without ligand; trimerizes upon ligand binding	Non-protein kinase, associated TRAF	26

[1]There are differences in the literature about the nomenclature used to describe receptor–ligand complexes. Here, we use the stoichiometry of the complex (i.e., the number of protomer chains of the receptor and ligand, respectively, involved in the complex). We identify two types of 2:2 complexes: the 2:2-I type, where each receptor chain contacts both monomeric ligands, and the 2:2-II type, where each receptor chain contacts both protomers of the dimeric ligand.

[2]The 2:2 complex is thought to form an intermediate receptor–ligand complex, whereas the 4:4 is the active receptor–ligand complex. Structural parameters (Fig. 2) have been computed for the former.

[3]These entries have not been included in the computational analysis shown in Fig. 2.

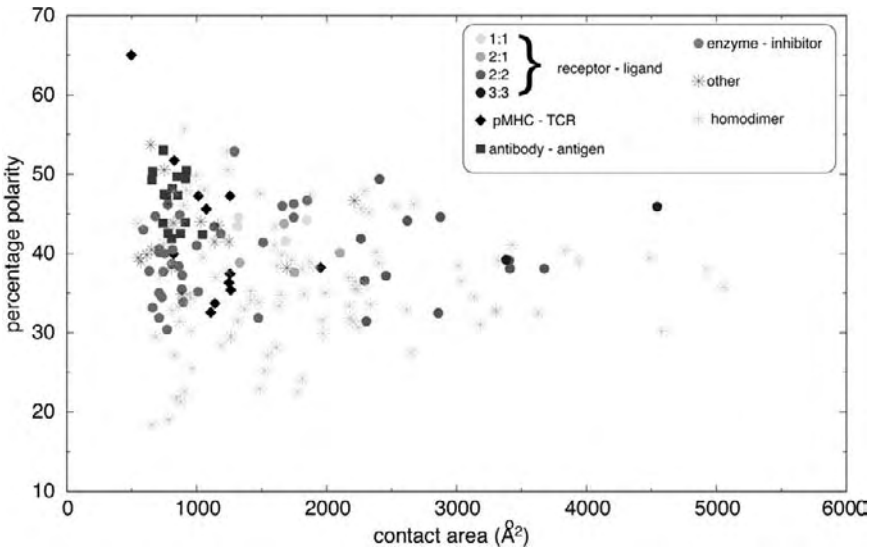


Figure 2 Correlation between the contact area of the interface and the percentage of the contact area that involves polar atoms for extracellular signaling complexes, diverse other nonobligate complexes, and homodimers. Structures used in this analysis are those studied previously [3,5] and more recent solved structures. Parameters have been calculated as described in Jones *et al*. [27]. When one or both of the proteins involved in the complex are multimers, the contact area is summed over all receptor–ligand interfaces to give a total for the complete assembly and the percentage polarity has been averaged respectively.

requirements of the individual molecules. Whereas the percentage of polar atoms in the interface is variable in the receptor–ligand and pMHC–TCR complexes, the antibody–antigen complexes consistently have more than 40% polar atoms in the interface and have a relatively small contact area (i.e., interface smaller than 1500 Å^2). The surface area buried in the specific non-obligate protein-protein associations is also highly variable. Large contact areas up to 5000 Å^2 are found for homodimers and various nonobligate complexes, such as multimeric receptor–ligand and large enzyme-inhibitor complexes (Fig. 2).

Structural rearrangements upon protein–protein association have been identified for many complexes, such as enzyme-inhibitor (e.g., thrombin–hirudin), intracellular signalling (e.g., G_α–$G_{\beta\gamma}$ protein) and receptor–ligand (e.g., receptor–human growth factor) complexes. Remarkably, protein–protein complexes that undergo structural rearrangements usually have large interfaces (i.e., >1500 Å^2). They involve disorder-to-order transitions, small changes in side-chain conformations (i.e., translational, rotational, and side-chain degrees of freedom), or gross conformational changes such as loop or domain movements. The monomeric human growth factor, for example, shows large helix movements upon binding into a cleft formed by the two subunits of the homodimeric receptor. Conformational changes are expected to play a major role in the transmembrane signaling process. Upon receptor–ligand complexation, side-chain flexibility may facilitate finding the complementary fit [8], whereas larger conformational changes may reveal hydrophobic surfaces or propagate a long-range structural rearrangement of the monomeric or oligomeric transmembrane receptor required for signal transduction. Residue spacing and molecular flexibility have been demonstrated to be important for protein–carbohydrate recognition in signalling, as well [9].

The structural basis of conformational flexibility is difficult to assess experimentally, as the current available experimental methods in structural determination (i.e., X-ray crystallography, nuclear magnetic resonance spectroscopy, electron microscopy) require a stable structure. Capturing the structures under different conditions (e.g., free and bound) or having thermodynamic and mutagenesis data can help to identify these changes or relate them to signal transduction activity. The current structural data in the Protein Data Bank (PDB) and a computational analysis of these protein–protein complexes in the PDB (Fig. 2) are probably biased toward structures that form stable structures. The atomic structures of many transmembrane domains or proteins have yet to be determined, which leaves the molecular mechanisms responsible for the signal transduction across the membrane still largely unknown.

Conclusion

The biology of signaling features a whole range of interactions, from weak to strong. Dissociation constants are found in the n*M* to m*M* range [10]. Both the complementarity of the physicochemical and geometrical properties of the interface and the flexibility of the surfaces of the receptor and signalling molecule contribute to the binding free energy. Structural data and currently available computational methods appear inadequate to estimate the binding energy or specificity of an interaction. The interaction

between molecules has usually been optimized throughout evolution to tune specificity and affinity to function and physiological environment (Fig. 1b). For example, high local concentrations of neurotransmitter can be reached in a chemical synapse that allows a low-affinity receptor–neurotransmitter interaction. Also, adjacent helper molecules or multiple interactions allow reduced affinity of isolated complexes by adding up weak interactions, generating a strong multicomponent complex. In comparison to other (cytosolic) protein–protein complexes, the anchoring of molecules in or at the membrane site is advantageous to localizing target subunits and substrate. In contrast, immune response complexes, such as antigen–antibody assemblies lack an extended period of a selective evolutionary optimization toward their physiological environment. Consequently, they exhibit a poorer shape complementarity than other molecular protein–protein associations [11]. In summary, proteins are versatile and the recognition process is different and unique to each molecular complex. Some of these will be discussed in more detail in the following chapters of this section.

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CHAPTER 5

Free Energy Landscapes in Protein–Protein Interactions

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Introduction

Specific protein–protein interactions provide a major part of the basic organization of living cells. Analysis of the structure of the complex provides a high-resolution static picture of the complex, while the affinity allows us to analyze the equilibrium thermodynamics of the interaction. However, understanding biological processes requires information on the nature of the full energy landscape of the complexation reaction. Analysis of the kinetics of association and dissociation allows characterizing the landscape in more detail. In combination with computational methods, the free energy landscape can be reconstructed based on kinetic data, as well as the transition state and intermediates along the pathway. Of special interest in analyzing the free energy landscape are "hot spot" residues, which make an outstandingly large contribution toward binding. The thermodynamics and kinetics of protein–protein interactions and the free energy landscape connecting the free and bound proteins are the subject of this chapter.

Thermodynamics of Protein–Protein Interactions

Specific protein–protein interactions provide a major part of the basic organization of living cells. The structure of a protein complex embeds the information about the relative mutual organization of two proteins in a frozen state; however, it does not intuitively provide information on the affinity between two proteins or the time-dependent process of complex assembly and dissociation. For a mechanistic understanding of biological processes and for engineering proteins that fulfill specific therapeutic tasks, we require physicochemical observables that describe the pathway of protein–protein interactions in detail. The binding affinity between proteins,

$$K_a = \frac{[AB]}{[A][B]} \tag{1}$$

given by the equilibrium concentrations of the proteins [A] and [B] and the complex [AB], is directly related to the free energy of interaction $\Delta G^\circ = -RT \ln K_a$. Thus, complex formation only takes place if $\Delta G^\circ < 0$. The free energy of the complex formation can readily be analyzed by measuring K_a (Fig. 1); for example, the energetic contributions $\Delta\Delta G_D$ of individual residues can be determined by measuring changes in the K_a upon mutation. According to the Gibbs–Helmholtz relation $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$, both the enthalpy ΔH° and the entropy ΔS° of the complex formation contribute to ΔG°. ΔH° reflects the strength of the interactions between two proteins (e.g., van der Waals, hydrogen bonds, salt bridges) relative to those existing with the solvent molecules, which are excluded from the binding interface. ΔS°, on the other hand, mainly reflects two contributions: changes in solvation entropy and changes in conformational entropy. Upon binding, the water released from the binding sites leads to a gain in solvent entropy. This gain is particularly important for hydrophobic patches on the protein surface (*hydrophobic effect*). At the same time, the proteins

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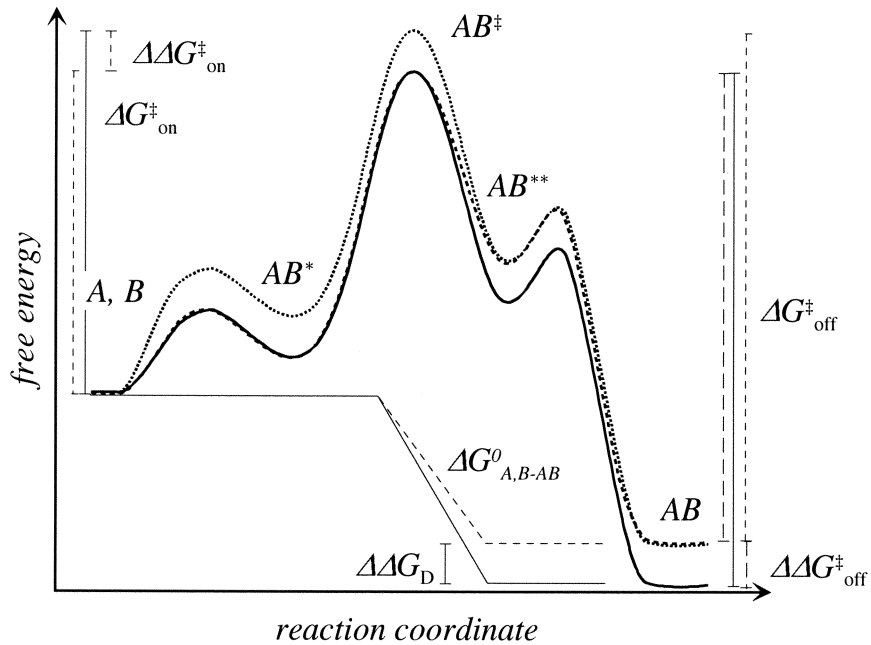


Figure 1 Free energy profile describing the pathway for the formation of a protein–protein complex (AB) from the free proteins A and B via the encounter complex AB*, the transition state AB‡, and the intermediate AB**. Comparison of the profiles for the wt proteins (—) with a mutant affecting long-range electrostatic interactions (·····) and a mutant affecting short-range interactions (------), respectively. The free energies, ΔG, are indicated for both the complex formation ΔG° and the transition state, as well as the changes in free energy of the encounter complex.

and individual residues within the proteins lose conformational freedom, resulting in a negative change in conformational entropy. The loss of conformational freedom was estimated to be on the order of 15 kcal/mol at 25° C, but values between 0 and 30 kcal/mol have been cited as well [1]. What do we know about the contributions toward entropy and enthalpy on the molecular level? Dehydration of nonpolar residues during association is always entropically favorable, while that of polar residues is unfavorable. The enthalpies are nevertheless negative, as they represent the energy of interaction of atoms at the interface relative to their interactions with water. As several partially canceling factors contribute toward the entropy and enthalpy of interaction, it is not surprising that for most mutant complexes the difference in free energy of binding is much smaller than the accompanying changes in $\Delta H°$ and $\Delta S°$. This has been emphasized by theoretical studies showing that, on forming a cavity in water to accommodate a solvent molecule, the change in the enthalpy of water (solvation) is exactly balanced by the entropy of the cavity; thus, changes in $\Delta H°$ and $\Delta S°$ cancel out each other in $\Delta G°$ [2]. Enthalpy–entropy compensations, then, seem to be a characteristic of weak noncovalent interactions, including protein–protein interactions.

Interaction Kinetics

While analysis of the K_a can provide an extensive thermodynamic picture of the complex, it does not allow any conclusion about the pathway that leads to the formation of the complex from the individual proteins. This is entirely determined by the shape of the full free energy landscape given by all possible states between the free proteins and the complex, most of which are not accessible experimentally. On this free energy landscape, the reaction itself most likely follows the pathway requiring the least free energy. This pathway is called the *reaction coordinate* and can be studied experimentally through the rates of association and of dissociation. Analysis of these kinetic parameters for several structurally and physicochemically well-defined protein–protein interactions allowed for establishing basic concepts of how proteins form complexes. In the following, we will give an overview about how kinetics can be used for analyzing the interaction pathway through the free energy landscape and how this can be understood on the molecular level.

In a general term, association of a protein complex (AB) from the unbound components (A + B) can be best described using a four state model:

$$\mathrm{A} + \mathrm{B} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{AB}* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{AB}** \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{AB} \qquad \text{(scheme 1)}$$

In this scheme, A and B are two proteins in solution, forming the complex AB. The reaction diagram of this interaction (Fig. 1) resembles that of protein folding, with the transition state being the most unstable species along the reaction pathway, which occurs at the highest peak of a reaction coordinate diagram. Two pre-complex states are formed along the reaction pathway. The encounter complex is positioned before the transition state for association (AB*) and

the intermediate complex (AB**), which is between the transition state and the final complex. In physical terms, the encounter complex tends to dissociate readily (with $k_{-1} >> k_2$), while the intermediate is already committed to form the final complex (thus, $k_3 >> k_{-2}$). It has to be emphasized that experimentally one often observes only the transition between A + B to AB and that the equilibrium dissociation constant (K_D) equals k_{off}/k_{on}. Yet, under certain experimental conditions, the pre-complexes are observable. A good example for such a case is the interaction between Ras and the Ras-binding domain of c-Raf1. Here, a two-step association process was suggested, with an initial rapid equilibrium step followed by an isomerization reaction occurring at the rate of several hundreds per second [3].

The intermediate (AB^{**}) is formed after the rate-limiting step for association, ($k_3 >> k_{-2}$); therefore, it does not affect the overall rate of association. The intermediate can be envisioned as a partially formed complex that has to reorganize to form the final complex. This reorganization step can be fast, such as for the interaction between cystatin A and papain (230 s^{-1}), or slow, such as for the interaction between lysozyme and HyHEL-10 and HyHEL-26 (~10^{-3} s^{-1}) [4,5]. A major problem in investigating this intermediate is to find a probe that can monitor independently the formation of the intermediate versus the formation of the final complex.

The Transition State

In the transition state, noncovalent bonds are in the process of being made and broken. At least one encounter complex can be found prior to the transition state, with additional intermediates occupying the energy landscape past the transition state. What is the molecular basis of the transition state for protein–protein interactions? The bound state of two proteins is characterized by local specific interactions (e.g., van der Waals, electrostatic) between widely desolvated binding sites, whereas the unbound state is characterized by complete solvation and higher translational and rotational freedom. During formation of the complex, the proteins have to pass through a free-energy maximum where translational–rotational entropy is reduced and the binding sites are partially desolvated, but short-range interactions and precise structural fitting have not yet been attained. This state is naturally the transition state. The transition state can be approached from the unbound state (association) and from the bound state (dissociation). Yet, by the principle of macromolecular reversibility, the nature and structure of the transition state should be the same.

The free activation energies required for reaching the transition state from the free proteins, $\Delta G^{\ddagger}_{on}$, and the complex $\Delta G^{\ddagger}_{off}$ (Fig. 1) are related to the rate constants of complex formation and complex dissociation, respectively. Thus, experimental information on the nature of the transition state is obtained from the rate constants of the interaction. Absolute values from bimolecular reactions are difficult to interpret; however, relative values of changes in the rate constants of association k_{on} or dissociation k_{off} upon mutation of individual amino acid residues allow for characterizing the features of the transition state. Mutation studies conducted on many protein interactions have clearly shown that rates of association are mostly affected by mutating charged residues [6]. Moreover, it is possible to introduce charge mutations at the periphery of the binding site that will affect only association, but not dissociation [7]. Mutations, which are neutral in respect to their charge, potently affect the dissociation rate constants. Masking electrostatic interactions between proteins by increasing the ionic strength has confirmed this observation; while the rate of dissociation is only marginally affected, the effect on the rate of association can be very large and is directly related to the electrostatic energy U of interaction between the two proteins according to Eq. (1):

$$ln\, k_{on} = ln\, k^0_{on} - \frac{U}{RT}\left(\frac{1}{1+\kappa a}\right) \tag{2}$$

where $In\, k^0_{on}$ is the basal rate of association in the absence of electrostatic forces, κ is the inverse Debye length, and a is the minimal distance of approach [6,7].

Direct information on the properties of the transition state could be obtained by double mutant cycle analysis of changes in activation free energies $\Delta\Delta G_{on}$, which are calculated from the association rate constants according to van't Hoff's isotherm:

$$\Delta\Delta G^{+}_{on} = RT\, ln \frac{k^{wt}_{on}}{k^{mut}_{on}} \tag{3}$$

$$\Delta\Delta G^{+}_{\mathrm{int}(on)} = \Delta\Delta G^{+}_{on(mut1,mut2)} - \Delta\Delta G^{+}_{on(mut1)} - \Delta\Delta G^{+}_{on(mut2)} \tag{4}$$

If the $\Delta\Delta G^{\ddagger}_{on}$ invoked by two individual mutations on each protein are additive, the two residues do not interact during the transition state; however, if the change is less or more than additive, one may assume that these two residues interact at the transition state. Probing the structure of the transition state of barnase/barstar and thrombin/hirudin by this method has shown that only charged residues, which are in close proximity in the final complex, already interact in the transition state. No significant interaction was measured between uncharged residues at this stage [8,9]. A somewhat different approach to probe docking trajectories experimentally uses the analysis of Φ values ($\Phi = \Delta\Delta G^{\ddagger}_{on}/\Delta\Delta G_D$). A Φ value close to one indicates that a specific interaction is formed at the transition state, while a Φ value close to zero indicates that the interaction is formed after the transition state. In a study of the HyHEL-10 Fab complex, multiple replacements were made in two positions, with most of the replacements having Φ values close to zero. This was

interpreted as the transition state being early along the reaction trajectory, before short-range interactions (which have the largest contribution on $\Delta\Delta G_D$) are formed [10]. The notion that short-range interactions affect k_{off}, while long-range electrostatic interactions affect k_{on}, was directly tested by introducing charged mutations at the vicinity, but outside the binding site of TEM1-BLIP. These mutations did increase specifically k_{on} by 250-fold but did not affect k_{off} (thus, the increase in k_{on} equals the increase in K_D and $\Phi = 1$) [7]. These data suggest that long-range electrostatic interactions increase the rate of association by lowering the free energy of the transition state by the same magnitude as the equilibrium constant (see Fig. 1). While mutations of non-charged residues do not significantly affect the transition state for association, they can significantly alter k_{off} and K_D. These data imply that the transition state is stabilized by electrostatic interactions and its structure already resembles that of the final complex, but the proteins are not yet close and oriented enough for short-range interactions.

Association of a Protein Complex

While the major part of the activation free energy is required for desolvation of the binding interface as a prerequisite for the formation of specific short-range interactions, further intermediate states are postulated to occur on the pathway of complex formation (Fig. 1). Prior to the transition state, the two proteins diffuse in solution statistically until they enter a steering region, in which the progression along the association pathway is actively steered toward complex formation (Fig. 2). The forces important within this region are mainly electrostatic in nature, with nonspecific hydrophobic interactions contributing as well to steer association. Analysis of the contribution of electrostatic forces to the rate of association clearly indicates that their contribution steams from guiding the two proteins toward the transition state; from stabilizing the pre-transition-state encounter complex, in which the binding interface is still largely solvated; and from lowering the free energy of the transition state. Calculations of a three-dimensional energy landscape of these forces shows electrostatic steering by charged residues, which provides an energy funnel directed toward formation of the final complex [6]. At physiological salt concentrations this funnel extends to less then 20 Å of inter-protein distance and fades rapidly upon rotation (at 60° rotation from the bound conformation, all electrostatic steering is lost; see Fig. 2). It was shown that charged "hot spot" residues have the largest effect on the size and depth of these energy funnels. Potential "hot spot" residues can now be identified computationally, making it possible to engineer pairs of proteins with much higher rates of association and affinity.

A second mechanism that potentially steers association is a partial desolvation of inter-protein hydrophobic surfaces. This effect plays a significant role in all association processes, but becomes particularly dominant for complexes, in which one of the reactants is neutral or weakly charged. The interaction provides a slowly varying attractive force over a small but significant region of the molecular surface. In complexes with no strong charge complementarity, this region surrounds the binding site, and the orientation of the ligand in the encounter conformation with the lowest desolvation free energy is presented in a conformation similar to the formed complex. While the electrostatic contribution toward faster association can be easily verified from mutational studies and the effect of the ionic strength on k_{on}, the contribution of desolvation effects can be assessed only from theoretical calculations [11]. The reason that mutation studies rarely identify noncharged residues, which significantly contribute to k_{on}, may be attributed to the small contribution of individual side chains to desolvation-induced association, as this is more of a global effect of the protein.

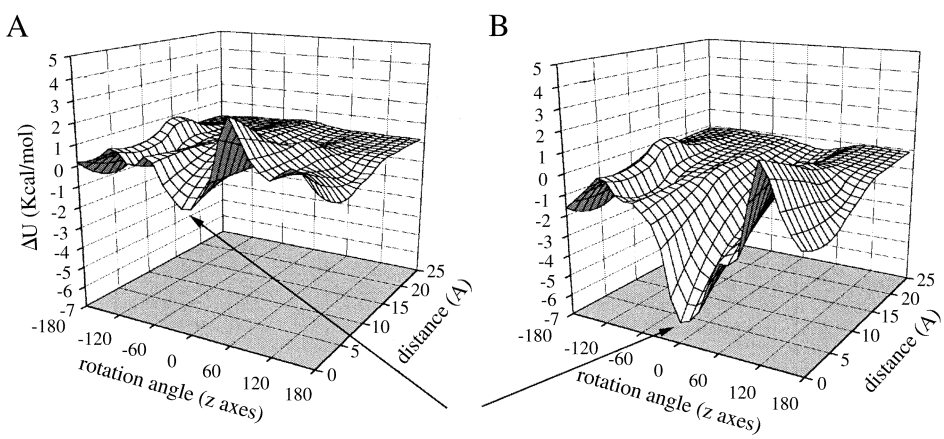


Figure 2 Three-dimensional energy landscape of the association between wt TEM1-β-lactamase with wt BLIP (A) and a much faster binding mutant (B). For demonstration, only the z-angle rotation is shown. The magnitude of the Debye-Hückel energy of interaction (ΔU) is plotted in three dimensions versus the distance and the relative rotation angle between the proteins. The arrows point at the 0° rotation angle, which is the X-ray crystallographic structure of the TEM1/BLIP complex. For details on the calculations employed, see Selzer and Schreiber [6].

Dissociation of a Protein Complex

Dissociation is a first-order reaction, the rate of which is independent of the concentration of the proteins. While the rate of association is a function of a fixed basal rate and a variable contribution of electrostatic nature, the rate of dissociation depends on the simultaneous breaking of many short-range interactions forming the protein–protein interface. These include hydrophobic and van der Waals interactions, H bonds, and salt bridges. The importance of the different interactions toward stabilizing the bound conformation depends on the location within the interface and its specific environment. Thus, individual charge–charge interactions seem to contribute little, as the gained interactions only barely compensate the free energy required for desolvation of the charged groups. However, if a network of charge–charge interactions is formed, a positive contribution toward binding is regained. H bonds seem to have a relatively small, but constant, contribution toward binding [12,13]. The effect of hydrophobic and van der Waals interactions was estimated from the buried surface area (nonpolar and total). However, no good absolute estimations have been obtained. The most intriguing question relates to the nature of "hot spots," which are residues that, upon mutation, cause a large shift in complex stability (reducing binding affinity by up to 10,000-fold). While no clear physical definition for "hot spot" residues has been formulated, they seem to be located at positions that are not water accessible [14,15]. Thus, the interface can be crudely divided into an outer ring of residues, which form some kind of a seal, and inner residues, which are fully stripped from solvent molecules. However, only a few of these fully buried residues are "hot spots," and the structural and energetic bases of "hot spot" residues are not yet clear.

Summary

This chapter discusses the energy landscape separating the unbound from the bound state of protein–protein interactions. Along the association pathway, an unstable diffusion encounter complex is formed prior to the transition state. Long-range electrostatic forces play a major role in stabilizing both the encounter complex and the transition state, thereby effectively steering the association process. In the transition state, the two proteins are correctly orientated toward each other and the interface is just being desolvated, so that subsequent short-range interactions can be formed to stabilize the complex. Accordingly, the free activation energy required to reach the transition state mostly stems from the energetically costly process of surface desolvation (especially of charged residues). Additional intermediates are located past the transition state. For these intermediates, part of the protein–protein interface is already formed, and the proteins are committed to evolving into the final complex. These pre-complexes are often difficult to track experimentally, thus it is reasonable to assume that they are more abundant than what has been reported so far.

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CHAPTER 6

Antibody–Antigen Recognition and Conformational Changes

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Introduction

Our understanding of the structural aspects of the recognition of antigens by antibodies has grown rapidly since the first structure of an antibody Fab fragment was determined in 1973 [1]. As of January, 2003, the Brookhaven Protein Data Bank [2] had 384 entries for X-ray or nuclear magnetic resonance (NMR) structures of antibodies, Fab, Fv, V_L, or V_H fragments. Of these entries, 197 are for Fab or Fv fragments bound to their antigens, and 43 of the Fab or Fv structures are available in both their free and antigen-bound forms. Five structures are now available for intact immunoglobulins [3–7], two of which contain visible electron density for the highly flexible hinge region [6,7]. Structures have been determined for antibodies derived from humans, mice, rats, camels, and llamas, as well as for genetically modified antibody fragments that have been "humanized," "camelized," or engineered as single-chain Fv fragments. The antigens recognized by these antibodies include small haptens, peptides, DNA, carbohydrate, and protein. Some of these antibodies are of chemical or medical importance, such as those that catalyze chemical reactions [8–11], neutralize viruses [7,12–24], or recognize tumors [25–31]. This wealth of structural information has proven invaluable in the fields of antibody engineering and catalytic antibody generation and in the development of effective antibody-based drug therapies.

Antibody Architecture

Antibodies can be rapidly tailored to accommodate almost any foreign antigen by a remarkable process whereby a very large number of different light and heavy chains are formed and then paired to generate the intact antibody. The antibody heavy-chain genes are created by V(D)J recombination [32], where the gene is generated by the recombination of the variable (V_H), diversity (D), and joining (J_H) segments. Extra nucleotides (N and P) can also be added at each recombination site. The light-chain genes are assembled in a similar fashion from a V_L and a J_L segment, with further N additions. For humans, there are 51 V_H [33,34] , 6 J_H [35], and 27 D [36,37] segments, resulting in over 8000 different possible heavy-chain gene combinations. There are 40 Vκ [38], 5 Jκ [39], 30 Vλ [37], and 4 Jλ [40] genes, plus N additions, that can be combined for at least 200κ and 120λ chain combinations. These heavy and light chains can then be further modified by somatic mutations. The immunoglobulin G (IgG) class of antibodies is the best studied structurally of all the different antibody classes, although IgA and IgM Fab fragments have also been structurally elucidated.

In the IgG antibody, the light (~25,000 kDa) and heavy (~50,000 kDa) chains pair to form three distinct protein domains: the Fc fragment and two Fab fragments (Fig. 1). There is a high degree of mobility between these three modules, which has led to some difficulty in the crystallization of intact antibodies, although, as discussed previously, some progress has been made in the crystallization of these molecules. Most of the structures currently available are for Fab or Fv fragments. Within the Fab fragment, mobility is also possible between the variable (V_L–V_H) and the constant (C_L–C_H1) domains around the "elbow angle." This angle can vary between at least 127° and 224° [41]. There can also be some variability in how the V_L and V_H domains pair with each other [41]. The constant domain region of

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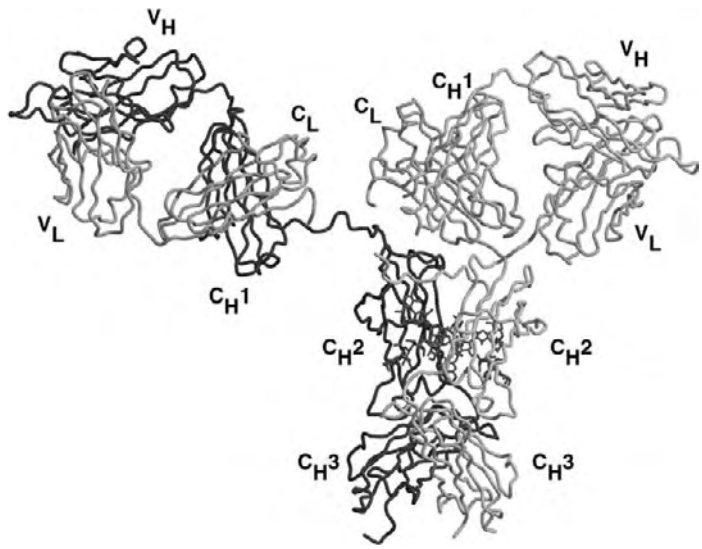


Figure 1 Crystal structure for the intact, HIV-1 neutralizing, human antibody b12 [7] (PDB code 1HZH). The two light chains are colored cyan, and the two heavy chains are colored blue and yellow; carbohydrate in the Fc portion is colored red. The two Fab domains are shown at the top of the figure (V_L, V_H, C_L, C_H1), connected to the Fc domain (C_H2, C_H3 dimer) by flexible protein linkers that allow the two Fab arms a great deal of flexibility with respect to each other and the Fc domain. All figures were prepared with MOLSCRIPT [70] and rendered with Raster3D [71,72]. For color figures, see CD-ROM version of *Handbook of Cell Signaling*.

the Fab fragment is fairly rigid, but the Fc fragments can show intra-fragment mobility (see Chapter 8). The Fab fragment is the portion of the antibody that recognizes antigen, and it does this via six loops termed the *hypervariable* or *complementarity-determining region* (CDR) loops. These loops can vary in sequence and length in order to optimize their specificity and affinity for antigen. Some studies have suggested that different size antigens may be recognized preferentially by distinct combinations of CDR loops that result in different binding site topologies [42,43]. For example, small haptens are frequently bound into deep pockets, peptides are bound in grooves, and large proteins usually bind a relatively flat binding site, but many examples exist that are exceptions to these broad generalizations. Although the CDR loops are the most variable portion of the Fab fragment, the structures of the L1, L2, L3, H1, and H2 loops have been classified into a limited number of defined or canonical conformations that can be predicted by the occurrence of particular amino acids at key structural positions [44–47]. The conformation of the base of the H3 loop can also be predicted with some reliability [46,48]; however, the portion of H3 that extends beyond the framework region of the Fab is too variable in sequence and structure to be predicted yet.

Conformational Changes

Examination of Fab or Fv structures in both the bound and free forms shows that, in some but not all antibodies, conformational changes accompany antigen binding. It is not known for certain whether these changes are always induced by antigen binding, or whether the unliganded Fab fragment can exist in multiple conformations in solution, with the binding of antigen stabilizing one of these already preferred conformations. Kinetic stopped-flow fluorescence experiments support the theory of a flexible antigen combining site [49], although no evidence for multiple conformations of unliganded antibodies has been observed yet in crystal structure analyses. The conformational changes can consist of side-chain rearrangements, CDR main-chain rearrangements, segmental movements of the CDR loops, changes in the relative orientation of the V_L–V_H domains, and combinations of some or all of the above.

Side-Chain Rearrangements

An interesting example of a side-chain rearrangement is seen in the anti-progesterone Fab DB3 [50–52]. In this antibody, the Trp^{H100} side chain occupies the antigen-binding site in the unliganded Fab and then moves out of the way in order to allow progesterone to bind (Fig. 2a). Here, this Trp side chain acts as a "surrogate" ligand for this antibody in the absence of progesterone, and its movement completely alters the shape of the binding site when comparing the free and bound forms (Fig. 3).

Main-Chain Rearrangements and Segmental Shifts

Main-chain CDR movements have been seen in all CDR loops with the exception of L2. These main-chain movements

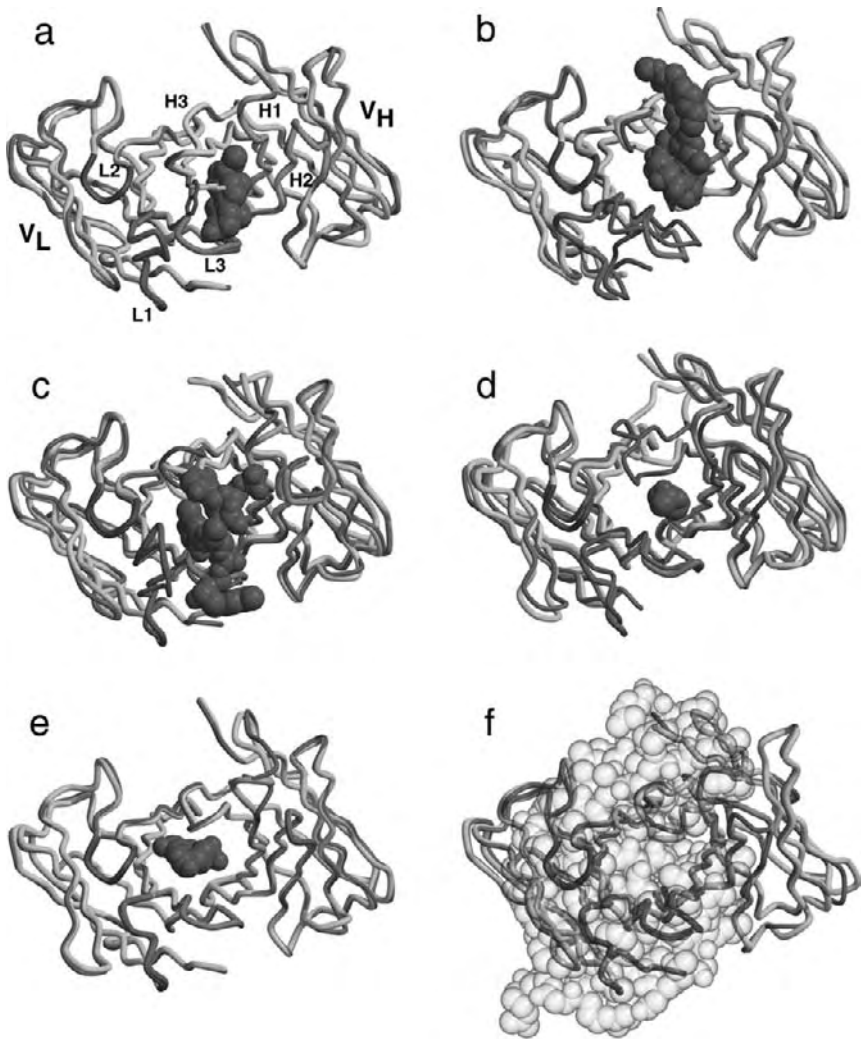


Figure 2 Side-chain and main-chain conformational changes in antibodies. (a) Superposition of the native and antigen-bound coordinates for the anti-progesterone Fab DB3 [50–52] (PDB codes 1DBA, 1DBB). The view is looking down into the antigen-binding site, with the Fab light chain on the left, the unliganded DB3 in light gray, and the liganded DB3 in darker gray. The CDR loops for the antigen-bound Fab are colored in red, while the CDR loops for the unliganded Fab are colored by CDR, with CDR L1, L2, L3, H1, H2, and H3 being colored blue, purple, green, cyan, pink, and yellow, respectively. The progesterone antigen is shown in a red CPK rendering. In DB3, there are no significant main-chain movements upon antigen binding; however, Trp^{H100} is located in the binding site in the unliganded Fab (yellow), while it rotates out of the binding site when progesterone is bound (red). (b) Superposition of the native and antigen-bound coordinates for the anti-tumor Fab BR96 [28,30] (PDB codes 1UCB, 1CLY). The view and coloring for this and all the panels are as for (a). The bound antigen (red CPK) is the Lewis Y nonoate methyl ester. BR96 shows large conformational changes in both the L1 (blue) and L3 (green) CDR loops. (c) Superposition of the native and antigen-bound coordinates for the anti-ssDNA Fab BV04-01 [55] (PDB codes 1NBV, 1CBV). The bound antigen (red CPK) is tri-thymidine. BV04-01 shows large conformational changes in the L1 (blue) and H3 (yellow) CDR loops. (d) Superposition of the native and antigen-bound coordinates for the catalytic antibody CNJ206 with antigen para-nitrophenyl methyl-phosphonate [59,65] (PDB codes 2GFB, 1KNO). This Fab shows large conformational changes in CDRs L3 (green) and H3 (yellow). (e) Superposition of the native and antigen-bound coordinates for the mature catalytic antibody 48G7 [60] (PDB codes 1AJ7, 2RCS). The bound antigen (red CPK) is 5-(*para*-nitrophenyl-phosphonate)-pentanoic acid. H2 (pink) undergoes large conformational changes. (f) Superposition of the native and antigen-bound coordinates for the anti-lysozyme antibody HyHEL-63 [64] (PDB codes 1DQQ, 1DQJ). The lysozyme antigen is shown as a transparent CPK model in order to see the footprint of the protein on the CDR loops underneath. This Fab has conformational changes in the H2 (pink) and H3 (yellow) loops.

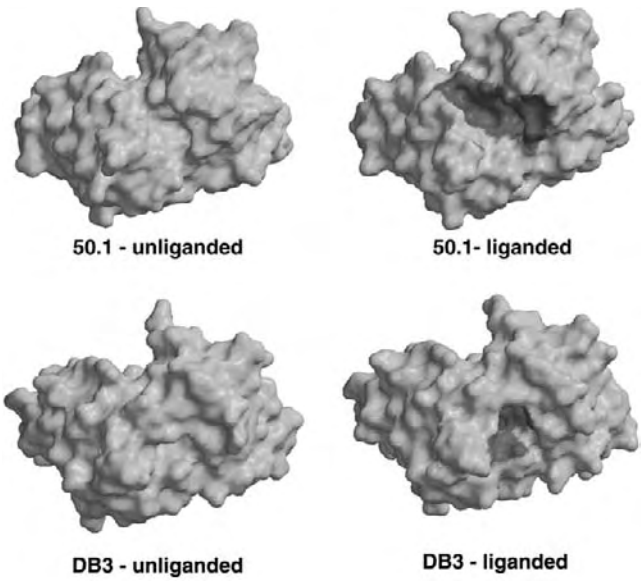


Figure 3 Topographical changes in antigen binding sites due to conformational changes. Fab 50.1 (top panel) is shown before (left) and after (right) antigen binding. The view is that looking down into the antigen binding site, as in Fig. 2. The peptide binding site is highlighted in purple on the liganded antibody surface, with peptide antigen omitted from the figure for clarity. The combination of an H3 rearrangement and a large V_H–V_L domain rearrangment have combined to substantially lengthen and widen the groove for peptide binding. Fab DB3 (bottom panel) is shown before (left) and after (right) antigen binding. The approximate binding site for progesterone is highlighted in purple. The structural change required for its small hapten to bind is the opening of a small pocket for the binding or steroids such as progesterone. This change is mainly due to a tryptophan residue that fills the pocket in the unliganded Fab but then moves out of the way to allow steroids to bind. Solid surfaces were calculated with GRASP [73].

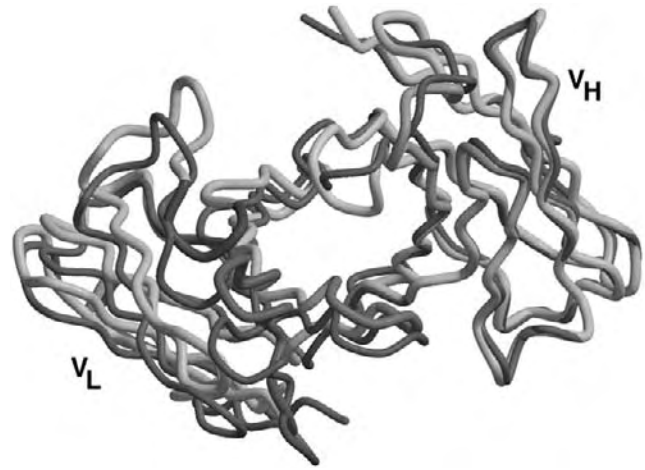


Figure 4 Conformational changes in Fab 50.1 [20,61] (PDB codes 1GGI, 1GGB). The unliganded and liganded Fab 50.1 structures are superimposed using V_H framework residues. The view is looking down into the antigen-binding site, with the Fab light chain on the left and the unliganded and liganded V_H domains in light and dark gray, respectively. CDR loops for the V_H domain are colored by CDR as in Fig. 2. The V_L domains from the liganded and unliganded Fabs are colored blue and cyan, respectively. The V_L domains differ in their relative orientation to the V_H domain by about 15°. Large conformational changes in the H3 CDR loop (yellow and red) are also visible. These combined conformational changes serve to widen and lengthen the binding groove for the peptide antigen.

can either consist of a rearrangement of CDR conformation, or a simple rigid-body, segmental shift of the CDR loop. The largest conformational changes in L1 are seen for BR96 [28,30], where the L1 CDR loop moves as much as 10 Å at the tip as it folds toward the antigen (Fig. 2b). Other Fabs showing L1 CDR loop movements upon antigen binding include the anti-hemaglutinin peptide antibody 17/9 [53,54], the anti-DNA antibody BV04-01 [55] (Fig. 2c), the anti-myohemerythrin peptide antibody B13I2 [56], the anti-HIV protease antibody F11.2.32 [57], and the anti-rhinovirus antibody 17-IA [18]. These antibodies all have rather long L1 CDRs (inserts after residue 27 of 4–6 residues), with the exception of 17-IA, which has no inserted residues. Small changes have been seen in the L3 CDR loops, where again BR96 shows the largest movement (up to 2.6 Å for the main chain; Fig. 2b), with the anti-hapten B1-8 [58] and catalytic antibody CNJ206 [59] (Fig. 2d) also showing conformational changes here. The largest changes in the H1 CDR loops are observed in the catalytic antibody 48g7 [60] in both its germline and mature forms (Fig. 2e), and also in the anti-HIV-1-peptide antibody 50.1 [61] (Fig. 4). The largest H2 changes are seen for the germline catalytic antibody AZ-28 [62], the anti-lysozyme antibodies HuLys11 [63] and HyHEL63 [64] (Fig. 2f), and the feline peritonitis virus antibody 409.5.3 [13]. The largest and most frequent CDR conformational changes are found in CDR H3. In an analysis of the 36 Fab or Fv fragments that exist in both free and bound forms, out of 115 total comparisons (some Fabs or Fvs have more than one molecule in the crystallographic asymmetric unit or have been solved in multiple crystal forms) 13, 5, 9, 13, and 38 pairs of structures were shown to have significant conformational changes (total rmsd for CDR residues >1.0 Å) in CDR loops L1, L3, H1, H2, and H3, respectively. The largest H3 movement seen thus far is for the catalytic antibody CNJ206 [65], where the tip of the H3 loop has a main-chain rearrangement of about 16 Å. Catalytic antibody 5C8 [66] and the anti-HIV-1-peptide antibody 50.1 [61] also show large changes in this CDR loop. The H3 CDR loops of CNJ206, 5C8, and 50.1 are not long; CNJ206 and 5C8 both have only two amino-acid inserts after residue H100, while 50.1 has a three amino-acid deletion in this region.

V_L–V_H Rearrangements

Rearrangements are also observed in the relative orientation of the V_L to the V_H domains upon antigen binding. The largest seen thus far is for Fab 50.1 [61], with a change of around 15° (Fig. 4). Other large changes are seen for CNJ206 (6.8°) [65], BV04-01 (7.5°) [55], and 13B5 (8.1°) [67]. The domain rearrangements, in combination with the changes in CDR conformation discussed in the previous section, can combine to create changes in the size and shape of the antigen-binding site (Fig. 3). These changes can be small, such as the creation of a small binding pocket for progesterone in DB3 [50–52], or larger, such as the elongation and widening of a binding groove for the HIV-1 V3 loop peptide in 50.1 [20, 61].

Conclusion

Antibodies are wonderfully malleable molecules that can vary in both their sequence and structure in order to recognize an infinite number of potential antigens. The extensive diversity of the V(D)J recombination along with the fixed pool of variable genes leads to a sufficient number of different specificities to bind almost any antigen with high affinity. Structural studies of these molecules have led to a better understanding of their mechanism of action and allowed for improvements in their natural design in order to produce antibodies that are useful in many fields, such as catalytic antibody production. Catalytic antibodies are now available to carry out many different chemical reactions, some of which have no naturally occurring enzyme catalyst and are being used more and more widely as tools by synthetic chemists [8–10,68,69].

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CHAPTER 7

Binding Energetics in Antigen–Antibody Interfaces

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Introduction

Antibodies may be regarded as the products of a protein engineering system developed by nature for generating a virtually unlimited repertoire of complementary molecular surfaces and, as such, constitute an excellent model for elucidating the principles governing macromolecular recognition. We have used X-ray crystallography and site-directed mutagenesis to understand how structural features contribute to the affinity and specificity of antigen–antibody binding reactions.

Antibody molecules are composed of two identical polypeptide chains of approximately 450 amino acids (the heavy, or H, chains) covalently linked through disulfide bridges to two identical polypeptide chains of about 250 residues (the light, or L, chains). Based on amino-acid sequence comparisons, the H and L chains may be divided into N-terminal-variable (V) and C-terminal-constant (C) portions. Each H chain contains four domains (V_H, C_H1, C_H2, C_H3), each of which contains two anti-parallel β-sheets connected by a disulfide, while each L chain consists of two such domains (V_L, C_L). These β-sheet domains are structurally very similar and hence have been termed the *immunoglobulin fold* [1]. The V_H and V_L domains each contain three segments, or loops, which connect the β-strands and are highly variable in length and sequence among different antibodies. These so-called complementarity-determining regions (CDRs) lie in close spatial proximity on the surface of the V domains and determine the precise conformation of the combining site. In this way, the CDRs confer specific binding activity to the antibody molecule. The central paradigm of antigen–antibody recognition is that the three-dimensional structure formed by the six CDRs recognizes and binds a complementary surface, or epitope, on the antigen.

X-ray crystallographic studies of over 30 antigen–antibody complexes involving protein antigens [2–12] have provided much valuable information on the molecular architecture of protein–protein interfaces, including the identity of contacting residues, the amount of buried surface area, the number and type of hydrogen bonds, and the magnitude of conformational changes associated with complex formation. However, the basic principles governing antigen–antibody and protein–protein interactions have remained elusive [13–20], with important fundamental problems relating to the recognition process still to be solved: What are the relative contributions of hydrophobicity, surface complementarity, and hydrogen bonding to the energetics and mechanism of binding? To what extent do the strengths of individual bonding interactions depend on their local environment and overall location in the interface? What is the role of solvent in complex stabilization? What is the contribution of conformational flexibility, or structural plasticity, in antigen–antibody recognition? Is productive binding mediated by a distinct subset of combining site residues, or are complex cooperative interactions involving both contacting and non-contacting residues responsible for the observed affinities? What determines whether an interface residue is a so-called "hot spot" for ligand binding (i.e., a residue that contributes a disproportionately large fraction of the binding free energy)? How are potentially disruptive amino-acid changes in the interface (for example, ones that create "holes") accommodated?

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What is the structural basis of affinity maturation, whereby somatic mutations in antibody genes that confer increased affinity for antigen are selected? Finally, is it possible to predict *ab initio* the effects of a given amino acid substitution on antibody affinity and specificity? In this review, we describe recent attempts to construct energetic maps of antigen–antibody interfaces using X-ray crystallography coupled with site-directed mutagenesis.

Thermodynamic Mapping of Antigen–Antibody Interfaces

In contrast to the wealth of structural information on antigen–antibody and other protein–protein interfaces, the available data on the thermodynamics of the association reactions are far more limited. Indeed, our current view of the energetics of protein–protein association is largely based on detailed mutagenesis and binding studies of only a few complexes [15]. We have studied the binding of monoclonal antibody D1.3 to two structurally distinct ligands: its cognate antigen, hen egg white lysozyme (HEL), and the anti-D1.3 antibody E5.2. The crystal structure of the complex formed by D1.3 with HEL has been determined to a nominal resolution of 1.8 Å [21]. In addition, the structure of the complex between D1.3 and E5.2 is known to 1.9-Å resolution [12]. Surprisingly, D1.3 contacts HEL and E5.2 through essentially the same set of combining site residues (and most of the same atoms). Thus, of the 18 D1.3 residues that contact E5.2 and the 17 that contact HEL, 14 are in contact with both E5.2 and HEL. In this review, we will focus on the D1.3-HEL and D1.3-E5.2 complexes, as these currently represent the most extensively studied models for antigen–antibody recognition.

To evaluate the relative contribution of individual residues to stabilization in the D1.3–HEL and D1.3–E5.2 complexes, alanine-scanning mutagenesis was performed in the D1.3 combining site. In total, 16 single alanine substitutions were introduced and their effects on affinity for HEL and for E5.2 were measured using surface plasmon resonance detection, fluorescence quench titration, or sedimentation equilibrium [22]. Mutagenesis of D1.3 residues in contact with HEL in the crystal structure of the D1.3–HEL complex revealed that residues in V_LCDR1 and V_HCDR3 contribute more to binding than residues in V_LCDR2, V_LCDR3, V_HCDR1, and V_HCDR2. By far the greatest reductions in affinity ($\Delta G_{mutant} - \Delta G_{wild\ type} > 2.5$ kcal/mol) occurred on substituting three residues: V_LTrp92, V_HAsp100, and V_HTyr101. By replacing V_LTrp92 with residues bearing increasingly smaller side chains and determining the crystal structures and thermodynamic parameters of binding for each of the resulting mutant D1.3–HEL complexes, we demonstrated a correlation between the binding free energy and the apolar surface area that corresponds to 21 cal mol^{-1} $Å^{-2}$ [23]. This estimate of the hydrophobic effect in a protein–protein interface is in excellent agreement with predictions based on transfer free-energy values for small hydrophobic solutes. Significant effects on HEL binding (1.0 to 2.0 kcal/mol) were also seen for substitutions at D1.3 positions V_LTyr32 and V_HGlu98, even though the latter is not involved in direct contacts with HEL. Mutations at nine other contact positions (V_LHis30, V_LTyr49, V_LTyr50, V_LSer93, V_HTyr32, V_HTrp52, V_HAsp54, V_HAsp58, and V_HArg99) had little or no effect (<1.0 kcal/mol). Therefore, the binding of HEL by D1.3 is largely mediated by only 5 of the 14 residues tested.

For the interaction of D1.3 with E5.2, affinity measurements showed that V_HCDR2, V_HCDR3, and V_LCDR1 of D1.3 are more important for binding E5.2 than V_HCDR1, V_LCDR2, and V_LCDR3 [22]. Overall, D1.3 V_H residues appear to contribute more to the free energy of binding than V_L residues, as the most destabilizing alanine substitutions (>2.5 kcal/mol) are located in V_HCDR2 (Trp52Ala and Asp54Ala) and V_HCDR3 (Glu98Ala, Asp100Ala, and Tyr101Ala). Significant effects (1.0 to 2.0 kcal/mol) were also observed for the following contact residues: His30 and Tyr32 in V_LCDR1, Tyr49 in V_LCDR2, Tyr32 in V_HCDR1, Asn56 and Asp58 in V_HCDR2, and Aeg99 in V_HCDR3. Mutations at positions V_LTyr50, V_LTrp92, and V_HThr30 had little or no effect (<1.0 kcal/mol). Thus, of the 15 contact residues tested, 12 make significant contributions to binding E5.2.

On the basis of extensive mutational analysis of the complex between human growth hormone and its receptor, Wells and colleagues [24–26] proposed that the formation of specific protein–protein complexes is mediated by only a few productive interactions or "hot spots" that dominate the energetics of association. Consistent with this idea, our analysis of the D1.3-HEL interaction revealed that only a small subset of the total combining site residues of D1.3 appears to account for a large proportion of the binding energy; most residues (9 of 14) make little or no apparent net contribution (<1.0 kcal/mol). This contrasts with the interaction of D1.3 with E5.2 in which nearly all the contacting residues play a demonstrable role in binding ligand (>1.0 kcal/mol), even though a number of hot spots ($\Delta\Delta G > 2.5$ kcal/mol) are clearly present. Therefore, stabilization of the D1.3–E5.2 complex is achieved by the accumulation of many productive interactions of varying strengths over the entire interface between the two proteins.

The functional surfaces of D1.3 involved in binding HEL and E5.2 mapped onto its three-dimensional structure are shown in Figs. 1A and 1B, respectively. With the exception of V_LTrp92, which lies at the periphery, the residues of D1.3 most important for binding HEL (V_HTyr101, V_HAsp100, V_LTyr32, and V_HGlu98) are located in a contiguous patch at the center of the combining site. Residues at the periphery make only minor contributions to the binding energy. A similar pattern is observed for the D1.3–E5.2 complex, with the most important residues (V_LTyr32, V_HTrp52, V_HAsp54, V_HGlu98, V_HAsp100, and V_HTyr101) forming a central band of key contacts. For the most part, however, the hot spots for the two interactions do not overlap. For instance, alanine substitution at position V_LTrp92 of D1.3 produces a 100-fold decrease in affinity for HEL but does not appreciably affect binding to E5.2. Conversely, the V_HTrp52Ala

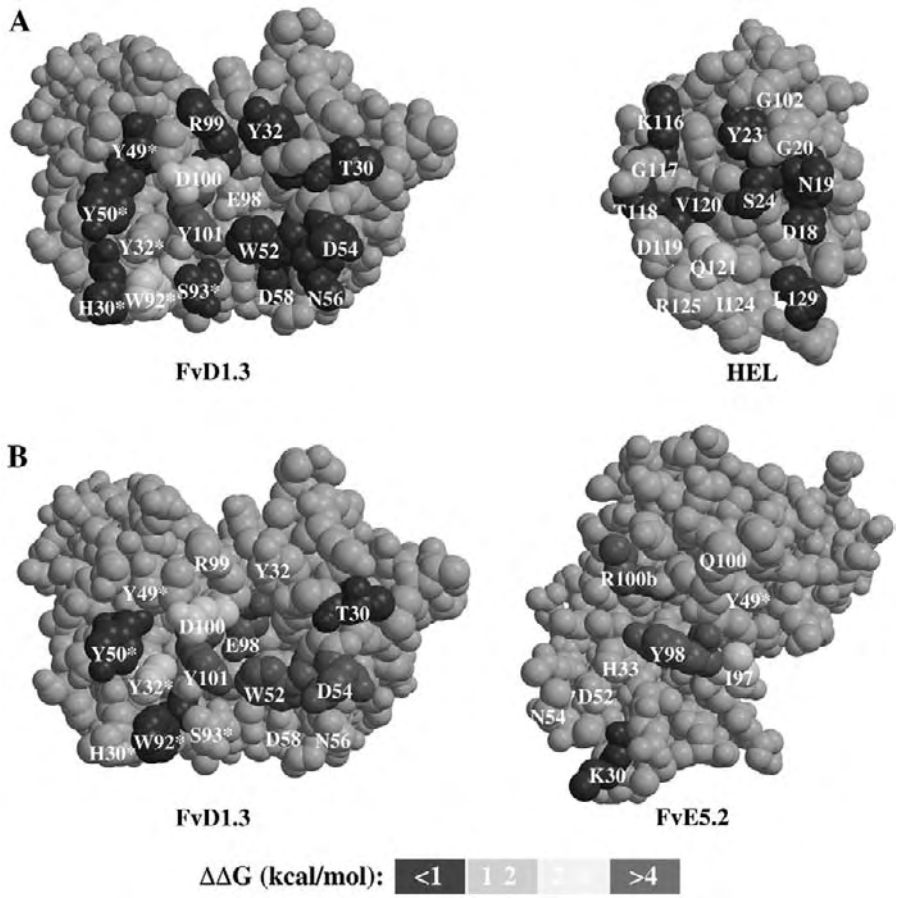


Figure 1 Energetic maps of antigen–antibody interfaces. (A) Space-filling model of the surface of D1.3 (left) in contact with HEL and of the surface of HEL (right) in contact with D1.3. V_L residues are marked with asterisks. The two proteins are oriented such that they may be docked by folding the page along a vertical axis between the components. Residues are color-coded according to the loss of binding free energy upon alanine substitution. (B) Model of the surface of D1.3 (left) in contact with E5.2 and of the surface of E5.2 (right) in contact with D1.3. Residues are colored as in (A).

substitution decreases affinity for E5.2 1000-fold but has virtually no effect on binding to HEL. Only substitutions V_HAsp100Ala and V_HTyr101Ala greatly affect the binding to both HEL and E5.2. We therefore conclude that a single set of contact residues on D1.3 binds HEL and FvE5.2 in energetically different ways. Thus, although D1.3 recognizes these two proteins in ways that are structurally very similar, this similarity extends only partially to the functional epitopes.

To probe the relative contribution to binding of HEL residues in contact with D1.3 in the crystal structure of the FvD1.3–HEL complex, 12 non-glycine HEL residues were individually mutated to alanine and their affinities for wild-type D1.3 measured [27]. Significant decreases in binding ($\Delta\Delta G > 1$ kcal/mol) were only observed for substitutions at four contact positions: Gln121, Ile124, Arg125, and Asp119. The most destabilizing mutation was at position Gln121 ($\Delta\Delta G = 2.9$ kcal/mol). In the wild-type structure, Gln121 penetrates a hydrophobic pocket, where it is surrounded by the aromatic side chains of V_LTyr32, V_LTrp92, and V_HTyr101 [16]. Mutations at the remaining eight contact positions (Asp18, Asn19, Tyr23, Ser24, Lys116, Thr118, Val120, and Leu129) had little or no effect ($\Delta\Delta G < 1$ kcal/mol). Therefore, for both the D1.3 and HEL sides of this interface, only small subsets of the total contacting residues appear to account for a large portion of the binding energy.

As shown in Fig. 1A, the residues of HEL most important for binding D1.3 (Asp119, Gln121, Ile124, and Arg125) form a contiguous patch located at the periphery of the surface contacted by the antibody [27]. Hot spot residues on the D1.3 side of the interface generally correspond to hot spot positions on the HEL side. For example, HEL hot spot residues Gln121 ($\Delta\Delta G = 2.9$ kcal/mol) and Arg125 (1.8 kcal/mol) contact D1.3 hot spot residue V_LTrp92 (3.3 kcal/mol); in addition, $Gln121_{HEL}$ contacts V_LTyr32 (1.7 kcal/mol) and V_HTyr101 (>4.0 kcal/mol). Similarly, functionally less important D1.3 and HEL residues tend to be juxtaposed in the antigen–antibody interface: $Asp18_{HEL}$ ($\Delta\Delta G = 0.3$ kcal/mol) and Thr118 (0.8 kcal/mol) interact with D1.3 V_LTyr50 (0.5 kcal/mol) and V_HTrp52 (0.9 kcal/mol), respectively.

To investigate the apparent contribution of E5.2 residues to stabilization of the D1.3–E5.2 complex, single alanine substitutions were introduced at 9 of 21 positions in the combining site of E5.2 involved in contacts with D1.3, and the affinity of the mutants for wild-type D1.3 [28] was measured. The most destabilizing substitutions are located at positions V_HTyr98 and V_HArg100b ($\Delta\Delta G > 4.0$ kcal/mol). Substitutions at the other 7 positions tested (V_LTyr49, V_HLys30, V_HHis33, V_HAsp52, V_HAsn54, V_HIle97, and V_HGln100) also resulted in significant effects on binding (1.2 to 2.8 kcal/mol). When the residues of D1.3 and E5.2 important in complex stabilization were mapped onto the three-dimensional structure of each antibody, we observed that hot spot positions on the E5.2 side of the interface generally corresponded to hot spots on the D1.3 side (Fig. 1B), as in the D1.3–HEL interface (Fig. 1A). This complementarity of functional epitopes is in agreement with the observation that energetically critical regions on human growth hormone match those on its corresponding receptor [24–26]. In the hormone receptor case, however, the functional epitopes pack together to form a hydrophobic core surrounded by hydrophilic residues, with substantial reductions in affinity occurring only on substitution of the nonpolar ones. In contrast, our analysis of the D1.3–E5.2 and D1.3–HEL systems shows that both polar (e.g., D1.3 residues V_HAsp54, V_HGlu98, and V_HAsp100) and nonpolar residues (e.g., D1.3 residues V_LTrp92 and V_HTrp52) play a prominent role in complex stabilization and that there is not a clear segregation of polar residues at the periphery of the interface and nonpolar ones at the core (Fig. 1).

Conclusions

On the basis of these, and related [15,17,20], studies, two broad categories of protein–protein interfaces may be defined: (1) ones in which ligand binding is mediated by a small subset of contact residues, and (2) ones in which the free energy of binding arises from many productive interactions distributed over the entire protein–protein interface. In addition, each of these categories may be further subdivided into: (1) ones that resemble cross-sections through folded proteins in which hydrophobic residues are in the interior and hydrophilic ones at the periphery and in which productive binding is mediated largely by the former, and (2) ones in which polar and nonpolar residues are evenly distributed throughout the interface and in which both residue types make comparable contributions to complex stabilization.

These results demonstrate that considerable caution should be exercised when attempting to estimate the strengths of specific interactions in an antigen–antibody (or other) protein–protein interface on the basis of three-dimensional structures alone. The simple fact that two residues make direct contacts in a protein–protein interface does not necessarily imply that there exists a net productive interaction between them. Rather, the majority of such contacts may be energetically neutral, as in the D1.3–HEL complex. Although recent computational methods for predicting the strengths of these interactions appear promising [20,29–32], information on the relative contribution of individual residues to complex stabilization can only be reliably obtained at the present time through actual affinity measurements of site-directed mutants of the interacting species.

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CHAPTER 8

Immunoglobulin–Fc Receptor Interactions

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Introduction

Antibodies are multifunctional protein molecules capable not only of recognizing and binding to foreign antigens but also activating a range of molecular and cellular responses in the host that lead to neutralization or destruction of the invading organism or foreign material. Antibodies, or immunoglobulins, are built upon a common four-chain structure of two heavy and two light chains, as exemplified by immunoglobulin G (IgG) (Fig. 1A), each chain consisting of a tandem array of domains. Antigen binding occurs at the V (variable) domains, which determine the specificity of the antibody for antigen, but the C (constant) domains of the heavy chain (in the Fc region; Fig. 1A) are responsible for the subsequent effector functions of the antibody.

The five classes of antibody (IgA, IgD, IgE, IgG, and IgM) are distinguished by the C domain sequences of their heavy chains (α, δ, ε, γ, and μ), each with a distinct range of effector functions and a specialized role in the body's immune system. Many of the cell-surface receptors for the Fc regions of these antibodies have been identified, but here we shall be concerned only with those for which the three-dimensional structures and their complexes with the antibody Fc are known, namely IgG- and IgE–receptor interactions. IgE, the antibody responsible for antiparasitic responses but nowadays better known for its association with allergic disease, differs from IgG, the principal serum antibody responsible for the secondary immune response to infection, in having an additional pair of domains in its Fc region in place of the flexible hinge region of IgG (Fig. 1B). The Fc regions of IgA and IgM, which can form dimers and pentamers, respectively, of the basic four-chain unit (and are stabilized by additional polypeptide chains), are more complex still, but the three-dimensional structures of these uncomplexed antibody Fc regions are still unknown.

To date, structural information is available for three distinct types of cell-surface Fc receptors. The first of these is the family consisting of the IgG Fc receptors, FcγRI, II (a and b), and III (a and b), as well as the IgE receptor FcεRI and IgA receptor FcαRI. All consist of an α-chain with two (three for FcγRI) extracellular, Ig-like, ligand-binding domains, either alone (FcγRIIa and b, IIIb) or associated with β and/or a pair of γ-chains (FcγRI, IIIa, FcεRI and FcαRI). (For reviews, see references [1–4].) The single-chain FcγRIIIb and four-chain FcεRI (the two for which crystal structures of their extracellular domains complexed with Fc are known) are shown schematically in Figs. 1C and D. Distinct from this family of receptors, however, are the neonatal IgG Fc receptor, FcRn, which is responsible for the transport of IgG across the placenta, and the low-affinity receptor for IgE, FcεRII (or CD23), which is involved in both allergen uptake by antigen-presenting cells and regulation of IgE synthesis by B cells. Whereas FcRn belongs to the class I major histocompatibility complex (MHC) family (Fig. 1E) [5], FcεRII is a trimeric C-type lectin with a wholly different molecular architecture and oligomeric structure (Fig. 1F) [6]. The nature of the interactions between these three different types of receptor and

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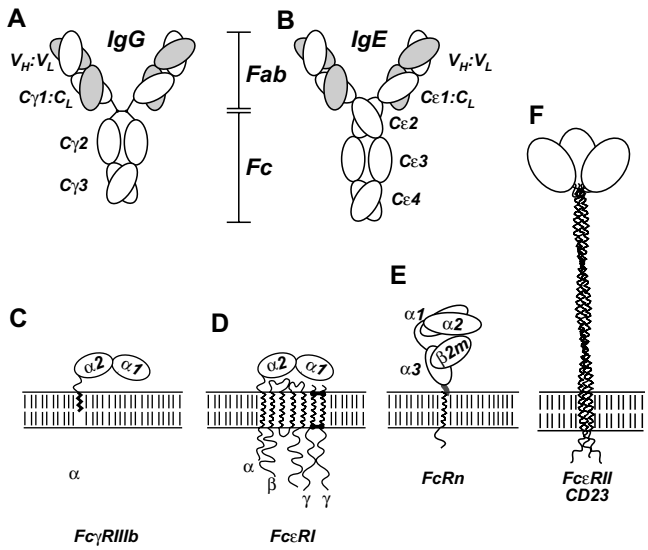


Figure 1 Schematic representations, drawn to scale, of the domain structures of: (A) IgG; (B) IgE; (C) GPI-anchored, low-affinity IgG receptor FcγRIIIb; (D) high-affinity IgE receptor FcεRI; (E) neonatal IgG receptor FcRn; and (F) low-affinity IgE receptor FcεRII (CD23). Molecules A through D consist of Ig or Ig-like domains, FcRn is class I MHC-like, and FcεRII consists of C-type lectin domains linked to the membrane and trimerised through an α-helical coiled-coil stalk.

Fc will be discussed first for the IgG and then for the IgE receptors.

IgG–Receptor Interactions

FcγR

Several crystal structures are now available for the extracellular domains of FcγRII [7–9] and FcγRIII [10,11], which show (together with FcεRI [12,13]) that they are all remarkably similar, as expected from their highly homologous sequences. There is an acute angle between the two Ig-like domains, which pack against each other around a hydrophobic interface. In the structure of the complex between FcγRIII and IgG Fc, first determined by Sondermann *et al*. in 2000 [10], loops from the α2 domain and part of the linker region between the two domains of the receptor protrude into the space between the two Cγ2 domains of the Fc just below the hinge region. Thus, there are two distinct regions of interaction, one on each of the heavy chains, which involve residues of the lower hinge and Cγ2 domains (Fig. 2A). Upon binding, the IgG Fc opens up slightly and the Cγ2 domains move apart compared with uncomplexed IgG Fc; at the same time, the angle between the two receptor domains increases (from 70 to 80°), compared with the free receptor structures [10]. The uncomplexed IgG Fc was initially two-fold symmetric and thus in principle might have been expected to bind to two receptors, but the distortion of this symmetry upon binding to the receptor, and the fact that the receptor lies on the approximate two-fold axis of the complex (Fig. 2A), ensures a stoichiometry of 1:1. This is essential if free IgG is not to cross-link receptor in the absence of antigen [14]. The orientation of the IgG Fc bound to the receptor clearly implies that the Fab arms of the IgG molecule must be bent at the hinge; the overall topology is depicted in Fig. 2A.

The structure of the FcγRIII/IgG Fc complex has subsequently been determined by others in a different crystal form [15], and the structure is virtually identical. In neither structure do the two N-linked oligosaccharide chains (at Asn297 in each Cγ2 domain) contribute directly to receptor binding, but they probably serve to stabilize the Fc domains in the binding region, as removal of carbohydrate from IgG Fc severely reduces its receptor binding capacity [16,17].

Because the extracellular Fc-binding domains of the FcγRI and FcγRII are so similar, (as are the Fc regions of the different subclasses of human IgG), the FcγR/IgG Fc complexes are all likely to be essentially similar in structure [9]. There are however, differences in affinity and binding kinetics between the different FcγR and the various human subclasses of IgG [1,2,18], and these presumably result from minor differences in the nature of the residues at the interface [9]. More intriguing is the striking difference between the affinity and kinetics of binding of IgE to FcεRI compared with IgG to its receptors; we shall return to this issue later.

FcRn

The neonatal Fc receptor closely resembles a class I MHC molecule, complete with a β2-microglobulin chain (β2-m), but the peptide-binding groove formed between the α1 and α2 domains is too narrow and is nonfunctional [5]. In the complex with IgG Fc, recently determined at higher resolution [19] following an earlier low-resolution study [20], FcRn interacts through residues of the α2 domain, with some contacts from β2-m. The region on Fc to which it binds is the cleft between the Cγ2 and Cγ3, distant from the FcγR binding site (Fig. 2B). IgG binds to FcRn with nanomolar affinity at acidic pH (in transport vesicles), but releases it at neutral pH (in the blood). The binding interface accounts for these properties, as it is both very extensive (1870 $Å^2$) and includes four salt bridges, three of which involve histidine residues on Fc and either aspartic or glutamic residues on FcRn. At $pH \leq 6.5$, the histidines are protonated and form salt bridges, whereas at $pH \geq 7.0$ they are neutral and the salt bridges are lost. The crystal structure also appears to show that quaternary structural differences resulting from binding to one Fc heavy chain alter the binding site on the other chain, thus accounting for the observed negative cooperativity in binding a second IgG molecule to the receptor. A common feature of IgG binding to both FcγR and FcRn, therefore, is a distortion of the two-fold symmetry inherent in the Fc region of the free antibody molecule, resulting in either a partial (FcRn) or total loss of Fc binding at the second site. Another similarity is the orientation of the Fc relative to the membrane (Fig. 2B), again implying a bend between the Fc and the Fab arms of the antibody.

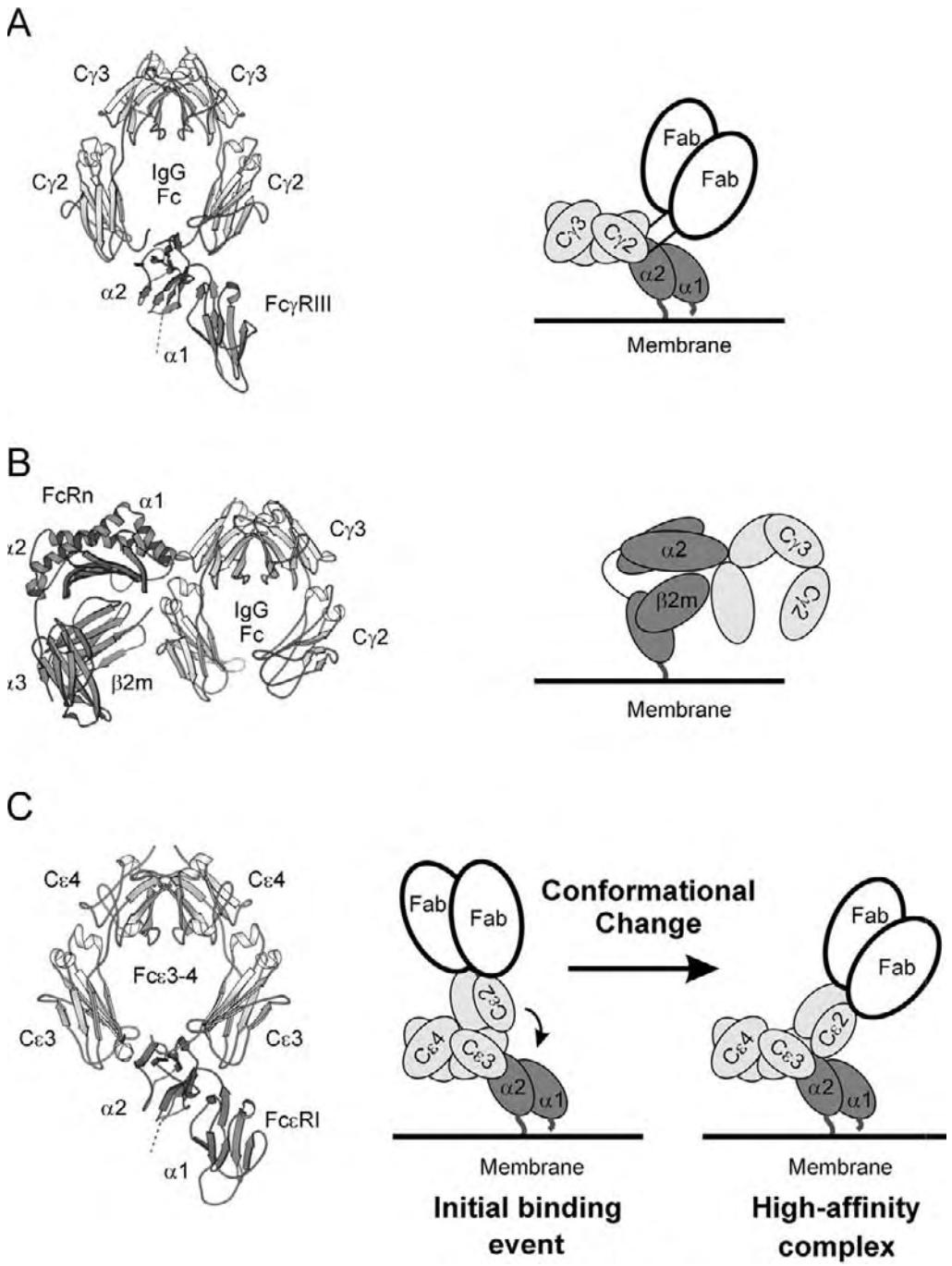


Figure 2 (A) IgG Fc/sFcγRIII complex (PDB code 1E4K) and schematic (rotated view) to show the disposition of the IgG molecule and its Fab arms relative to the membrane. (B) IgG Fc/FcRn complex (PDB code 1I1A) and schematic (same orientation) to show the disposition of the Fc relative to the membrane, again implying that the IgG must be bent. (C) IgE Fcε3-4/sFcεRIα complex (PDB code 1F6A) and schematic (rotated view) to show the complete Fc (with Cε2 domains), the Fab arms, and the conformational change that is proposed to lead to high-affinity binding.

IgE–Receptor Interactions

FcεRI

The crystal structure of the complex between the two extracellular domains of FcεRI α-chain and a subfragment of IgE Fc consisting of the Cε3 and Cε4 domains [21] (termed here Fcε3-4 to distinguish it from the whole Fc that includes the Cε2 domains) showed essentially the same topology of interaction (Fig. 2C) as seen for the IgG Fc/FcγRIII complex. The Cε3 domains of Fc open up upon binding to the receptor, compared to the uncomplexed state [22,23], but there is little change in the angle between the two receptor domains (refer to previous description of FcγRIII).

However, there is movement in one of the β-strands at the edge of the α2 domain, which moves over from one β-sheet (as a D strand alongside E) in structures of the free receptor [12,13,23] to the other (as a C′ strand alongside C) in the complex.

As in the IgG Fc complex, there are two subsites of interaction, one on each heavy chain, and the total buried surface area is extensive (1850 Å^2). However, indirect evidence from kinetic studies comparing the binding of Fcε3-4 and whole Fc to FcεRI [24,25] and direct evidence from nuclear magnetic resonance (NMR) [26] indicate that the Cε2 domains also contribute to receptor binding. The structure of the complete IgE Fc has recently been solved [27], revealing that the Cε2 domains are not only bent back onto the Cε3 domains, away from the receptor binding region, but also prevent access to one of the two Cε3 subsites required for receptor binding. The clear inference from this structure and modelling of the complex between the complete Fc and FcεRI (based upon the crystal structure of the Fcε3-4/FcεRI complex) is that a substantial conformational change in Fc involving both Cε2 and Cε3 domains, together with the change in the CC′ region of the receptor, must accompany receptor binding (shown schematically in Fig. 2C) [27]. It is these conformational changes, more extensive than those accompanying IgG Fc binding to its receptor, together with the additional receptor contacts provided by Cε2, which may account for the significantly enhanced affinity (by several orders of magnitude) and reduced dissociation rate of IgE compared to IgG (IgG binding to FcγRIII: $K_a = 5 \times 10^5\ M^{-1}$ and $k_d = 1\ s^{-1}$ [18]; IgE binding to FcεRI: $K_a = 1 \times 10^9\ M^{-1}$ and $k_d = 2 \times 10^{-4}\ s^{-1}$ [26]). A feature in common with the IgG–receptor interaction, however, is the asymmetry induced in IgE Fc which, together with steric inhibition across the approximate local two-fold axis of the Fc, prevents free IgE molecules from activating mast cells by cross-linking receptors and triggering an immediate allergic reaction.

FcεRII/CD23

No crystal structure is yet available for FcεRII, but homology models have been built for the trimer of C-type lectin domains, based upon crystal structures of highly homologous members of this family [28–30]. The interaction site on IgE Fc is contained within Cε3, but, despite the fact that this domain is glycosylated and binds to the lectin-like domain of FcεRII, carbohydrate is not involved [31]. The site in Cε3 is distinct from that of FcεRI, and the stoichiometry of binding is 2:1 (sFcεRII:IgE Fc) [32]. It may be that when an IgE molecule binds to the FcεRII trimer at the cell surface it simultaneously contacts two domains, as thermodynamic analysis of the binding identified two distinct interactions [32], and oligomerization of FcεRII enhances its affinity for IgE tenfold [33,34]. Furthermore, IgE Fc (with a valency of two) can in principle cross-link FcεRII (with a potential valency of three) at the cell surface, and it may be that receptor aggregation contributes to the mechanism of IgE homeostasis, as membrane-bound FcεRII is known to deliver a downregulatory signal for IgE synthesis [35]. Intriguingly, a soluble form of FcεRII consisting of the lectin heads and a part of the α-helical coiled-coil stalk (refer to Fig. 1F) can upregulate IgE synthesis, presumably by interacting with surface IgE on B cells committed to IgE synthesis, in a reversal of the conventional orientation in which IgE is the soluble ligand (soluble forms of the FcγR also exist, but their functions are not known) [35].

Summary

The IgG/FcγRIII and IgE/FcεRI crystal structures reveal homologous interactions, and it is likely that the other IgG receptors, and perhaps also the homologous IgA receptor, will interact in a similar manner. Within this similar topology however, the kinetics and affinity of binding can vary over several orders of magnitude, although the exceptionally slow dissociation rate of IgE from FcεRI is due in part to additional interactions from the Cε2 domain which has no counterpart in IgG. Thus, IgE bound to mast cells in the tissue has a half-life of the order of weeks and accounts for the persistent sensitization that is characteristic of allergic hypersensitivity; the half-life of IgG bound to its receptors, in contrast, is of the order of minutes. However, the stoichiometry of both of these interactions is 1:1, thus the trigger for receptor signaling is cross-linking of the antibody–receptor complex—for example, by a multivalent or aggregated antigen in an immune complex.

The IgG/FcRn complex presents a different binding topology with a potential stoichiometry of 2:1, although the induced conformational change may limit this to 1:1. However, the role of this receptor is to transport monomeric IgG as ligand, and there may be no requirement for receptor aggregation. The location of the receptor binding site in IgG Fc, between the Cγ2 and Cγ3 domains, overlaps remarkably with the binding sites for a number of other proteins, including the bacterial Fc-binding proteins A and G, and human rheumatoid factor autoantibodies (which as surface IgM are in effect B-cell antigen receptors with specificity for IgG Fc). The crystal structures of these three complexes are known [36–38], and all display 2:1 stoichiometry; a fourth Fc-binding protein, the membrane glycoprotein Fc receptor from herpes simplex virus, binds to this same region but with 1:1 stoichiometry [39]. It has therefore been proposed that this binding cleft has particular physicochemical characteristics that render it such an attractive site [40], and apparently bacteria and viruses have evolved Fc-binding proteins directed at this region in order to interfere with antibody-mediated clearance mechanisms.

Finally, FcεRII offers yet another topology in which receptor oligomerization in the absence of ligand enhances affinity through an avidity effect, and multivalency in both ligand (bivalency) and receptor (trivalency) may lead to receptor aggregation as the trigger for transmembrane signalling.

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CHAPTER 9

Plasticity of Fc Recognition

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THE HINGE REGION BINDING SITE ON THE IMMUNOGLOBULIN G CONSTANT FRAGMENT (FC) DEMONSTRATES THE REMARKABLE PLASTICITY THAT SOME PROTEIN SURFACES CAN EXHIBIT IN THEIR MOLECULAR INTERACTIONS. LOCATED AT THE INTERFACE BETWEEN TWO β-SANDWICH DOMAINS (C_H2 AND C_H3), THIS BINDING SITE CLOSELY RESEMBLES THOSE FOUND ON MANY EXTRACELLULAR HEMATOPOIETIC CYTOKINE RECEPTORS; HOWEVER, IT IS DISTINGUISHED BY THE LARGE VARIETY OF UNIQUE PROTEIN FOLDS THAT HAVE EVOLVED TO INTERACT WITH IT. RANDOM DISULFIDE-CONSTRAINED PEPTIDES SELECTED FROM A PHAGE DISPLAY LIBRARY FOR BINDING TO FC ALSO SPECIFICALLY SEEK OUT INTERACTIONS IN THIS REGION. THIS INDICATES THAT THE MECHANISM BEHIND SUCH CROSS-REACTIVE BINDING IS PHYSIOCHEMICAL IN NATURE AND NOT MERELY A REFLECTION OF THE BIOLOGICAL ROLE OF THE SITE. ANALYSIS OF THE AVAILABLE FC CO-COMPLEX CRYSTAL STRUCTURES PROVIDES CIRCUMSTANTIAL EVIDENCE THAT CONVERGENT EVOLUTION IN BINDING TO THE HINGE REGION IS FACILITATED BY ITS HYDROPHOBIC, ACCESSIBLE, AND ADAPTIVE NATURE. SUCH FEATURES MAY BE GENERAL INDICATORS OF A POTENTIAL FOR PLASTIC BINDING ON PROTEIN SURFACES.

Introduction

Immunoglobulins play an essential role in targeting the response of the mammalian immune system to foreign matter. Free antibodies, such as immunoglobulin G (IgG), possess a variety of binding sites on their surfaces that directly link molecular recognition events to specific biological consequences (Fig. 1a) [1]. Most well studied are the antigen binding sites located within the variable complementarity determining regions (CDR) present in each Fab fragment. No less important are the binding sites for complement and effector activation, which are located on the N-terminal region of the C_H2 domain of Fc [2,3].

Here, we focus on a third binding site on IgG, the recognition site for the neonatal Fc receptor (FcRn), which is located at the C_H2/C_H3 hinge region of the Fc fragment (Fig. 1a) [4,5]. Named for its discovery in neonatal tissues, FcRn plays an important role in the shuttling of IgGs from mother to child in development of the immune system. However, the "neonatal" classification for FcRn now appears overly narrow, as it has been implicated in immune system functions beyond the fetal stage [6], including being a contributor to the unusually long serum half life of IgG [7].

Our primary interest in the FcRn binding site, however, arises not from its biological role, but rather from the great diversity of other natural molecules that have evolved to recognize this same region [8]. Specifically, three other genetically and structurally unrelated protein domains have arisen independently to interact with this shared surface on Fc. Two of these are small bacterial binding domains, and the third is an autoimmune antibody involved in rheumatoid arthritis.

The C_H2/C_H3 hinge region on Fc is also particularly interesting from a cell signaling standpoint because of its structural similarity to a variety of extracellular cytokine receptors (Fig. 1b), wherein the intra-strand loops of two Ig-superfamily β-sandwich domains [9] comprise a protein-binding site. Though there is no close sequence homology between the fibronectin type III domains found in these receptors and the Ig constant domains found in Fc, the similarity in their three-dimensional architecutures is quite apparent. Thus, the mechanism of plasticity in Fc may be

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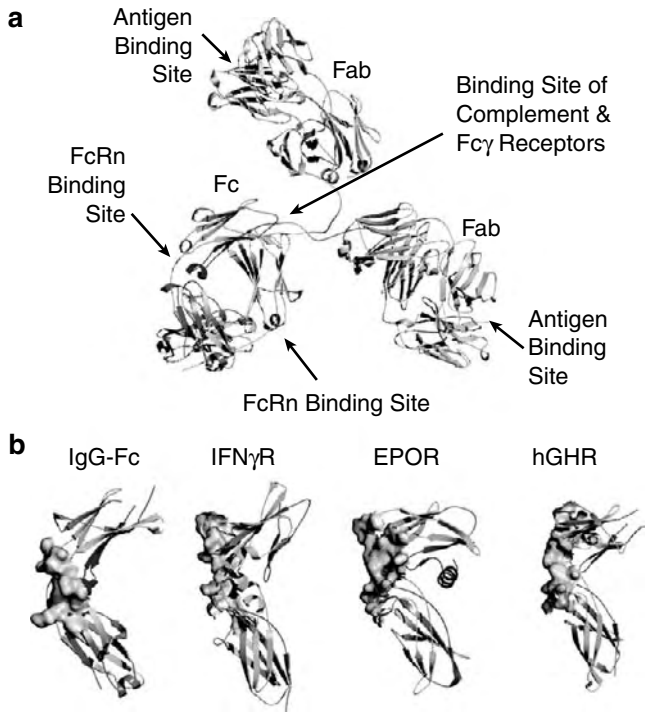


Figure 1 The structure of immunoglobulin G (a) contains several biologically important binding sites on its surface [26]. The neonatal Fc receptor (FcRn) binding site, located at the hinge region of two β-sandwich domains, is structurally analogous to binding sites found on extracellular hematopoetic cytokine receptors (b) in that many of these receptors use loops from two adjacent β-sandwich domains to create a binding site. Several of these receptors surfaces are known to exhibit binding plasticity, through binding of symmetric receptors to asymmetric hormones [27,28], through hormone cross-reactivity [10], or through binding to low-molecular-weight protein mimetics at the hormone binding site [29].

informative and helpful in understanding the cross-reactive binding behavior of certain cytokine receptors [10].

Structures of the Natural Fc Binding Domains

Although FcRn is a homolog of major histocompatibility complex (MHC) class I, the FcRn binding site for IgG is unrelated to the MHC peptide binding groove. Instead, FcRn uses a set of loops displayed from its two structural domains to contact Fc (Fig. 2a). Rheumatoid factors, which are autoantibodies associated with rheumatoid arthritis, also use loop regions to interact with Fc and often target the C_H2/C_H3 hinge region [11]. However, as revealed by the structure of one such antibody in complex with Fc (Fig. 2c) [12], rheumatoid factor CDR loops do not resemble the Fc binding loops found in FcRn. Also unlike FcRn, rheumatoid factors are not known to have any beneficial role, but are instead thought to arise as part of the disease pathology [13].

A variety of of infectious organisms have also been found to express proteins capable of binding human immunoglobulins [14–16]. However, only two such proteins, protein A and protein G, have been characterized by X-ray crystallography. Protein A is found on *Staphylococcus aureus* [17],

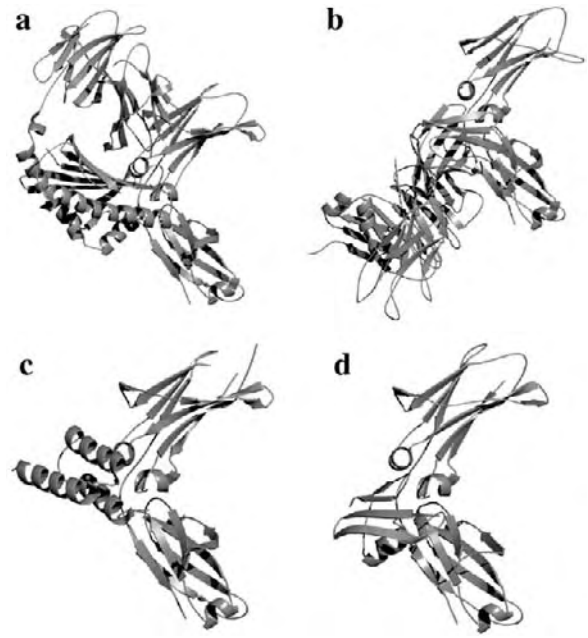


Figure 2 Natural IgG–Fc binding proteins that interact with the Fc C_H2/C_H3 hinge-region binding site include: (a) FcRn [5], (b) rheumatoid factor [12], (c) protein A [19], and (d) protein G [20]. (Adapted from DeLano *et al.*, *Science*, 287, 1279–1283, 2000. With permission.)

whereas protein G is an analogous but structurally unrelated protein expressed by *Streptococcus* G148 [18]. Both proteins contain a series of small, repeated protein binding domains, each capable of binding immunoglobulins. Here, we focus on domain B1 of protein A and domain C2 of protein G, both of which exhibit binding affinity for the Fc fragment of IgG. The structures of the complexes are shown in Figs. 2c and 2d [19,20]. The Fc binding domain of protein A consists of three helices, two of which make direct contact with Fc. In contrast, the protein G binding domain utilizes a single helix and two strands of a β-sheet to make such contacts. Neither protein domain shows structural homology to the Fc binding region of either FcRn or rheumatoid factor.

With these four Fc co-complexes in mind, it is apparent that nature has independently solved the same molecular recognition problem four times and in four different ways. Other natural solutions are also believed to exist [21] but have yet to be resolved by X-ray crystallography. Such profound cases of evolutionary convergence are rare at the molecular level, and they provide a unique opportunity to study the properties that promote binding on protein surfaces.

The Consensus Binding Site on Fc

Superposition of the binding site footprints of the four natural IgG–Fc binding domains reveals the presence of a common surface patch on the Fc surface (Fig. 3a). Just six side chains are involved in forming this saddle-shaped consensus site between the 250's loop of the C_H2 domain and the 430's loop of the C_H3 domain. Together these side chains

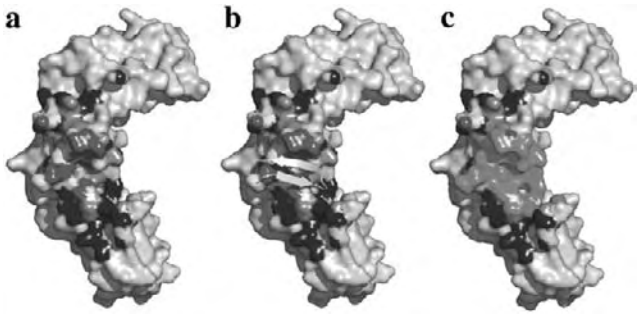


Figure 3 Superposition of the binding site footprints on IgG–Fc for the natural Fc binding proteins (a). Surface atoms are colored red, yellow, light blue, and dark blue, reflecting their participation in four, three, two, or one of these protein interfaces, respectively. Atoms in residues 252 to 254 and 434 to 436 form a nearly contiguous patch of the Fc surface that is common to all of the crystallographically characterized interactions. The crystal structure of an Fc-binding peptide (white) is shown in (b), and atoms in the solvent-protected footprint of the peptide are shown in (c). The peptide covers the same consensus set of atoms as the natural binding domains. (Adapted from DeLano *et al.*, *Science*, 287, 1279–1283, 2000. With permission.)

account for a contiguous surface patch of approximately 500 $Å^2$.

What is the driving force that has led nature to target this binding site repeatedly with so many diverse molecules? A trivial explanation would be that an important biological function of IgG, such as binding of the neonatal Fc receptor, is innately coupled to these residues. If disruption of this particular function is somehow beneficial to bacterial infection, then this hypothesis would explain why protein A and protein G binding domains have co-evolved to bind this site; however, it would not explain why rheumatoid factors are also specifically attracted to the hinge region.

An alternative hypothesis is that the innate physiochemical composition of this site is inherently "sticky" and promotes binding as well as antigenicity. If the biological role for protein A and G is primarly to localize IgGs to the bacterial surface, perhaps to evade immune system surveillance, then it might simply be the case that the C_H2/C_H3 hinge region was the most evolutionarily efficient IgG surface to target for binding. Likewise, if this region is an innately attractive part of the protein surface, then that would explain the frequent emergence of autoimmune rheumatoid factors targeting Fc. We chose to evaluate this hypothesis by evolving novel IgG–Fc binding domains in the laboratory to discover if molecules selected purely for binding would indeed target the C_H2/C_H3 hinge region.

Evolution of an Fc Binding Peptide

Phage display of small peptide libraries is a powerful technique for developing novel binding parters to proteins of interest [22]. We began with a library of approximately 4 billion random, 20-amino-acid, disulfide-constrained peptides displayed in a multivalent fashion on the surface of M13 bacteriophage [23]. This library was screened for binding to an immobilized Fc fragment. After several rounds of selection and amplification, a dominant peptide, ETQRCTWHMGELVWCEREHN, emerged from the library. Repetition of the experiment gave the original peptide and another with a closely related sequence KEASCSYWL-GELVWCVAGVE. Both of these peptides were synthesized and shown to compete with a protein A binding domain for binding to Fc with dissociation constants of about 5 μM [8].

Two subsequent rounds of monovalent phage-display optimization, sequence analysis, and manual truncation led us to identify a smaller 13-amino-acid peptide, DCAWHLGELVWCT, which bound Fc with a dissociation constant of approximately 25 nM and retained competitive binding activity with protein A [8]. Because this peptide shared a highly conserved –C———GELVWC– sequence pattern with the original peptides, all three peptides were assumed to bind in a related manner.

We solved the X-ray crystal structure of this 13-mer peptide in complex with Fc (Fig. 3b) [8]. The structure confirmed that it interacts with the same hinge region binding site of Fc as the other proteins, and the β-hairpin conformation of the peptide itself provides a fifth example of a unique protein fold capable of binding to the Fc hinge. Remarkably, the footprint of this small peptide on the surface of Fc covers virtually all of the consensus atoms derived from the much larger proteins domains (Fig. 3c). This result supports the hypothesis that the Fc hinge is attractive and that molecules in search of productive binding interactions will be drawn to this region, even in the absence of a specific biological function.

Factors Promoting Plasticity

What is the physiochemical basis for the attractiveness of this site, and how is it that so many diverse scaffolds are able to find productive interactions with it? Several characteristics stand out about this region of the Fc molecule. First, this binding site is located at an adaptive hinge region between two protein domains which are apparently free to move relative to one another, as there are no direct contacts between the protein components of the C_H2 domains observed in crystal structures of the Fc dimer. Relative rotations of up to 11 degrees between the C_H2 and C_H3 domains can be seen across the various structures. Such flexibility makes it possible for main-chain atoms on one hinge loop to move through a distance of over 2.5 Å relative to the other hinge loop, and this intrinsic adaptability presumably facilitates formation of complementary surfaces with several diverse partners.

A second notable feature is the highly exposed nature of the residues in this consensus site. The 250's and 430's loops protrude from the protein surface, and the side chains of Ile253, Ser254, and Asn434 all point outward, making few intramolecular contacts with other side-chain or main-chain atoms. Thus, they are highly available to form productive intermolecular interactions. Indeed, several binding partners

possess concave pockets on their binding surfaces which engulf these convex features.

A third aspect is the hydrophobic nature of the C_H2/C_H3 hinge. Quantitative surface patch analysis of the Fc surface has shown that this region is one of very few highly accessible hydrophobic regions on the Fc binding site surface [8,24]. Interestingly, one of the other large accessible, hydrophobic surfaces patches is the shared binding site for the family of Fcγ receptors and for C1q, which lead to effector and complement activation after antigen binding has occurred [1].

Conserved and Functionally Important Molecular Interactions

Although the Fc hinge region binding proteins are unrelated at the secondary and tertiary structural levels, patterns do emerge when one compares the detailed atomic interactions made by these molecules. Superposition of the available crystal structures enables identification of a set of conserved molecular interaction sites in the consensus region (Fig. 4). Although the overall folds of each of these Fc binding domains are distinct, there are numerous similarities in the geometric arrangements of the specific functional groups presented by each partner (Fig. 5).

Mutagenesis experiments with the Fc binding partners also support the notion that the consensus binding site serves as a shared "affinity handle" across the different receptors. FcRn binding can be disrupted by alanine substitutions at positions 252, 253, 435, and 436 [2]. Likewise, the binding of many rheumatoid factors is sensitive to truncation of the side-chain atoms in the consensus binding region [11,25]. Binding of protein A is disrupted by alanine substitutions at positions 253 and 435, and the Fc binding peptide can be blocked by alanine substitutions at 434, 435, or 436 [8]. In each case, there are functionally important binding interactions in the consensus region, though the relative importance of individual residues appears to be non-uniform.

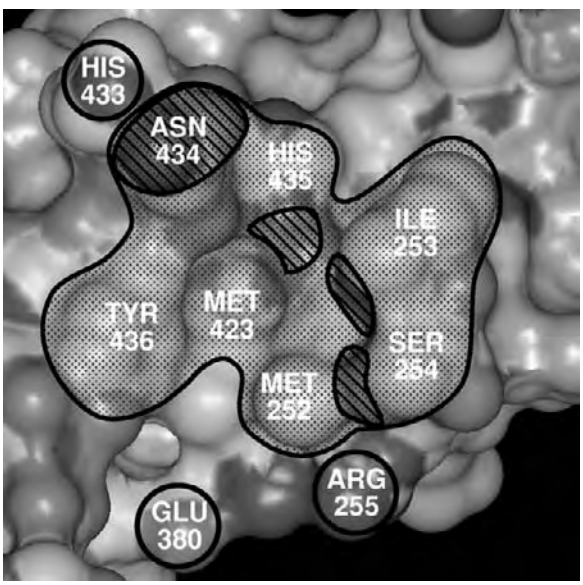


Figure 4 Topology of converserved interaction sites in consensus binding region on Fc. The predominantly hydrophobic consensus region is shaded. The hydrogen bonding sites are shown with diagonal lines, and salt bridging locations are denoted by open circles. Nitrogen and oxygen are colored blue or red, respectively, and carbon and sulfur atoms are colored green. Hydrogens are not shown. (Adapted from DeLano *et al.*, *Science*, 287, 1279–1283, 2000. With permission.)

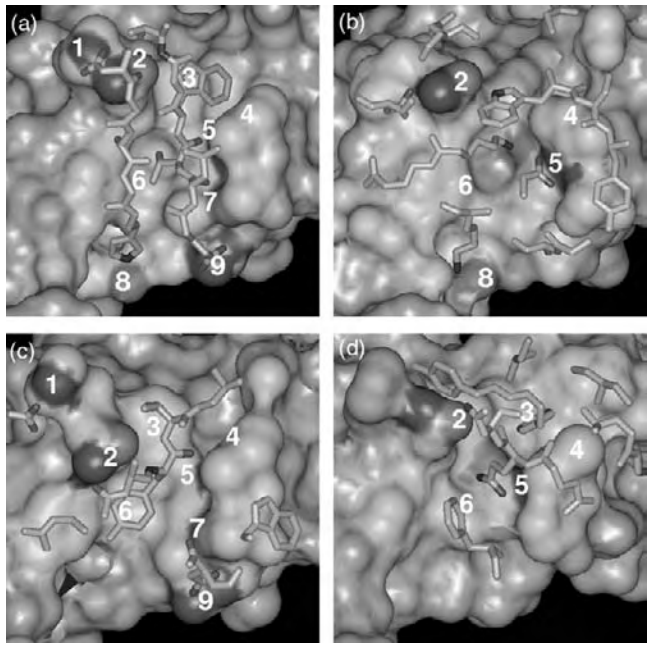


Figure 5 Comparison of the Fc binding interactions of (a) the Fc binding peptide, (b) domain C2 from protein G, (c) rheumatoid factor, and (d) domain B1 of protein A. Numbers indicate the following conversed interactions: (1) salt bridges with His433, (2) hydrogen bonding to Asn434, (3) hydrophobic packing onto His435, (4) burial of the hydrophobic "knob" formed by Ile253 and Ser254, (5) hydrogen bonding to main chain (N–H) of Ile253, (6) hydrogen packing onto Met252 and Tyr436, (7) hydrogen bonding to Ser254, (8) salt bridges with Glu380, and (9) salt bridges with Arg255. For clarity, only interfacial atoms are shown, and only nitrogen and oxygen atoms involved in conserved polar interactions are colored blue or red, respectively. The remaining contacts are colored yellow and green. (Adapted from DeLano *et al.*, *Science*, 287, 1279–1283, 2000. With permission.)

Conclusion

From studying the consensus binding site on Fc, it is evermore apparent that the complementary "lock-and-key" model for specific binding events does not apply to protein–protein interactions in the way it does to interactions between an enzyme and its substrate. The Fc hinge region teaches us that there may be many solutions to binding to a protein surface and that vastly different protein scaffolds can apparently give rise to equivalent sets of molecular interactions. Given that small peptides can be evolved to bind such a site, it seems reasonable to think that small drug-like organic compounds might also be able to bind adaptive protein–protein interaction surfaces and give rise to news classes of therapeutics capable of modulating cellular signaling.

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CHAPTER 10

Ig-Superfold and Its Variable Uses in Molecular Recognition

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Introduction

The immunoglobulin (Ig)-superfold is one of the most common structural motifs in the proteins of multicellular organisms. Typically, this fold mediates specific protein–protein interactions within the extracellular environment. As such, it is a hallmark of molecular structures that perform recognition and signaling functions. In this chapter, the standard features and variants of the basic Ig-fold are briefly detailed, and the ways in which this motif is incorporated into the overall molecular architecture of monomeric and multimeric proteins are discussed. The available three-dimensional structural information is surveyed for complexes involving Ig-fold interactions, and various examples are compared and contrasted. These complex structures illustrate the diversity of the interaction modes by which the Ig-fold can participate in functional recognition.

The Immunoglobulin Superfamily

The β-sandwich topology, first identified in the domains of immunoglobulin (Ig) structures and hence termed the Ig-fold, is one of the most common structural motifs in the proteins of multicellular organisms. The immunoglobulin superfamily (IgSF) is comprised of proteins that contain at least one Ig domain. This motif characteristically occurs within the extracellular portions of proteins and frequently mediates recognition events. Indeed, the evolution of IgSF proteins appears to be linked to the development of multicellular organisms. A dramatic increase in the number of IgSF genes is observed in vertebrates (for example, more than 750 in the human genome) when compared to the numbers in flies (140 genes) and in worms (64 genes) [1].

Ig-Superfold Structures and Assemblies

THE IG-SUPERFOLD

The Ig-superfold is characterized by a primary sequence motif that spans some 100 amino acids. In three dimensions, this sequence motif translates into a compact domain structure that comprised of two anti-parallel β-sheets packed face to face (Fig. 1). Although there is a defined topology and connectivity for the Ig-superfold, the number of β-strands is variable. To take account of this variability Ig-like domains have been classified into different sets, according to the number and arrangement of the β-strands [2, 3]. The nomenclature is standardized with the β-strands labeled sequentially from A to G, and structurally equivalent β-strands in different sets retain the same letter. The I set is defined as having strands ABED in one β-sheet and A′GFCC′ in the other. The V set has an extra C″ strand in the latter β-sheet, while sets C1 and C2 lack strands A′, and A′ and D, respectively. For all of the sets, primarily hydrophobic residues form the core of the β-sheet sandwich, and there is commonly an inter-sheet disulfide bond present (usually between β-strands B and F) to add extra stability to the fold. Additional disulfide bonds can also be present both within an Ig-like domain and between Ig-like domains.

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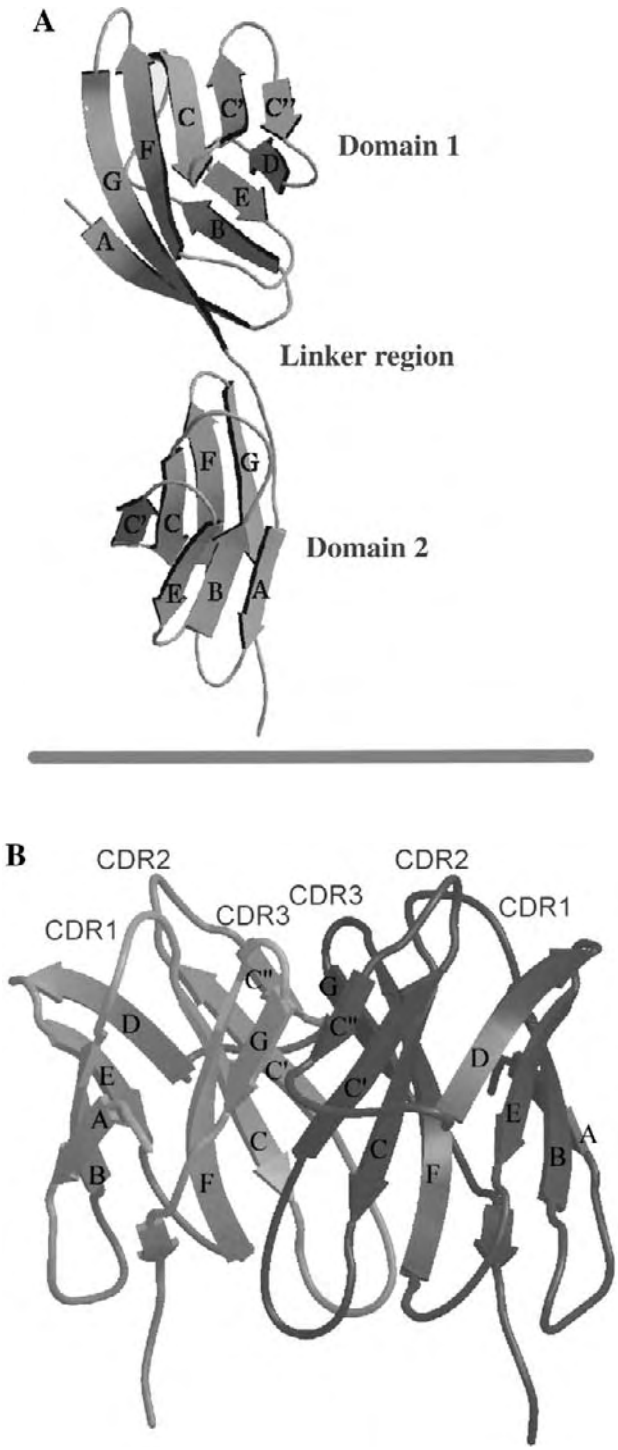


Figure 1 (a) CD2 structure. An example of the "beads on a string" IgSF architecture. (PDB code 1HNF [8]), and (b) CD8 structure. An example of a dimeric IgSF molecule (PDB code 1CD8 [12]).

Other Topologically Related Folds

Several separately cataloged types of structural motif have a β-sheet sandwich topology similar to that of the Ig-superfold. These will not be discussed in this chapter, as phylogenetically they do not appear to be related to the IgSF, but rather to be the result of different evolutionary paths converging to the same stable fold topology. They include the motif characterized by the type III domains of fibronectin (FnIII), the bacterial C3 subset, and the actinoxanthine-like C4 subset [4].

Multiple Ig-Like Domain Assemblies in Molecular Architecture

IgSF proteins are typically multi-domain structures, and these domains may or may not be exclusively Ig like. Multiple copies of the Ig-superfold can occur sequentially within the same polypeptide chain, resulting in a "beads on a string" type of linear arrangement of the domains (Fig. 1a); an extreme example of this molecular architecture is exhibited by the giant, multidomain muscle protein titin [5]. At the cell surface, the longest of the linear-type IgSF proteins identified to date is sialoadhesin (siglec-1), with an extracellular region consisting of 17 Ig-like domains [6]. More typically, the extracellular regions of IgSF proteins contain 1 to 4 Ig-like domains, often intermingled with fibronectin type III domains [7]. The linker regions between domains in the "beads on a string" type of structures can be short and relatively rigid (for example, between the two Ig-like domains of CD2 [8]) or longer, introducing more degrees of freedom at certain points in the molecular structure, as in the link between domains 2 and 3 of CD4 [9].

For a subset of IgSF cell adhesion molecules, such as axonin/TAG-1 and hemolin, an extended linker region allows the molecule to double back on itself, pairing Ig domains to create an overall horseshoe or U-shaped type of architecture [10,11]. A pair-wise packing of Ig domains is reminiscent of the archetypal immunoglobulin structure, and, more generally, within the IgSF two separate polypeptide chains bearing Ig-like domains homo- or heterodimerize (Fig. 1b) to form the stable molecular structure—for example, CD8 [12].

The size of IgSF molecules, their flexibility, the positioning of the Ig-like domain(s) within the overall structures, and the formation or absence of pairwise domain interactions within the molecules all contribute to the mode of function of the Ig-superfold in each particular case.

Ig-Superfold-Mediated Recognition

The Ig-superfold provides a stable platform that can be adapted to mediate a myriad of specific homophilic and heterophilic interactions ranging from small molecule recognition through to recognition of proteins and glycans. For cell–cell type interactions, the functional capacity of the Ig-like domain is frequently modulated by its position within the overall molecular architecture, as well as within any supra-molecular assemblies at the cell surface.

Modes of Ig-Superfold Interaction

The archetypal Ig-superfold in immunoglobulins mediates specific recognition through the complementarity-determining region (CDR) loops at one end of the variable

domain β-sandwich. However, subsequent IgSF structures and functional studies have highlighted the diversity of potentially functional surfaces on the Ig-superfold. Ig-like domains can interact with a ligand via their β-sheets, via their loop regions, or via a combination of both. A single Ig-like domain may be functional in isolation or two or more may be required, either as consecutive domains of the "beads on a string" molecular architecture or as homo- or heterodimers. Finally, the combination of protein–protein contacts mediated by a single IgSF molecule can result in the formation of extended interaction arrays at and between cell surfaces.

THE CLASSICAL IMMUNOGLOBULIN TYPE OF IG-SUPERFOLD INTERACTION

Several IgSF molecules that act as cell-surface receptors or coreceptors in the immune system employ interaction modes similar to those used by immunoglobulins for antigen recognition. One obvious example is the T-cell receptor, an IgSF molecule that is discussed in detail elsewhere in this handbook (see Chapter 11). A second, closely related example is provided by the cytotoxic T-cell coreceptor CD8. Like the T-cell receptor, CD8 interacts with major histocompatibility complex (MHC) class I molecules. Both TCR and CD8 have an MHC class I binding surface composed of the two sets of CDR-like loops (BC, C′C″, and FG) from a dimer of variable domains. The structure of the CD8αα–MHC class I complex (Fig. 2) for both human and murine molecules reveals CD8 binds one MHC class I molecule, interfacing with the MHC α_2 and α_3 domains as well as contacting β_2-microglobulin [13,14]. The focal point of the interaction is the DE loop of the MHC class I α_3 Ig-like domain, which is clamped between the CDR-like loops of the two CD8 subunits. This mode of interaction is analogous to that used by immunoglobulins when binding to antigenic surfaces containing a single prominent loop.

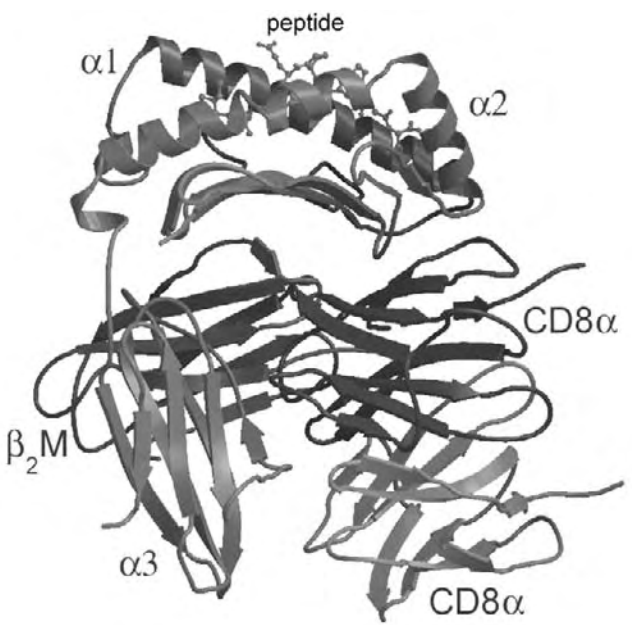


Figure 2 The MHC class I–CD8αα complex (PDB code 1AKJ [13]).

HETEROTYPIC IG-SUPERFOLD INTERACTIONS

Monomeric IgSF cell-surface molecules mediate interactions with a broad spectrum of ligand types, many discussed in detail elsewhere in this handbook. The diversity of ligands is matched by the diversity of interaction modes. For completeness, three of the most distinctive interaction mechanisms are briefly reviewed here.

At present, there is no complex structure to illustrate definitively the mode of interaction of IgSF members such as ICAM1 and ICAM2, VCAM, and MadCAM with integrins; however, the key contribution of an aspartic acid residue that is prominently exposed on a loop in the N-terminal Ig-like domain of these molecules is well established [15–20]. It is believed that this acidic residue may contribute to the coordination of a divalent cation within the integrin structure [21].

Several Ig-superfold-mediated protein–protein interactions involve contributions from the linker region between two sequential Ig-like domains within a receptor structure. This mode provides a common theme for the otherwise diverse interactions of the FGF receptor to the cytokine FGF [22–24] and the KIR family of natural killer (NK) cell receptors to MHC class I molecules [25,26]. Both families of interaction have been characterized by crystal structures of representative complexes and are discussed in detail in subsequent chapters.

In addition to mediating protein–protein interactions, the Ig-superfold can adapt to function in glycan recognition. This property is exemplified by cell-surface receptors of the siglec (sialic acid binding IgSF lectin) family [27]. Members of this IgSF subgroup are characterized by the sialic acid binding function of their N-terminal, V-set, Ig-like domain. The key features of the siglec-style binding site for sialic acid have been revealed by the crystal structure of the N-terminal domain of sialoadhesin (siglec-1) in complex with 3′ sialyllactose [28]. The binding is centered on the N-terminal portion of β-strand G at the edge of the β-sandwich (Fig. 3) and utilizes interactions with side chains from three residues (an arginine and two tryptophans) that are conserved across the siglec family.

HOMOTYPIC IG-SUPERFOLD INTERACTIONS

trans Interactions Mediating Cell-to-Cell Contacts

Many of the cell adhesion molecules responsible for cell-to-cell interactions conform to the "beads on a string" type structure with Ig-like domains arrayed in a linear fashion in the N-terminal extra-cellular region prior to a single membrane spanning section. For such structures, the functional Ig-like domains are frequently those most distal to the cell surface (i.e., the N-terminal domains). Domain 1 of such molecules usually belongs to the I-set or V-set class of Ig-superfold and the functional interactive surface commonly encompasses part of the A′GFCC′(C″) β-sheet. This in particular holds for interactions involving IgSF ligands. Examples of crystal structures for recognition complexes of this type

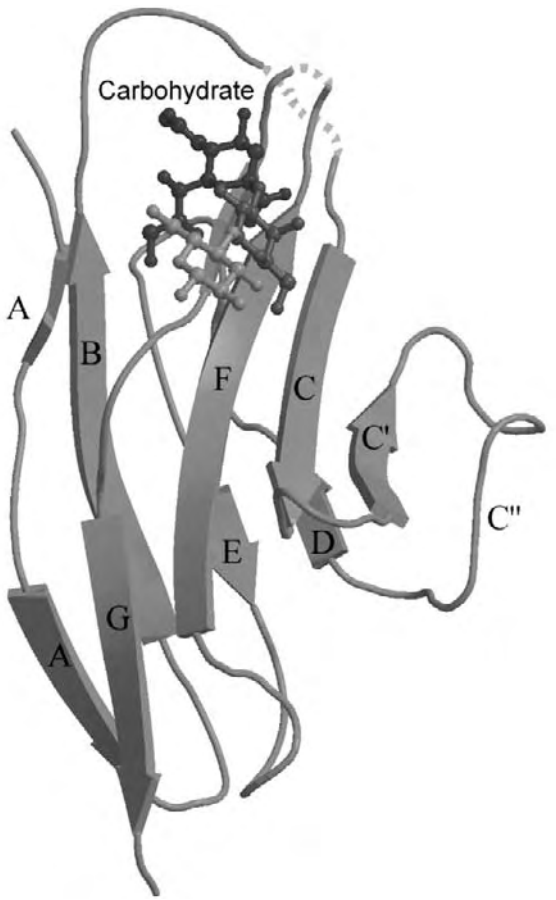


Figure 3 The N-terminal Ig-like domain of sialodhesin in complex with the carbohydrate 3′ sialyllactose (PDB code 1QFO [28]).

include P0–P0 (where molecular contacts within the crystal lattice are representative of homophilic IgSF–IgSF interactions [29]) and the structures of CD2–CD58 and B7-1-CTLA4 (representative of heterophilic IgSF–IgSF interactions [30,31]).

The homophilic interactions of the neural cell adhesion molecules show some variations on the above theme. A crystal structure of the first two N-terminal Ig-like domains of NCAM revealed a propensity for this molecular fragment to interact as a cross-shaped antiparallel dimer with residues from the B and E β-strands of domain 1 in molecule 1 interacting with those of the FG loop in domain 2 of the second molecule [32]. Functional data suggest that interactions mediated by domains 1 and 2 may not represent the whole story, but this dimer structure does provide a compelling mechanism for one mode of NCAM-mediated cell–cell adhesion. The four N-terminal Ig-like domains of chicken axonin-1 [10], and the distantly related insect protein hemolin [11], form a U-shaped structure due to intramolecular contacts between domains 1 and 4 and domains 2 and 3 that acts as the functional interactive unit. Lattice contacts within the axonin-1 crystals suggest that these U-shaped units mediate cell–cell interaction via an edge-to-face type of packing involving the CE loop in domain 3 and the FG loop in domain 2.

cis Interactions Modulating Avidity In general, cell adhesion molecule interactions are individually low affinity interactions. However, several crystal structures have provided significant evidence for the occurrence of homophilic *cis* interactions between IgSF molecules, interactions that could mediate the formation of stable, zipper-like arrays in the context of a cell–cell interface. The crystal structure of P0, the major structural protein of peripheral nerve myelin, provided one of the first such examples, with crystal lattice contacts suggesting that *cis* interactions mediate formation of P0 tetramers that in turn mediate an array of *trans* interactions to clusters of tetramers on the opposed membrane [29]. Array-wise interactions have also been proposed for the neural cell adhesion molecules exemplified by axonin-1 [10]. Heterophilic examples include the dimerization of B7-1 [33], which, when combined with the dimeric molecular structure of its IgSF ligand CTLA-4, could result in the formation of extended arrays between T cells and antigen-presenting cells in the immune system [31,33,34] (Fig. 4).

IgSF Molecular Architecture and Interactions in the Context of Function

The Ig-superfold appears to provide a stable structural platform capable of supporting many variations on the theme of specific ligand recognition. The interactions it mediates can be high affinity (nanomolar range, as in cytokine receptor interactions, such as between FGF and FGF receptor), medium affinity (micromolar to nanomolar range, as immunoglobulin–antigen complexes), or weak affinity (millimolar range, as exemplified by many of the cell adhesion molecule interactions), but always a high degree of specificity is retained. For the cell adhesion type of interaction, it has been proposed that a predominance of electrostatic, in particular hydrogen-bond-based, binding provides the mechanism for generating only low affinity while maintaining specificity [35]. In each case, the binding affinities, kinetics, and avidity are matched to the requisite functional role of the interaction.

In addition to the adaptability of the interaction surface it can provide, the modular nature of the Ig-superfold also lends itself well to the formation of large multi-domain or multi-molecular assemblies. Such assemblies provide additional mechanisms by which to modulate function. For example, the 17 Ig-domain extracellular region of sialoadhesin may serve to present the sialic acid binding N-terminal domain at sufficient distance above the cell surface that it avoids any *cis*-type interactions with glycan ligands on the same cell surface [6]. Conversely, the closely matched sizes of interaction complexes such as CD2–CD58, B7-1-CTLA4, and MHC–TCR may be integral to the formation of supramolecular assemblies between cells [36,37]—for example, the immunological synapse. Such assemblies increasingly are perceived as the deciding factor in the biological outcome of a cell–cell recognition event.

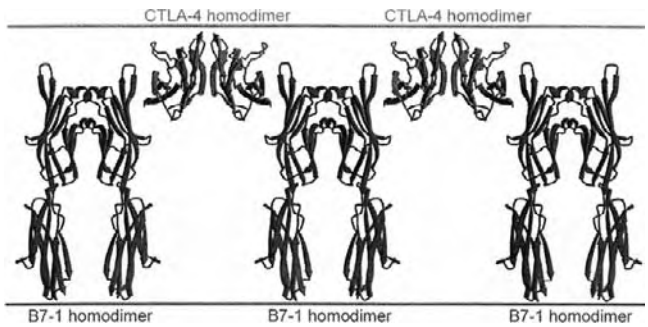


Figure 4 Crystal packing contacts for the structure of B7-1–CTLA-4 complex; an example of a zipper-like array compatible with cell–cell interaction. (PDB code 1I8L [31])

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CHAPTER 11

T-Cell Receptor/pMHC Complexes

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TCR Generation and Architecture

T cells bearing clonotypic T-cell receptors (TCRs) are generated from a pool of naïve progenitor cells by a two-stage process of positive and negative selection. The TCRs on these cells must recognize self peptides bound to self, or syngeneic, major histocompatibility complexes (MHCs) before they can differentiate from "double positives" into $CD4^+$- or $CD8^+$-expressing "single positives." However, positively selected T cells that are reactive against self-pMHCs are destroyed by negative selection. Positive selection establishes two subclasses of TCRs that associate with either of the two coreceptors CD8 (Fig. 1), and CD4:$CD8^+$ T cells recognize pMHC class I molecules, while $CD4^+$ T cells are activated by peptides bound to MHC class II.

αβ TCRs are heterodimeric cell-surface glycoproteins that consist of disulfide-linked α and β chains and have a domain organization similar to antibodies (Fig. 2). Each chain is composed of an immunoglobulin (Ig)-like variable (V) and constant (C) domain, a transmembrane region, and a short cytoplasmic tail. The C domains serve to anchor the TCR in the membrane of the T cell and to interact with accessory signaling molecules such as CD3. The variable domains carry the complementarity-determining regions (CDRs), with which the TCR binds pMHC antigen with a generally low affinity, but moderate specificity.

Peptide Binding to MHC Class I and II

In the cellular immune response, peptides are displayed to T cells in complex with class I or class II MHC molecules. Both classes of MHC are heterodimers of similar structures; they are composed of three domains, two Ig-like and one α/β domain (MHC fold) that forms the peptide binding site. Whereas in class I MHC molecules, the peptide binding site is constructed from the heavy chain only, in class II MHC, it is formed by both chains. A β-pleated sheet forms the floor of the binding groove, which is flanked by α-helices (Fig. 2). Polymorphic residues in the α-helices and β-sheet floor cluster at the center of the binding groove and change its shape and chemical properties, thus accounting for the peptide-specific motifs that have been identified for each MHC allele [1–3].

Class I MHC molecules bind peptides in an extended conformation with the C terminus and the other main anchor residues buried in allele-specific pockets, leaving the upward-pointing peptide side chains available for direct interaction with the TCRs. Thus, the peptide lengths are usually 8 to 10 residues [4,5]; substantially longer peptides can bind but, due to the fixing of their N and C termini, they must bulge out of the binding groove [6].

In class II MHC, the peptide termini are not fixed, and the bound peptides can be significantly longer than in class I MHC; the peptide backbone is confined to repeating polyproline type II, helical, ribbon-like conformations [7]. The peptides also lie slightly deeper in the binding groove. Thus, the peptide has the potential to dominate the TCR/pMHC interface more in class I due to the ability to bulge out of the groove depending on the length of the peptide and the pMHC [6]. Additionally, extensive ridges in some MHCs force the peptide to bulge even higher out of the groove and provide more intimate contact with the TCR [8,9].

TCR/pMHC Interaction

Whereas in humoral immunity antibodies identify antigenic molecules as distinct entities, in the cellular response TCRs recognize antigenic peptide fragments only when presented by an appropriate MHC molecule. A fundamental

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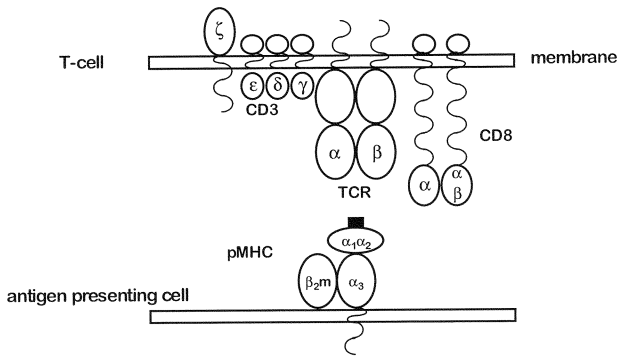


Figure 1 Schematic representation of the components in a class I TCR/pMHC/CD8/CD3 signaling complex. The heavy chain consists of the α_1–α_3 domains, to which the light chain β_2-microglobulin (β_2m) is noncovalently attached. The peptide–MHC (pMHC) complex is anchored to the plasma membrane of the antigen-presenting cell via its α_3 domain while the $\alpha_1\alpha_2$ super-domain binds the peptide (□). The CDR loops of the αβ TCRs recognize the pMHC complex, while the coreceptor CD8 binds simultaneously to the α_3 domain either as an αα homodimer or an αβ heterodimer. The signal from the pMHC complex (if any) is then transmitted through the T-cell plasma membrane by the CD3 signaling modules. Phosphorylation of the CD3ζ chain by the ZAP70 kinase (not shown) is an early step in this signal transduction cascade.

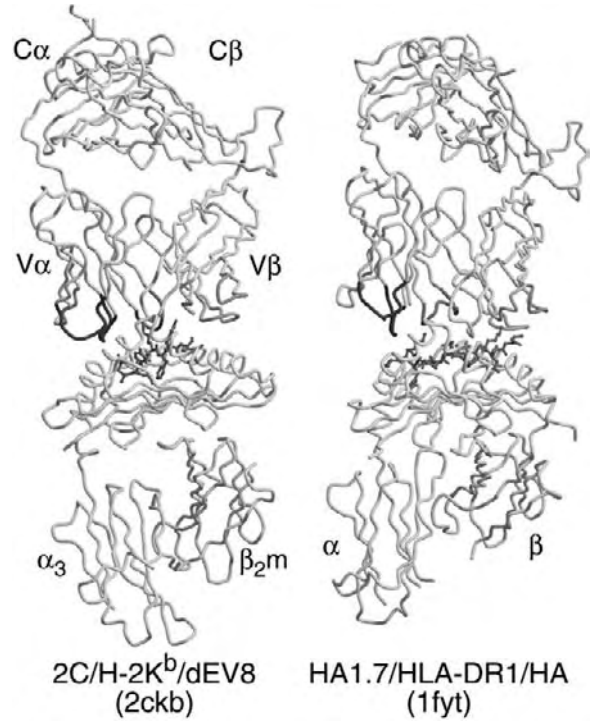


Figure 2 Similar structural architecture of class I and class II TCR/pMHC complexes. The Cα traces of the TCRs [34,51] are shown on top with the colored CDR loops contacting the pMHC at the complex interfaces. The V_α and V_β domains are positioned over the N-terminal and C-terminal halves of the peptide, respectively. The peptides are drawn as red ball-and-stick representations and have fixed termini in class I MHC but can extend out of the binding groove in class II MHC. The CDR loops are colored as follows: CDR1α (residues 24–31): dark blue, CDR2α (48–55): magenta, CDR3α (93–104): green, CDR1β (26–31): cyan, CDR2β (48–55): pink, CDR3β (95–107): yellow, and HV4 (69–74): orange.

difference between antibody/antigen and TCR/pMHC recognition is that the specificity of the former is dependent on high affinity (K_d is nanomolar) for the free antigen, whereas in the latter low affinities predominate (K_d is ~0.1–500 μ*M*); thus, specificity must be ensured by a different mechanism. Possible mechanisms are outlined in the following sections.

Orientation of the TCR in TCR/pMHC Complexes

The seven independent TCR/pMHC complexes determined to date (reviewed in references [10] to [12]; Table 1) confirm that the TCR heterodimer is oriented approximately diagonally relative to the long axis of the MHC peptide-binding groove [13,14]. The Vα domain is located above the N-terminal half of the peptide, while the Vβ domain can contact the C-terminal portion of the peptide (Fig. 2). The fluctuation in the TCR orientation has been described generally as diagonal [13,14] and, in one case, orthogonal [15], but it appears that the TCR orientation, or twist, on MHC class I and class II shows a relatively restricted spread of about 35° (Fig. 3). However, the TCR deviates not only in its twist, but also in its roll and tilt, which can be gleaned from the angle of the pseudo two-fold axis between the TCR Vα and Vβ domains and the MHC β-sheet floor (Fig. 3). In addition, the TCRs can differ in their αβ chain pairings, such that the pseudo- Vα/Vβ two-fold angle can also contribute to the variation in TCR orientation on the pMHC. As a result of the various TCR orientations, the buried surface for the TCR/pMHC complex can vary extensively between 1240 and 1930 Å^2, with the peptide contributing a relatively restricted range of 21 to 34% to the pMHC side of that interface. Vα can contribute from 37 to 74% (average 57%) and Vβ from 26 to 63% (average 43%) of the TCR buried surface. This bias in chain usage has also been noted for antibodies, where V_H usually provides a larger contribution to the antibody–antigen interface [16].

Peptide Recognition by the TCR CDR Loops

The suggestion that only a few up-pointing peptide side chains contribute to the specificity of the TCR/pMHC interaction [17] was confirmed by TCR/pMHC crystal structures. In class I, these interactions are dominated by the peptide residues that extend or bulge most out of the groove and, hence, represent functional hotspots [18] in the TCR/pMHC interface. For nonamer and octamer peptides, these represent residues P5, P7, and P8 and P4, P6, and P7, respectively. For class II peptides, the key side-chain contributions are more uniformly dispersed (P1, P2, P3, P5, P8). On the other hand, the contribution of the peptide backbone to TCR interaction is very modest for both class I and class II, where none to only a handful of contacts are made. The only exception so far is for the HLA-A2/Tax complex, where the large P4–P5 bulge includes a glycine at P4 that enables the TCR to access the peptide backbone [14,19].

Analysis of the number of contacts reveals that CDR1β and CDR2β often make minimal contact with the pMHC

Table 1 Overview of TCR/pMHC Complex Structures (1996–2002)

Complex	PDB ID	Peptide activity	Constructs and expression systems	Ref.
2C/H-2K^b/dEV8	2ckb	Weak agonist	*D. melanogaster*, acidic/basic leucine zipper for specific TCR chain pairing	13, 34
2C/H-2K^b/SIYR	1g6r	Superagonist[a]	—	18
2C/H-2K^{bm3}/dEV8	1jtr	Weak agonist	—	39
scBM3.3/H-2K^b/pBM1	1fo0	Agonist	Myeloma cells for TCR, *E. coli* for MHC (refolded from inclusion bodies)	30
B7/HLA-A2/Tax	1bd2	Strong agonist†	*E. coli*, refolded from inclusion bodies	19
A6/HLA-A2/Tax	1ao7	Strong agonist†	*E. coli*, refolded from inclusion bodies	14
A6/HLA-A2/TaxP6A	1qrn	Weak antagonist	—	21
A6/HLA-A2/TaxV7R	1qse	Weak agonist	—	21
A6/HLA-A2/TaxY8A	1qsf	Weak antagonist	—	21
KB5-C20/H-2K^b/pKB1	1kj2	Agonist	Myeloma cells for TCR, *E. coli* for MHC (refolded from inclusion bodies)	12
scD10/I-A^k/CA	1d9k	Agonist	*E. coli* for TCR, refolded from inclusion bodies; CHO cells for MHC; peptide covalently connected to the MHC	15
HA1.7/HLA-DR1/HA	1fyt	Agonist	*E. coli* for TCR, refolded from inclusion bodies; *D. melanogaster* for MHC; peptide covalently connected to the TCR	51
HA1.7/HLA-DR4/HA	1j8h	Agonist	—	38

[a]The nomenclature *superagonist* or *strong agonist* is equivalent in these instances. Class I and class II complexes are separated by the horizontal line; sc: single-chain Fv fragment of the TCR. (Adapted from Rudolph, M. G. and Wilson, I. A., *Curr. Opin. Immunol.*, 14, 52–65, 2002.)

compared to CDR3β. In Vα, CDR2α tends to have fewer contacts with the pMHC than CDR3α, although an exception is found in the allogeneic BM3.3 complex, where CDR3α has almost no contacts (see above). However, in most cases, peptide contacts are made primarily through the central CDR3 loops, which also exhibit the greatest degree of genetic variability. In contrast, the majority of conserved MHC contacts are mediated by the CDR1 and CDR2 loops [20], particularly in Vα.

Discrepancy Between Magnitude of Structural Changes and Biological Outcomes

Altered Peptide Ligands: Antagonism and Superagonism

So far, no dramatic structural changes that could account for the magnitude of the different signaling outcomes of various altered peptide ligands (APLs) have been observed in the TCR/pMHC structures, when strong agonist, weak agonist, and antagonist peptides are presented by the same MHC to the same TCR [18,21]. Only slight readjustments occur in the TCR/pMHC interface to accommodate different up-pointing peptide side chains. In the A6 system, the number of peptide–TCR contacts does not correlate with the degree of agonism and antagonism [14,21]. Similarly, in the 2C system, the buried surface does not change much when weak and strong agonists are compared, but the complementarity [18] and the number of TCR/pMHC contacts increases despite the relatively minor substitution of an arginine (strong agonist) for a lysine (weak agonist) at P4. Again, no gross conformational changes in the TCR or pMHC are observed, but slight rearrangements in the CDR loops accommodate the different peptides [18].

The correlation of complex half life [22] with the degree of agonism or antagonism is also not clear cut. In both 2C and A6, the strong agonists (SIYR and Tax) have a longer half life (9.2 and 7.5 s) than do weak agonists (3.7 s for H-2K^b-dEV8 and 1.5 s for HLA-A2-V7R). However, by using surface plasmon resonance, agonists have been found in the A6 system that have shorter half lives than do antagonists [23]. An antagonist was converted to an agonist by stepwise filling of a cavity in the TCR/pMHC interface and the biological activity paralleled the TCR/pMHC affinity, not the half life of the complex [23]. Half-lives of TCR/pMHC complexes on the cell surface could be extended by interaction with the coreceptors CD4 and CD8 [24]. Lateral interactions among the TCR/pMHC signaling complexes or interactions with other costimulatory or inhibitory receptors, as in the immunological synapse, may thus form above a certain threshold of TCR/pMHC complex half life [25].

TCR Conformational Variation and Changes

Sufficient numbers of TCR structures are now available to assess the extent of conformational variation that arises in their antigen combining sites. As expected, the four TCR outer CDRs 1 and 2 adopt canonical conformations [26],

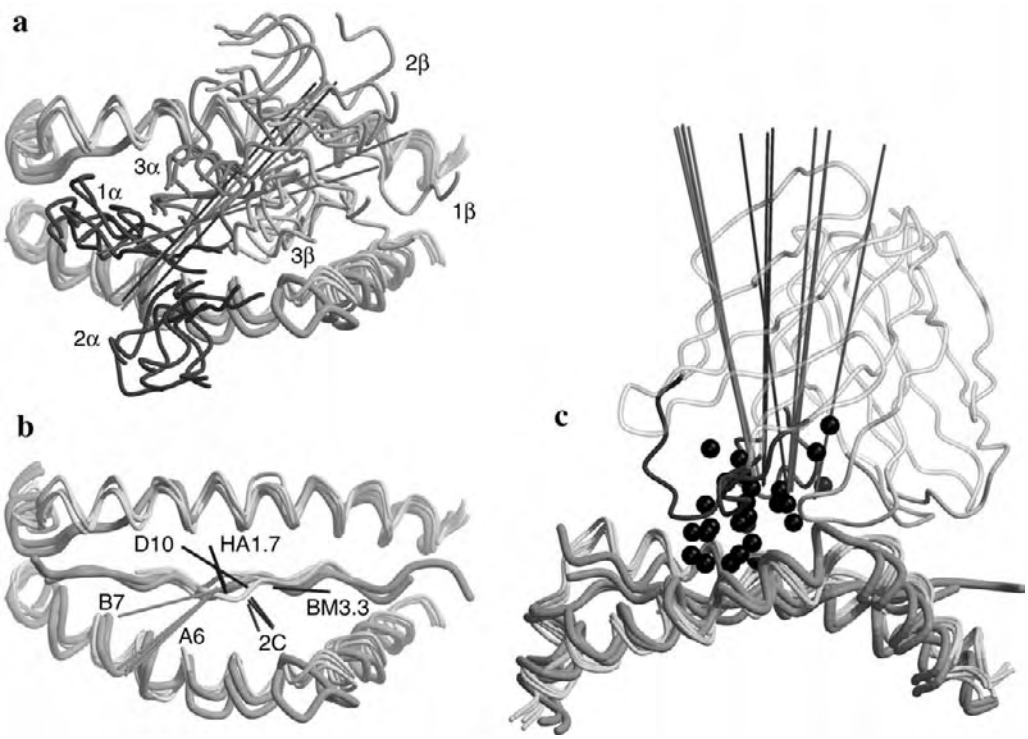


Figure 3 Relative orientation of the TCR on top of the MHC and comparison of peptide conformations in class I and class II TCR/pMHC complexes. The MHC helices are shown as light and dark gray tubes for class I and class II, respectively. The CDR loops are colored as in Fig. 2. Lines and axes are colored blue for class II TCRs and orange and red for human and mouse class I TCRs, respectively. (a) Variation in the diagonal (twist) orientation of the six independent TCR/pMHC complexes. The projection of a linear least-squares fit through the centers of gravity of the CDR loops is shown for the six different TCRs. (b) and (c) Variation in the tilt and roll of TCR/pMHC complexes. The pseudo two-fold axes that relate the Vα and Vβ domains of the TCRs to each other are shown for 12 TCR/pMHC structures. This gives a good estimate of the inclination (roll, tilt) of the TCR on top of the MHC, which is a function of the TCR, not the pMHC ligand. One extreme case is the allogeneic BM3.3 TCR, which is shown as a transparent Cα trace. Water molecules filling a large cavity between the TCR and pMHC in this complex are shown as black spheres. (Adapted from Rudolph, M. G. and Wilson, I. A., *Curr. Opin. Immunol.*, 14, 52–65, 2002.)

as first described for antibodies [27,28]. A small number of discrete canonical conformations may be able to describe most of the known sequences of the α1,2 and β1,2 loops. At present, three to four canonical structures have been defined for each of these loops [26]. What makes the TCR different from antibodies is the enormous variation seen in both of the central CDR3s (Fig. 3). In antibodies, CDR L3 adopts a well-defined set of canonical structures, but the equivalent CDR3α loop is highly variable in the current set of TCR structures, as well as the CDR3β loop [12]. Thus, the prediction [29] that these CDRs would be most variable and adapt to the pMHC primarily (but not exclusively [30]) through contact with the peptide has been borne out.

Two examples are available to assess the extent of conformational variation in the CDR loops in the presence of APL. For TCR 2C, only small variations are seen in CDR3β but, for TCR A6, these conformational rearrangements are much larger. Evidence for flexibility in the TCR has also been derived from kinetic and thermodynamic studies [31–33]. Whether these data support a model in which flexible CDRs stabilize or rearrange upon pMHC binding remains an unanswered question. What is consistent so far in both the structural and kinetic/thermodynamic experiments is that conformational rearrangements of the CDRs can provide better complementarity of the TCR to both the MHC [34] and the peptide [18,21].

ALLOREACTIVITY

Alloreactivity is the phenomenon in which a strong immune response can be generated against foreign pMHC molecules to which one's T cells have not been previously exposed [35,36]. Thus, an important practical corollary in defining the structural rules of T cell recognition is to explain alloreactivity [37]. So far, three complexes have addressed this issue [30,38,39]. The complex of the BM3.3 TCR with the allogeneic MHC H-2K^b is perhaps the most structurally distinct so far, but the corresponding syngeneic complex is currently not known. The BM3.3 TCR tilts substantially towards the β-chain side (Fig. 3), with the α-chain making few direct contacts with the MHC. In fact, the long central CDR3α is flared back such that it makes no contacts with the peptide and only two with the MHC. The majority of the interactions are with the β-chain, consistent with that proposed for the interaction of H-2L^d with TCR 2C, where an extreme bulge in the C-terminal half of the peptide is

likely to increase its interaction with the TCR β-chain [9]. Two recent studies [38,39] suggest that subtle changes in allogeneic MHCs can alter the peptide conformation and location such that the same peptide is presented differently to the TCR. Thus, these structural studies conclude that TCR interaction with the bound peptide strongly affects the alloresponse.

Role of Bound Water in TCR/pMHC Recognition

Several TCR/pMHC complexes contain bound water molecules in their TCR/pMHC interfaces. The ability of water molecules to provide additional complementarity by filling of cavities in the interface is well documented for antibodies [40]. The highest resolution TCR/pMHC complexes (2.4–2.5 Å) contain 17 (2C/H-2K^{bm3}/dEV8 [39]), 39 (BM3.3/H-2K^{b}/pBM1 [30]), and 15 (HA1.7/HLA-DR4/HA [38]) waters in their interface with 6, 12, and 6, respectively, mediating contact between the TCR and pMHC. Thus, these recent higher resolution TCR/pMHC structures indicate a strong involvement of bound water to provide complementarity and specificity to the recognition process. Yet, no specific waters are conserved among these structures, indicating that their presence is dependent on the individual sequences of both the TCR and pMHC. In the allogeneic BM3.3 complex, about 30 interfacial waters are sequestered in a cavity between the Vα and the pMHC, as a result of the TCR Vα domain lifting up from the pMHC surface [30].

Water molecules can also improve complementarity to (and, thus, stability of) pMHC interactions. Small sequence and structure changes in either the peptide (APLs) or the MHC (as in alloreactive complexes) can be amplified on the pMHC surface by redistribution or acquisition of bound waters in the TCR/pMHC interface. A good example is the allogeneic H-2K^{bm8} complex, where water can partially substitute for the loss of buried side-chain functional groups [41]. In addition, such buried MHC substitutions, which occur frequently in allogeneic MHC, can transmit their effects by altering the water structure and the electrostatic properties on the surface, even though their mutated residues are not directly "seen" by the TCR [39].

Conclusions and Future Perspectives

The evolution of a common docking mode that enables the αβ TCR to survey the contents of the MHC binding groove is remarkable. However, the seven independent complex structures determined so far have not yet revealed the basis for this conserved orientation. No absolutely conserved pairs of interactions are apparent in these different TCR/pMHC complex interfaces that would account for their relatively fixed docking orientations. The variability in the tilt, twist, and roll of the TCR indicates that the docking problem is solved in detail differently in each case to provide sufficient complementarity for binding (K_d in the micromolar range). With the exception of the alloreactive BM3.3 TCR, where most of the interactions with pMHC are due to the β-chain, the TCR Vα interactions with the pMHC seem to predominate, providing some basis for a conserved orientation. Additionally, glycosylation may play a role in facilitating docking, as both the TCR and MHC are highly glycosylated and, hence, could sterically restrict the range of possible orientations [42,43].

Another major unresolved issue is how the exceedingly small changes in the TCR/pMHC interface in response to different APLs can lead to such drastically different biological outcomes. Complementarity, buried surface area, or number of contacts in agonist versus antagonist complexes are very similar and are difficult to reconcile with the substantial differences in T-cell responses. Therefore, differentiation of strong from weak agonists, or agonists from antagonists, by visual inspection of the crystal structures seems impossible. Similarly, while the trend of increased half life for agonist versus antagonist TCR/pMHC complexes is so far maintained, exceptions have been found that belie this as a general rule.

In order to extract all of the general principles that govern TCR/pMHC recognition, further TCR/pMHC complex structures are needed. Although models of the TCR/pMHC/coreceptor(CD4/CD8) complex can be assembled from the component pieces [42] that include the distal globular domains of CD8/pMHC class I complexes [44,45], the recent low-resolution CD4/pMHC class I complex [46], and the CD3εγ NMR structure [47], perhaps the most important breakthrough would be the determination of a complete αβ TCR signaling complex, that includes the αβ TCR, CD3γδεζ, pMHC, and CD4 or CD8. This more complex assembly would lay open any global changes that may influence TCR signaling events. However, the lack of the membrane-anchoring domains in the constructs used for the current structure determinations will remain a problem until intact membrane proteins can be routinely crystallized. Future studies will also reveal how bulky ligands, such as bulged peptides [6], glycopeptides [48,49], or glycolipids in the case of CD1 [50] can be accommodated in the TCR/pMHC interface.

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CHAPTER 12

Mechanistic Features of Cell-Surface Adhesion Receptors

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Living cells constantly interact with their environment. As a consequence, a number of sensory systems have evolved for the collection, processing, and integration of a remarkable range of environmental stimuli arising from cell–cell and cell–substrate interactions. For instance, developmental and morphological processes in higher eukaryotes rely on the orchestrated migration of cells in response to specific physical and chemical cues; T-cell activation relies on the localization and compartmentalization of cell-adhesion and signaling molecules; and adherent cells must respond to a variety of intracellular and extracellular mechanical forces. All of these processes rely on the engagement of specific cell-surface receptors with the appropriate extracellular ligand to report on the immediate physical environment by transducing extracellular signals across the plasma membrane. This review examines the diversity of mechanisms thought to be involved in adhesion and signaling and highlights some of the shared principles that must be considered for all signaling pathways utilizing cell-surface receptors.

Mechanosensory Mechanisms

The ability to detect and respond to alterations in applied mechanical force is required for a number of cellular and developmental functions. This is particularly critical for adherent cells that directly contact the extracellular matrix (ECM) and are subject to considerable physical deformation. For example, sheer forces associated with blood flow are major determinants of arterial tone and vascular reorganization. At the cellular level, morphology and orientation are optimized to minimize mechanical stress and damage associated with variations in flow-related forces (see, for example, references [1] to [3]). Similarly, fibroblasts must be highly responsive to the mechanical forces associated with alterations in the ECM (reviewed in Schwartz and Ginsberg [4]).

Considerable evidence points to focal adhesions, the sites of cell–substrate contact, as the sensors of mechanical force. Central to focal adhesion assembly and function are the integrins, a family of α–β heterodimeric transmembrane glycoproteins that provide essential adhesive functions for cell migration and the establishment and maintenance of normal tissue architecture. At least 18α and 8β chains allow for the formation of multiple integrin heterodimers that are able to display a spectrum of specificities for cell-surface adhesion molecules and for a range of ECM components, including laminin, collagen, and fibronectin. The integrin cytoplasmic domains bind a variety of scaffolding and actin regulatory proteins, which in turn recruit a large number of adaptor and signaling molecules. These physical links couple the integrins to the downstream activation of numerous signaling molecules, including MAP kinase, focal adhesion kinase, Src, and PI3-kinase (see, for example, references [4] and [5]). Furthermore, integrin affinity is modulated by the activation state of the particular cell in question, and this "inside-out" signaling is thought to control the tertiary and quaternary structural rearrangements required for high-affinity ligand binding. The focal adhesion may thus be viewed as a highly dynamic sensory organelle that exploits the direct linkage between the ECM and actin cytoskeleton to respond to mechanical force through a wide range of signaling pathways.

The mechanisms underlying integrin-associated signaling rely on the determinants of mechanical strain, including tension provided by cytoskeletal motor proteins, such as myosin-II,

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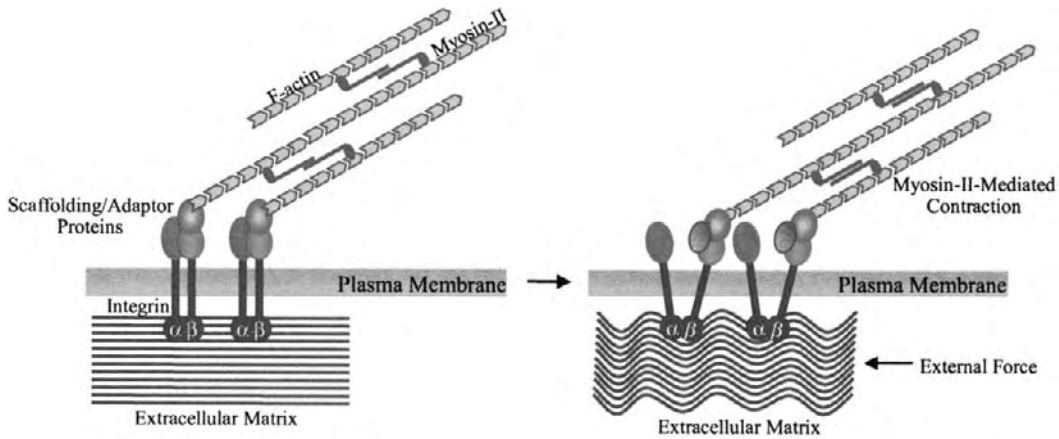


Figure 1 Model for the mechanochemical signaling mechanism of integrins at focal adhesions. The extracellular domain of integrin binds ECM components, such as collagen and fibronectin. The cytoplasmic domain of integrin contacts a series of scaffolding proteins and cytoskeletal regulatory proteins (pink ellipse), including talin and paxillin, which provides a direct physical linkage between the ECM and the actomyosin cytoskeleton. Alterations in the ECM generate tension that may result in tertiary and quaternary structural changes (illustrated here as a scissor-like motion between the α- and β-integrin chains). These structural changes are propagated to the cytoplasm, which may uncover cryptic binding and recruitment sites for additional signaling molecules (blue ellipse). The ability to couple force generation to alterations in the composition of integrin-associated focal adhesion molecules provides a direct mechanism for mechanochemical signaling.

and the intrinsic mechanical properties of the underlying ECM (Fig. 1). For example, the growth of cells on soft, or pliable, surfaces does not support integrin signaling nor the formation of focal adhesions [6], while "stretching" of these substrates supports both focal adhesion formation and integrin signaling [7,8], presumably by allowing for a sufficient level of tension to be achieved. At the molecular level, mechanical force may be transduced into a cytoplasmic signal through a number of possible mechanisms. The application of force may disrupt or distort various intermolecular binding interfaces, resulting in the reorganization of focal adhesions by enhancing the entry or exit of specific signaling molecules through either free or facilitated diffusion. A related potential mechanism is the force-induced conformational reorganization of integrin-associated focal adhesion molecules, which may uncover cryptic binding and recruitment sites for additional signaling molecules. This notion is consistent with the fact that a number of focal adhesion components, including vinculin and ERM proteins, exist in multiple conformations (see references [9] and [10] and references therein). Of special note are a series of structural [11–15] and biochemical studies (reviewed in references [12,13,16]) describing the localized ligand-induced conformational rearrangements and a model for integrin activation [12,13]. This model suggests that a large-scale conformational reorganization, including a scissor-like motion, may be required for high-affinity ligand binding. Some aspects of this conformational plasticity may also play a role in transducing mechanical force into cytoplasmic signals. These mechanisms, whether affecting the dynamic assembly/disassembly properties of the focal adhesion as a whole or directing conformational reorganization of a specific focal adhesion protein, can provide a direct linkage between cell surface–ECM adhesive interactions, focal adhesion composition, and cytoplasmic signaling. Furthermore, recent studies demonstrate a complex relationship between valency and geometric organization of the ligand and the strength of integrin-associated signaling [17], suggesting some mechanistic similarities with the c described below. Thus, integrin-associated signaling provides one of the clearest couplings of signaling and the adhesive properties of a receptor–ligand pair.

Cell–Cell Adhesions/Adherens Junctions

The cadherins are a family of cell-surface receptors that form calcium-dependent homophilic interactions between the surfaces of adjacent cells. These interactions result in the formation of intercellular adhesions, adherens junctions, which play essential roles in the establishment and maintenance of cell polarity and tissue architecture and in the recognition and migratory events associated with developmental and morphological processes. These adhesive interactions are supported by a catenin-mediated linkage to the underlying actin cytoskeleton, as the carboxy-terminal cytoplasmic tail of cadherin binds β-catenin, and via an interaction with α-catenin is linked to the cortical actin network (Fig. 2) (see Conacci-Sorrell *et al.* [18] and references therein). The importance of this cytoskeletal connection is highlighted by the observation that disruption of normal catenin function prevents the formation of mature adherens junctions and is associated with increased motility and invasiveness of tumor cells (reviewed in Okegawa *et al.* [19]).

β-Catenin plays a dual role in cell physiology, as in addition to being an essential structural component of the adherens junction it serves as a transcriptional activator of several genes involved in cellular proliferation and

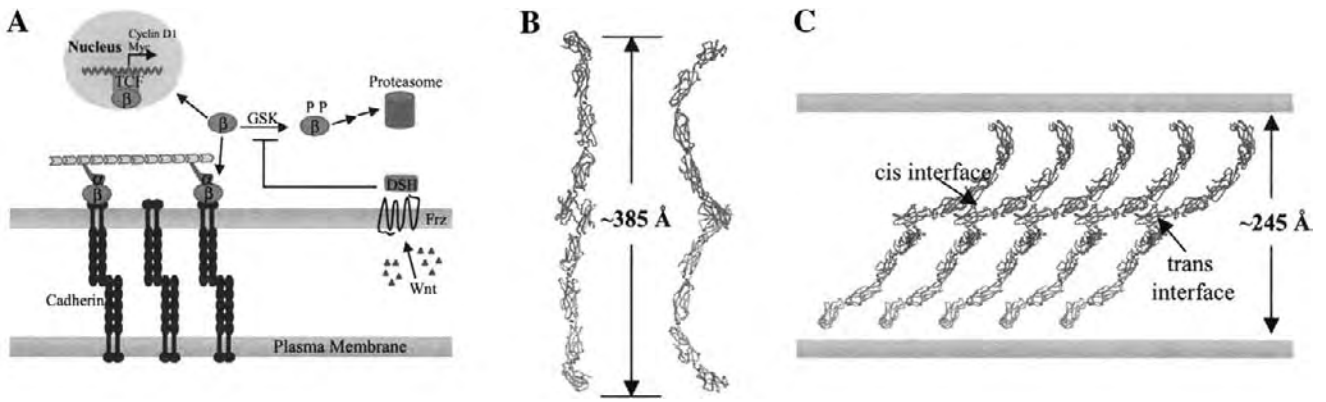


Figure 2 Adherens junctions and cadherin function. (A) Schematic of adherens junction organization and associated signaling pathways. The catenins provide a direct physical linkage between the homophilic cadherin-mediated cell–cell contacts and the underlying actin cytoskeleton and support the integrity of the adherens junctions. In turn, the actin cytoskeleton provides β-catenin docking sites that serve to modulate β-catenin signaling by buffering the soluble concentration of β-catenin. (Adapted from Conacci-Sorrell, M. *et al.*, *J. Clin. Invest.* **109**, 987–991, 2002.) (B) Structure of C-cadherin showing the arched arrangement formed by the five individual cadherin domains (EC1–EC5) and a model for the *trans* (cell–cell) interaction from abutting EC1 domains. Two orthogonal views are shown, with the arched nature of the structure evident in the right figure. (C) A model of the *trans* and *cis* interactions at the adherens junction based on contacts present in the C-cadherin crystal structure. In this model, the individual cadherin molecules in the adherens junction are tilted by ~45° with respect to the plasma membrane, implying an intermembrane separation of ~245 Å.

invasion, including Myc, cyclin D1, metalloproteinases, and fibronectin [18]. A number of regulatory mechanisms modulate β-catenin signaling. In the absence of Wnt signaling, cytoplasmically disposed soluble β-catenin is a substrate for phosphorylation by glycogen synthase phosphorylase, which serves to mark it for degradation by the 26S proteasome; however, activation of the Wnt pathway inhibits this phosphorylation and β-catenin is shunted to the nucleus, where it forms a complex with the T-cell factor (TCF) to activate selected genes. The formation of normal adherens junctions appears critical for control of β-catenin signaling, as a loss of cadherin expression correlates with increased nuclear β-catenin. Thus, there appears to be a close linkage between cadherin-mediated adhesion and β-catenin-mediated signaling pathways, with the adherens junction acting as a buffer of soluble β-catenin (Fig. 2) [20].

Structural studies have suggested several models for the homophilic adhesive interactions formed by the cadherins at adherens junctions. The recent report of the structure of the entire extracellular domain of C-cadherin by Boggon, *et al.*[21] provides new insights into both the *cis* (intracellular) and *trans* (intercellular) interactions that are essential for the formation and maintenance of adherens junctions (Fig. 2). The structure shows that the five extracellular cadherin domains (EC1–EC5) form an arched structure, and the abutment of two N-terminal EC1 domains in the crystal provides a model for the *trans* adhesive interaction. Additional crystal contacts suggest a model of the *cis* contact, and together the interactions observed in the crystalline state provide a detailed model for the periodic organization of cadherin molecules within the adherens junction. Of particular note is the suggestion that the cadherin molecules in the adherens junction are tilted by ~45° with respect to the plasma membrane, implying an intermembrane separation of ~245 Å. This feature of the model is particularly noteworthy, as there is a strong bias to view intrinsic membrane proteins as projecting perpendicular to the plane of the plasma membrane; *a priori* there is no fundamental reason for this assumption.

T-Cell Costimulation

An optimal T-cell response requires the integration of a number of distinct extracellular signaling and adhesive events at the T-cell–antigen-presenting cell (APC) interface, which has been termed the *immunological synapse*. Engagement of T-cell receptors (TCRs) on the surfaces of T cells with major histocompatibility complex (MHC)/peptide complexes displayed on the surfaces of APCs is essential, but not sufficient, for complete T-cell activation [22]. The subsequent engagement of a series of costimulatory receptor–ligand pairs provides the additional signals needed for efficient T-cell activation, as well as the negative signals required to attenuate the immune response (Fig. 3) [23–25]. The most extensively characterized T-cell costimulatory receptors are CD28 and CTLA-4, which share ~30% identity and bind the B7-1 and B7-2 ligands presented on APCs. Together with signaling through the TCR, the engagement of CD28 by the B7 ligands leads to optimal T-cell activation [22], while the interaction of B7 with CTLA-4 provides inhibitory signals required for downregulation of the response.

Initial TCR engagement is followed by a remarkable reorganization and compartmentalization of signaling and adhesive molecules at the immunological synapse. The central zone of the synapse contains the receptor–ligand pairs, including the TCR–CD3/MHC–peptide complex, CD28/B7 costimulatory complex, and CD2/CD58 complexes, as well as noncovalently associated intracellular signaling molecules, such as fyn, lck, and PKC-theta [26]. The central zone is bordered by the peripheral zone, which is composed of large adhesion molecules, including LFA-1 and ICAM-1,

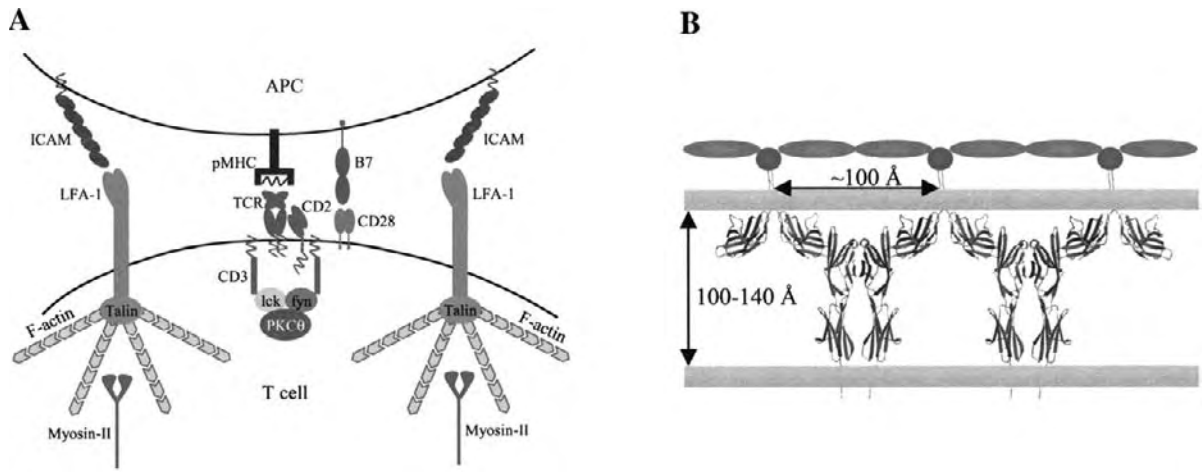


Figure 3 T-cell activation and the immunological synapse. (A) Schematic of the immunological synapse highlighting the compartmentalization of specific signaling components into discrete zones. The central zone is enriched in cell-surface signaling molecules (i.e., TCR, MHC/peptide complex, and costimulatory receptors and ligands) and cytoplasmically associated scaffolding and signaling proteins (i.e., Src family kinases, etc.). Surrounding this signaling complex is the peripheral zone, which is composed of large adhesion molecules and cytoplasmically associated cytoskeletal components required for the observed pattern of localization. (B) Model for the costimulatory signaling network at the T-cell–APC interface. The disulfide-linked CTLA-4 dimers are shown in red, while the noncovalent B7-1 dimers are blue. The interactions between these two dimeric bivalent molecules in the crystal result in a periodic array of CTAL-4 and B7 homodimers with a characteristic spacing of ~100 Å. This periodicity may result in the organized recruitment of signaling molecules (pink and red) and may in some circumstances provide further adhesive interactions required for productive signaling.

and components of the actin cytoskeleton (Fig. 3) [26]. This organization appears to be dependent on an uncompromised actomyosin cytoskeleton, thus providing another example of the intimate involvement of the actin-based cytoskeleton in a fundamental signaling pathway. A number of potential functions have been proposed for the molecular organization in the synapse, including the polarized secretion of cytokines, TCR recycling, and the promotion of costimulatory receptor–ligand engagement [27,28]. In addition, the B7 ligands appear to control APC function, as crosslinking the B7 isoforms modulates both B-cell proliferation and antibody production [22,29–31]. Thus, engagement of the costimulatory receptor–ligand pairs represents an outstanding example of bidirectional signaling.

Of particular note are the recent structural descriptions of the CTLA-4/B7 receptor–ligand complexes, which exhibit an alternating arrangement of bivalent CTLA-4 and B7 dimers (Fig. 3) [32,33]. The observation of this linear periodic array suggests a model for the organization of these cell-surface molecules at the immunological synapse. Importantly, the observed spacing between the extracellular receptor domains is also imposed on any cytoplasmically associated signaling molecules, and suggests that the oligomerization of multiple (i.e., at least two) CTLA-4 dimers may be required to afford a biologically optimal organization and local concentration of intracellular signaling molecules.

In considering the types of assemblies that are formed *in vivo* by multivalent receptor–ligand pairs, it is essential to bear in mind the relative concentrations of the binding partners (Fig. 4). For example, a large excess of either receptor or ligand will favor the formation of "isolated" signaling complexes. In the case of limiting ligand, a cell-surface complex composed of two receptor dimers (e.g., CTLA-4)

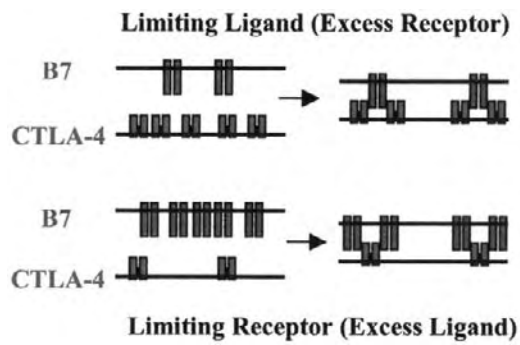


Figure 4 Effect of stoichiometry on the signaling complexes formed by multivalent receptor–ligand pairs. (Top) Limiting ligand will favor the formation of cell-surface complexes composed of two receptor dimers (e.g., CTLA-4) linked by a single ligand dimer (e.g., B7-1). This assembly would impose a constraint between the two adjacent receptors and any associated cytoplasmic signaling molecules (i.e., ~100 Å in the case of the CTLA-4/B7 complex). (Bottom) Excess ligand would favor complexes composed of a single receptor linking two independent ligand dimers. This association would not enforce any specific spatial relationship between individual receptor molecules but would still direct the localization of the receptor and ligand to the immunological synapse and could result in a sufficiently high local concentration of individual receptor dimers to support signaling.

linked by a single ligand dimer (e.g., B7-1) would be favored, and such an assembly would impose an ~100-Å constraint between the two adjacent receptors and any associated cytoplasmic signaling molecules. In contrast, the presence of excess ligand would favor complexes composed of a single receptor linking two independent ligand dimers. This association would not enforce any specific spatial relationship between individual receptor molecules but would still direct the localization of the receptor and ligand to the

immunological synapse and could result in a sufficiently high local concentration of individual CTLA-4 dimers to support signaling. Finally, equivalent amounts of receptor and ligand at a cell–cell interface would favor the formation of more extensive periodic networks. Importantly, this is a general consideration relevant to all multivalent receptor–ligand pairs.

In addition to playing a direct role in signaling, cell-surface receptor–ligand engagement constrains the approach of the adjacent plasma membranes (as in the case of the adherens junction discussed above) and may play a role in directing the organization of molecules at the cell–cell or cell–ECM interface. The maximal dimension of the CTLA-4/B7 complexes (~100–140 Å) is compatible with those of other receptor–ligand pairs present in the central zone of the synapse (i.e., MHC/TCR [34,35] and CD2/CD58 [36]). In contrast, the adhesive complexes present in the peripheral zone (e.g., the LFA-1/ICAM-1 complex) are significantly larger in maximal extent, and this difference has led to the suggestion that the compartmentalization observed in the immunological synapse is the consequence of a mechanical sorting mechanism based on relative molecular dimension [37,38]. While this is an appealing hypothesis, it is based on the assumption that intrinsic membrane proteins extend perpendicular to the plasma membrane and ignores the possibility that a molecule of large extent can be accommodated within the central zone by tilting with respect to the plasma membrane, as was suggested in the model of C-cadherin in the adherens junction (Fig. 2).

While the adhesive functions of ICAM and LFA-1 are essential to synapse formation and T-cell function, engagement of these molecules is also likely to play a direct signaling role in T-cell activation and function. Recent studies have shown that ICAM-1 binding is associated with LFA-1 clustering, enhanced actin polymerization, and F-actin bundling within T cells [39]. Conversely, crosslinking of ICAM-1 in lymphocytes stimulates calcium signaling and PKC activity, which results in cytoskeletal rearrangements associated with migration [40]. These observations indicate a strong coupling between adhesive and signaling functions and suggest that reciprocal bidirectional signaling may be associated with ICAM/LFA-1 adhesive interactions (see, for example, Lupher *et al.* [41]).

As the localization of adhesive partners at cell–cell and cell–ECM interfaces necessarily results in the localization of cytoplasmically associated species, it is relevant to ask whether situations exist in which adhesive functions are fully uncoupled from signaling events. For instance, the one-dimensional lattice observed in the CTLA-4/B7 crystal structures exhibits considerable similarities to the adhesive assembly formed by the cadherins (Fig. 2), and on this basis it is tempting to suggest that costimulatory receptor–ligand engagement might also provide adhesive interactions required for efficient T-cell function. Although no data bear directly on the adhesive properties of CTLA-4, recent studies indicate that CD28 does not make any significant contributions to the adhesive properties of naïve T cells [37]. These results differ from earlier studies indicating that the CD28/B7 interaction significantly enhanced adhesion. However, these earlier studies utilized systems in which either receptor or ligand was overexpressed [42,43], again stressing the importance of accurately knowing the cell surface densities of the binding partners in order to correctly predict mechanism. These recent studies also indicated that only ~30% of the CD28 molecules exhibited free lateral diffusion in the plasma membrane [37], implying that only a fraction of the total population may be available to bind B7 at the immunological synapse. While no evidence supports limited diffusional freedom as a general feature of cell-surface proteins, these studies nonetheless stress the potential importance of considering the "available" receptor and ligand concentrations, as opposed to total cellular concentrations.

Axon Guidance and Neural Development

The Eph family of receptor tyrosine kinases and their associated ephrin ligands play a central role in neural development by providing repulsive guidance cues that direct axonal targeting. Specifically, a migrating growth cone expressing a given Eph receptor will turn away from cells expressing cognate ephrin ligands, as a result of the disassembly or redistribution of filamentous actin networks at the leading edge [44]. Two classes of ephrins are defined on the basis of their mode of cell surface attachment. The ephrin A ligands utilize a glycophosphatidylinositol (GPI) linkage for cell-surface attachment and bind the EphA receptors, while ephrinB ligands are transmembrane proteins that bind EphB receptors.

Recent structural characterization of the ephrin-B2/EphB2 receptor complex provides new insights into the potential signaling mechanisms utilized (Fig. 5) [45]. This structure provides details of the receptor–ligand binding site and of a "circular" 2 : 2 receptor–ligand complex that is thought to be relevant to signaling. The organization observed in the crystal structure is consistent with ligand-induced clustering of the EphB2 receptor, resulting in the *trans*-autophosphorylation required for activation and subsequent recruitment of signaling molecules, including src family kinases and GTP-activating proteins (GAPS) [46]. Engagement also results in clustering of the ephrin ligand, providing another example of bidirectional signaling, as the cytoplasmic domain of ephrin-B2 is required for normal angiogenesis and vascular morphogenesis [46]. Furthermore, consistent with the propensity to form higher order oligomers, the crystal structure suggests the formation of an extended two-dimensional signaling complex (supercluster) of receptors and ligands at the cell–cell interface (in contrast to the one-dimensional array proposed for the CTLA-4/B7 complexes), which might afford enhanced signaling.

The proposed long-range organization suggests that, in addition to a direct role in signaling, engagement of the Eph



Figure 5 Structure of the ephrin-B2/EphB2 receptor complex. (A) Circular tetramer formed by the interaction of two EphB2 receptors (green) with two ephrinB2 ligands (yellow) thought to represent the favored receptor–ligand organization *in vivo*. Note that each ligand contacts two receptor molecules, but there are no ligand–ligand or receptor–receptor contacts. (B) Crystal packing results in another tetramer (elliptical), in which an extensive interface is formed between two receptor molecules. The physiological relevance of this binding interaction remains to be proven but may be consistent with the propensity of Eph/ephrin molecules to form higher order oligomers. (C) A "layer" from the ephrin-B2/EphB2 receptor complex crystal structure showing the long-range, two-dimensional ordered array formed by the combination of both the circular (highlighted in red) and elliptical (highlighted in blue) tetramers. Such an organized network could potentially play roles in signaling and/or adhesion.

receptor–ligand pairs may also provide essential adhesive functions. The first evidence supporting this notion came from the observation that ~17% of mice defective in ephrinA5 exhibit neural tube defects, which is not consistent with the classical repulsive effects attributed to ephrin/Eph receptor function [48]. These studies also revealed that the expression of splice variants of an ephrinA5 receptor (i.e., EphA7) which lack the intracellular kinase domain support direct adhesive interactions with ephrinA5-expressing cells[48]. This provides yet another example of the close linkage between signaling and adhesive interactions.

Conclusions

As illustrated, biology depends on a vast array of information processing activities that are coordinated by diverse cell-surface adhesion receptors and their cognate ligands. Though these receptor–ligand pairs differ in chemical and structural terms, there are common principles that must be carefully considered in order to construct viable molecular and atomic mechanisms for signaling. The engagement of receptor–ligand pairs leads to an increase in their local density/concentration at cell–cell and cell–ECM interfaces, and in many cases may support a natural coupling between signaling and adhesive function. Of particular importance is the quantitative understanding of both cell-surface oligomeric state and the available concentration of receptor and ligand on their cell surfaces, as they dictate the relative stoichiometries and the type of signaling complexes that can be formed at cell–cell and cell–ECM interfaces. Finally, as a general cautionary note, while direct structural information, in the form of X-ray and nuclear magnetic resonance (NMR) structures, may provide enormous insights into function and mechanism, in the absence of confirmatory biochemical data great care should be exercised in extrapolating intermolecular contacts observed in crystal structures to physiologically relevant protein–protein interfaces.

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CHAPTER 13

The Immunological Synapse

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Introduction

The immunological synapse (IS) is a specialized cell–cell junction between a thymus-derived lymphocyte (T cell) and an antigen presenting cell (APC) [1,2]. Activation of T cells is based on the interaction between T cell receptors (TCRs) and major histocompatibility complex proteins that have bound antigenic peptides (MHCp) [3,4]. Because the TCR and MHCp are attached to the surface of the T cell and antigen-presenting cell (APC), respectively, the initiation of an immune response requires a molecular grasp between the T cell and APC—a synapse. A current focus of research on the IS is to determine how this supramolecular structure contributes to T-cell sensitivity and the fidelity of the T-cell response. Four areas in which the IS concept is contributing to our understanding of T-cell activation are (1) coordination of antigen recognition and T-cell migration, (2) role of the cytoskeleton in T-cell activation, (3) mechanism of sensitive antigen recognition by T cells, and (4) integration of the adaptive and innate immune responses.

Migration and the Immunological Synapse

T-cell activation requires a sustained signal. The duration of signaling required to initiate proliferation of T cells is a minimum of 2 hr [5–7], but it may be much longer to achieve appropriate helper T-cell differentiation [8]. T cells migrate continually between the blood and the secondary lymphoid tissues where they encounter APCs. In the absence of an immune response, the T cell completes this cycle about once a day. During the initiation of an immune response, the T cells are held in the antigen-exposed lymph nodes or the spleen for 2 to 3 days, and then effector cells are released after the third day. *In vitro* T-cell recognition of agonist MHCp in the context of the adhesion molecule ICAM-1 delivers a stop signal to migrating T cells [9]. This stop signal is the first stage in forming an IS [2]. The signaling pathways required for the stop signal may include the adapter protein ADAP [10,11]. The environment of the T-cell/APC interaction regulates the stop signal. One example of this is that APCs with agonist MHCp do not stop T cells in three-dimensional collagen gels *in vitro* [12]. The mechanism of this effect is not known but may involve chemokine gradients [13] or interactions with extracellular matrix that prevent T-cell polarization toward the APCs. In lymph nodes, however, T cells are not exposed to extracellular matrix, which is sequestered in reticular fibers [14]. This non-adhesive reticular scaffold is decorated with APCs and defines corridors through which the T cells migrate. Based on the lymph node environment and *in vitro* data, it is most likely that the IS coordinates T-cell migration and the antigen-recognition process to allow full activation of T cells by small numbers of APCs that express the appropriate MHCp. Having the APCs with agonist MHCp stop the T cell is more efficient than the movement of the T cell from APC to APC when the number of APCs with agonist MHCp is small because the interactions with irrelevant APCs are minimized in the former. This view is supported by *in vivo* data demonstrating clustering of polarized T cells around dendritic cells [15,16].

The Cytoskeleton and the Immunological Synapse

Our expectations about molecular interaction in the IS have been shaped by early molecular definition of the molecules involved in this process [17]. The complex of the LFA-1

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with ICAM-1 (~48 nm) is larger than the complex of the TCR with MHCp (~15 nm) by over threefold [18–20]. Therefore, the LFA-1/ICAM-1 and TCR/MHC interactions segregate into different compartments within the contact area [21]. This receptor segregation forms receptor aggregates with the size and organization determined by the rigidity of the membrane, the kinetics of the interactions, and the degree of differences in molecular size of the participating receptor–ligand pairs [22]. This immediate segregation may be the initial trigger of receptor clustering and signaling in the nascent IS [23]. These events happen in seconds and set the stage for mature synapse formation.

The formation of the IS has been followed over time in live T cells [2] and studied at specific time points in fixed cell–cell conjugates [24]. The T cell forms an adhesion zone with the antigen-presenting bilayer, which is then surrounded by areas of close contact where TCR can reach the MHCp. If the TCR engagement exceeds a threshold rate and level, the T cell stops migrating and forms a ring of engaged TCR at the periphery of the nascent IS (Fig. 1A). This pattern takes about 30 sec to form and corresponds to the peak of TCR-associated tyrosine phosphorylation and Ca^{2+} mobilization. Within a few minutes, the sites of TCR engagement move from the periphery of the contact area to the center of the contact area to form the mature IS (Fig. 1B). During this time, the disk-like region of LFA-1/ICAM-1 interaction appears to give way to the centrally moving TCR, but the LFA-1/ICAM-1 interactions maintain the contact area and evolve into a ring of ~5-μm outer diameter (Fig. 1C). This last pattern can be stable for hours. The central region of TCR engagement is defined by Kupfer *et al.* [24] as a central supramolecular activation cluster (cSMAC) and the ring of LFA-1 engagement

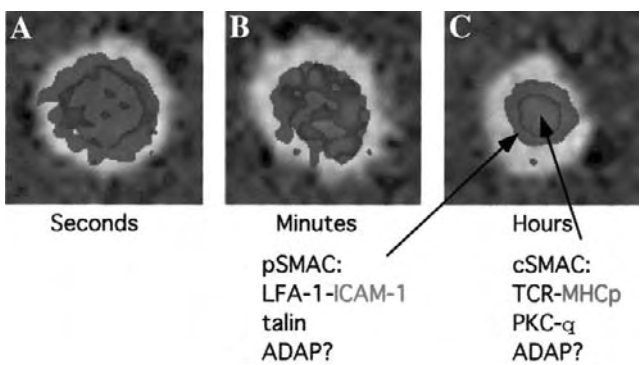


Figure 1 Development of the immunological synapse. Images are based on fluorescence microscope images of T-cell interaction with agonist MHC–peptide complexes (green) and ICAM-1 (red) in a supported planar bilayer with a T cell. The accumulation of fluorescence represents interactions in different time frames. Within seconds the T cell attaches to the substrate using LFA-1/ICAM-1 interactions in the center based on TCR signaling triggered at the periphery of the contact area (A). Over a period of minutes, the engaged TCRs are translocated to the center of the contact area (B). The final pattern with a central cluster of engaged TCR surrounded by a ring of engaged LFA-1 is stable for hours (C). Molecular markers for the cSMAC and pSMAC are indicated. For scale, the pSMAC is ~5 μm across. (Adapted from Grakoui, A. *et al.*, *Science,* **285**, 221–227, 1999.)

is defined as a peripheral supramolecular activation cluster (pSMAC). The other defining marker for the cSMAC is protein kinase C-θ, and an additional defining marker for the pSMAC is the cytoskeletal protein talin. The adapter protein ADAP links TCR signaling to LFA-1 activation so it may be expected to span these structures, but its physical location in the IS is not currently known. It is not clear if the same TCRs move from the outside to the center or if new TCRs are continually recruited. The interaction of the TCRs with agonist MHC–peptide complexes has a short half life (~5 sec) [25], and it is known that TCRs are degraded following effective engagement [26]. However, at some point in IS formation the interaction of the TCRs and the MHC–peptide complexes changes so they no longer dissociate. Thus, while serial engagement might dominate in the nascent IS, parallel engagement of at least 50 TCRs is a characteristic of the center of the mature IS. These observations have emphasized the concept that biochemical reactions are highly compartmentalized in the IS such that the location of receptor and signaling molecules must be considered to understand the biochemical basis of T-cell activation [27].

The formation of the synapse is highly active and depends on an intact actin cytoskeleton. The formation of the central cluster of TCR has a superficial similarity to antibody-mediated capping in that it requires an intact actino–myosin cytoskeleton. A plausible model based on this similarity has been proposed and initial results support some aspects of the model [28]. However, the IS has many elements that are completely absent in capping of cross-linked antigen receptors. For example, capping is based on a network of bivalent interactions on a cell surface that leads to extensive crosslinking, whereas receptor aggregation in a cell–cell contact is more likely to result from membrane fluctuations, receptor–ligand size differences, and interaction kinetics. These components have been incorporated in a physical model by Chakraborty and colleagues [22]. The predictions of this model are remarkably similar to the observations on the formation of the IS. This more physical view is compatible with an active role for the cortical cytoskeleton, because signaling-induced changes in cytoskeletal dynamics in activated T cells will profoundly regulate the Brownian bending movements of the membrane that are required for movement of the receptor interactions. This model could be described as a physical and mathematical elaboration on the kinetic-segregation model [23]. Thus, the early signals from the TCR that trigger increased actin polymerization may induce the membrane fluctuations that drive the maturation of the IS. Both the capping and the kinetic-segregation models predict that cytoskeletal dynamics are critical for IS formation.

The Role of Self MHCp in T-Cell Sensitivity to Foreign MHCp

Any single TCR interacts with a degenerate spectrum of MHCp. One way to study this spectrum is through altered peptide ligands in which an agonist peptide is mutated and

tested for biological activity: Null MHCp alone do not activate T cells, and agonist MHCp, the model foreign MHCp, induce full T-cell activation. Weak agonists induce a subset of T-cell responses, and antagonists interfere with T-cell responses to agonists. Approximately half of the TCR/MHCp binding energy comes from the TCR contacts with the MHC molecule [29]. Thus, the remaining peptides can be further divided. Naught peptides actively interfere with the TCR interactions and thus allow no interaction of the TCRs with MHC, while null peptides are neutral and allow the TCRs to interact with MHC. The interaction of null MHCp is too fast to induce a response in mature T cells [30]. Self MHCp that are agonists, weak agonists, or antagonists all induce apoptosis of immature T cells *in vivo* [30]. In contrast, null MHCp enhance positive selection. Thus, most mature T cells face APCs that are loaded with a mixture of null peptides (self). These mature T cells are triggered by APCs bearing a few agonist/weak agonist MHCp mixed with diverse null MHCp. Naïve T cells respond to approximately 300 agonist MHCp on APCs, while memory T cells require only 50 agonist MHCp [31]. A single agonist MHCp is sufficient to trigger cytotoxic T-cell killing [32].

How is the high sensitivity of immune recognition achieved? Can a single agonist MHCp achieve T-cell activation, or do other MHCp promote this process? Wülfing *et al.* tested the hypothesis that null (self) MHCp contribute to T-cell activation through analysis of proliferation and formation of the IS [33]. They demonstrated that null MHCp with a lysine-to-alanine mutation at a key TCR contact contributes to IS formation and T-cell activation triggered by subthreshold amounts of agonist MHCp. It was demonstrated that fluorescently labeled null MHCp were accumulated in the center of the IS and synergized with trace levels of agonist MHCp for T-cell activation. This was not true of all null MHCp, as a similar peptide with a lysine-to-glutamate mutation at the same TCR contact, most likely precluding TCR approach to the MHCp, did not have this coagonist activity. Therefore, the "null" classification of altered peptide ligands can be divided into coagonists, which synergize with agonist MHCp, and null peptides, which have no activity. Based on this result it can be proposed that agonist MHCp do not have to go it alone; they may be substantially helped by coagonist MHCp in the self peptide repertoire. The degree of help may vary with the specific TCR and MHC molecules and may have a role in autoimmune diseases. Help from coagonist MHCp may account for the remarkable sensitivity of T cells to agonist MHCp.

Integration of Adaptive and Innate Responses

The IS is not limited to adhesion molecules and MHC–peptide complexes. The process of naïve T-cell activation involves a system of checks and balances that are integrated to make activation decisions. An important aspect of this integration is that T cells test both the MHC–peptide complex and the status of the innate immune response in the APC. In response to evolutionarily conserved microbial products such as lipopolysaccharide, the APC is activated. This increases expression of a number of molecules including the MHCp, adhesion molecules, and ligands for costimulatory receptors. Ligands for costimulatory receptors include CD80 and B7-DC (also know at PDL2) [34]. CD28 is the receptor for CD80 and by binding CD80 it indirectly transduces an innate immune system signal that can be integrated with the TCR signal. CD28/CD80 interactions are very inefficient due to the low density of CD28 and its low lateral mobility on naïve T cells [35]. Upon immunological synapse formation CD28/CD80 interactions are facilitated and focused in the central region of the immunological synapse, very close to the site of TCR engagement. However, CD28/CD80 interaction does not help the TCR/MHCp interaction, which sets it apart from adhesion molecules such as LFA-1 and CD2 [35]. This suggests a sequential model for T-cell response to TCR and innate signals. The formation of the IS corresponds to the antigen signal. It is only when this signal is received that the T cell becomes competent to receive the signal through CD28. This is the first analysis of receptor interactions in adaptive–innate signal integration in the IS. It will be important to determine if other secondary signals are dependent on IS formation.

Summary

In summary, the IS concept provides a number of insights into the T-cell activation process. First, it provides a stop signal that coordinates antigen recognition and T-cell migration. Second, the essential role of the actin cytoskeleton in T-cell activation is related to the role of actin in IS formation. Third, the sensitivity of T cells to agonist MHCp is related to the role of weakly interacting, but probably more abundant, self MHCp in promoting IS formation. Finally, the IS provides a framework for orderly integration of the TCR and innate immune signals, such as in the case of CD28/CD80 interaction.

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CHAPTER 14

NK Receptors

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Introduction

Analogous to T cell receptors (TCRs) on the surface of T lymphocytes, natural killer (NK) cells function through cell-surface receptors (NCRs) that, unlike TCRs, can be any of a diverse array of molecules, either immunoglobulin-like or C-type lectin-like in structure. NCRs specific for classical and nonclassical major histocompatibility complex (MHC) class I proteins, expressed in complex patterns of inhibitory and activating isoforms on overlapping but distinct subsets of NK cells, play an important role in immunosurveillance against cells that have reduced MHC class I expression as a result of infection or transformation. Another NCR, NKG2D, an activating NCR first identified on NK cells but subsequently found on macrophages and a variety of T-cell types, is implicated in direct, antiviral, and antitumor immune responses. Recent crystallographic analyses of NCRs and NCR/ligand complexes reveal a range of recognition mechanisms that can be either similar to or quite distinct from TCR-mediated events.

Immunoreceptors

Recognition events between αβ T cell receptors (TCRs), expressed on the surface of T cells, and processed peptide fragments of endogenous proteins, presented on target cell surfaces as complexes with major histocompatibility complex (MHC) class I proteins, ultimately mediate activation of T-cell cytotoxic responses by the cellular arm of the adaptive immune system [1]. MHC class I proteins are integral-membrane, heterodimeric proteins with ectodomains consisting of a polymorphic heavy chain, comprising three extracellular domains (α1, α2, and α3), associated with a non-polymorphic light chain, β_2-microglobulin (β_2-m) [2]. The α1 and α2 domains together comprise the peptide- and TCR-binding "platform" domain; the α3 and β_2-m domains have C-type immunoglobulin (Ig) folds. Crystal structures of TCR/MHC complexes show that the TCR variable domains sit diagonally on the MHC platform domain, making contact with the peptide and the MHC α1 and α2 domains [3] (see Fig. 1). Binding studies show that the dissociation constants for these interactions range from one to tens of micromolar (see Table 1). Analysis of the kinetics of binding suggest that TCR/MHC binding is accompanied by a reduction in flexibility at the receptor/ligand interface [4].

Natural Killer Cells

Surveillance against cells undergoing tumorigenesis [5–9] or infection by viruses [10,11] or internal pathogens [12,13] is provided by natural killer (NK) cells, components of the innate immune system, thus helping to provide "covering fire" during the period that responses by the adaptive immune system are gearing up [14]. NK cells also act to regulate innate and acquired immune responses through the release of various immune modulators, chemokines, and cytokines, such as tumor necrosis factor α, interferon γ, MIP-1, and RANTES. Unlike T cells, NK cells function through a diverse array of cell-surface inhibitory and activating receptors.

Many NK cell surface receptors (NCRs) are specific for classical (such as HLA-A, -B, and -C in humans) and nonclassical (such as HLA-E in humans) MHC class I proteins and occur in paired activating and inhibitory isoforms [15–17]. Different NCRs, with different MHC class I specificities, are expressed on overlapping, but distinct, subsets of NK cells in variegated patterns—where the strength of the inhibitory signals may be stronger than stimulatory signals.

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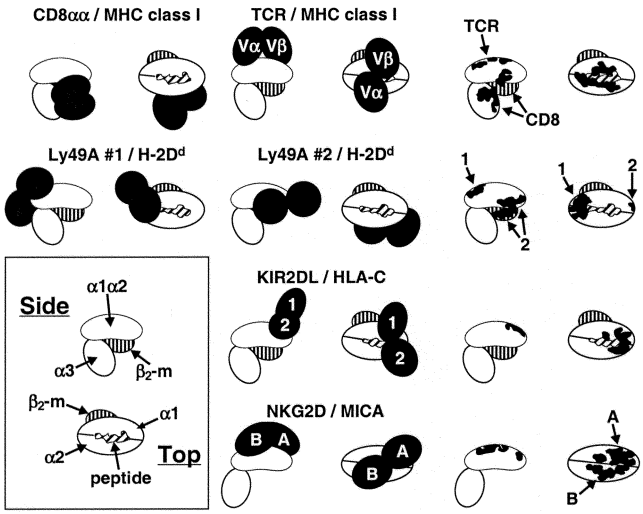


Figure 1 Schematic representations of structurally characterized NK receptor–ligand complexes. Each row shows two views of a receptor–ligand complex, first showing the organization of domains in the complex (receptor domains in black, labeled where a distinction between domains is significant; MHC class I ligand heavy chains in white and β_2-m in vertical stripes). The arrangement of domains in the ligands is detailed in the inset; the approximate solvent-accessible surface area of the bound peptide, if present, is shown as a cross-hatched area. The right-most columns show approximate footprints of receptors and coreceptors on the ligands as black patches, labeled by receptor component, subsite, or domain, as appropriate.

Thus, NK cell effector functions are regulated by integrating signals across the array of stimulatory and inhibitory NCRs engaged upon interaction with target cell surface NCR ligands [16,17], resulting in the elimination of cells with reduced MHC class I expression, a common consequence of infection or transformation [18]. Other NCRs, such as human and murine NKG2D, recognize divergent MHC class I homologs (ULBPs [19], MICA, and MICB in humans [20], and RAE-1 and H60 in mice [21,22] not involved in conventional peptide antigen presentation. Inhibitory receptors transduce signals through recruitment of tyrosine phosphatases, such as SHP-1 and SHP-2, and contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains [23,24]. Activating receptors associate with immunoreceptor tyrosine-based activation motif (ITAM)-bearing adaptor proteins, either DAP12 [25] or DAP10 [26,27], through a basic residue in their transmembrane domain.

Natural killer cell surface receptors can be divided into two groups based on structural homologies [28,29]. The first group includes the killer cell Ig receptors (KIRs) and consists of type I transmembrane glycoproteins with ectodomains containing tandem Ig domains. The second group, including the rodent Ly49 receptor family and the CD94/NKG2 and NKG2D receptor families found in primates and rodents, comprises homo- and heterodimeric type II transmembrane glycoproteins containing C-type lectin-like NK receptor domains (NKDs) [30]. NCR/ligand dissociation constants range from a hundred micromolar to tens of nanomolar (see Table 1). A series of recent results from X-ray crystallographic analyses detail the interactions for a number of NCR/ligand complexes.

Table I Immunoreceptor Affinities

Receptor	Ligand	K_D (μM)	Ref.
TCR	MHC class I	1–90	4,36
NKG2A–CD94	HLA-E	11.23	37
KIR	MHC class I	~10	38
huNKG2D	MICA	0.3	34
muNKG2D	H60	0.0189	39
muNKG2D	RAE-1α, β, γ, δ	0.345–0.726	39

Ig-Type NK Receptors: KIR

Two crystal structures of complexes between inhibitory KIR family NCRs and their MHC class I ligands, KIR2DL2/HLA-Cw3 [31] and KIR2DL1/HLA-Cw4 [32], show that the receptor binds in a 1:1 complex with HLA-C, making contact with both the α1 and α2 platform domains and the carboxy-terminal end of the bound peptide (see Fig. 1). (KIR receptor nomenclature identifies the number of Ig domains [2D(omains) or 3D, specific for HLA-C or HLA-B respectively], and whether the receptor is a long [L] form, containing ITIM repeats, or a short [S] form, interacting with ITAM-containing adaptor proteins.) Both complexes have interfaces showing both significant shape and charge complementarity, with the N-terminal KIR domains interacting primarily with the α1 domains of HLA-C, the C-terminal KIR domains contacting the α2 domains, and additional contacts provided by the interdomain KIR linker peptides (the "elbow"). The kinetics of binding, rapid on and off rates, are consistent with interactions dominated by charge–charge interactions.

Despite a high degree of conservation of binding surface residues between both KIR2DL2 and KIR2DL1, and HLA-Cw3 and -Cw4, few actual intermolecular interactions are conserved. This recognition flexibility is accomplished through altered side-chain conformations. KIR2D receptors distinguish between HLA-C allotypes on the basis of the residue at position 80; KIR2DL1 recognizes lysine and KIR2DL2 recognizes asparagine, and this specificity is conferred by the identity of the residue at position 44 in the receptor. In KIR2DL1, Lys80 is shape and charge matched to a distinct pocket on the surface of the receptor; while Asn80 is sensed through a direct hydrogen bond in the KIR2DL2 complex.

C-Type Lectin-Like NK Receptors: Ly49A

Ly49A is a disulfide-linked, symmetric, homodimeric, NKD-type NCR that is specific for the murine MHC class I protein H-2D^d (the human ortholog is nonfunctional). The crystal structure of the D^d/Ly49A complex [33] shows D^d homodimers binding to two distinct sites on the MHC protein (see Fig. 1). The first binding site positions Ly49A on the D^d platform domain, contacting both α1 and α2 and the

N-terminal end of the bound peptide—the opposite end from where KIR2D binds. The second binding site positions Ly49A in the cleft between the underside of the platform domain (the top being the peptide and TCR binding surface), the α3 domain and β_2-m. The second site is considerably more extensive than the first site, though less shape complementary and less dominated by charge–charge interactions, and is likely to be the immunologically relevant interaction on the basis of subsequent mutagenesis studies. The second site also overlaps the CD8 binding site on MHC class I proteins. As predicted, Ly49A clearly displays a C-type lectin-like fold, though failing to retain any remnant of the divalent cation or carbohydrate binding sites conserved in true C-type lectins. While the simplest binding mode for a symmetric homodimer is to interact with two monomeric ligands through two identical binding sites, each Ly49A interaction with D^d is with a single monomer because binding of ligand at one site sterically blocks binding at the second, homodimer-related site.

C-Type Lectin-Like NK Receptors: NKG2D

NKG2D is an activating, symmetric, homodimeric, NKD-type NCR. While highly conserved between primates and rodents, its ligands include very different molecules, both in humans and rodents. Crystal structures of two complexes, human NKG2D/MICA [34] and murine NKG2D/RAE-1 [35], show that NKG2D interacts with its MHC class I homologous ligands in a manner very similar to the way in which TCRs interact with classical MHC class I proteins (see Fig. 1), even though NKG2D contains NKDs while TCRs contain Ig domains. NKG2D retains the C-type lectin-like fold seen in Ly49A, with few variations, although the binding surface of NKG2D is much more curved than in Ly49A, matching the more curved surface of its ligands (which do not bind peptides), where the Ly49A and NKG2D binding surfaces encompass overlapping surfaces on the receptors. The interaction surfaces bury considerable solvent-accessible surface area and are highly shape complementary, but the human NKG2D/MICA interaction is markedly more so than the murine NKG2D/RAE-1 interaction. The reason that the human complex does not bind considerably more tightly than the murine complex (see Table 1) is likely due to the necessity of ordering a large loop on the surface of MICA concurrent with complex formation, reflected in the unusually slow on-rate for the human complex. Unlike KIR and Ly49A interactions at the first site, the NKG2D binding sites are much less dominated by charge–charge interactions. The stoichiometries of the NKG2D complexes are one homodimer binding to one monomeric ligand; however, unlike Ly49A, both homodimer-related binding sites on NKG2D contribute approximately equally to the interactions in both complexes, reflecting a binding site that has evolved to bind multiple target sites without the degree of side-change rearrangements seen in the KIR interactions. It has also been proposed that the NKG2D/MICA complex is likely a good model for the CD94/NKG2A/HLA-E complex.

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CHAPTER 15

Carbohydrate Recognition and Signaling

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Introduction

The recognition of extracellular and cell surface carbohydrates by specific carbohydrate-binding proteins, or lectins, is an important component of many biological processes. Here, we review the main principles of protein–carbohydrate recognition with particular reference to examples where structural data are available and signaling is known to be important. In conclusion, we explore the suggestion that carbohydrate-mediated interactions provide unique cell-signaling mechanisms.

Biological Roles of Carbohydrate Recognition

Carbohydrates, in the form of oligosaccharides or glycoconjugates, are found on the cell surfaces and extracellular proteins of virtually all living organisms. Although roles for carbohydrates in endogenous physiological interactions had long been suspected, it was not until the 1970s, with the discovery of the hepatic asialoglycoprotein receptor and Man-6-phosphate-mediated intracellular protein targeting, that firm evidence for such roles began to emerge. Since then, a number of animal lectin families have been identified [1,2] and their functions, in processes ranging from protein folding and quality control to leukocyte homing, have been the subject of considerable study.

The cloning of glycosyltransferases and the generation of null-mutant mice have also provided further clear evidence of roles for endogenous carbohydrate recognition in the development and functioning of the immune and nervous systems [3–5]. In addition, mutations affecting the elaboration of complex carbohydrates are now known to be the basis for a growing number of human diseases collectively known as the congenital disorders of glycosylation (CDGs) [6]. The discovery that aberrant glycosylation of dystroglycan results in various forms of muscular dystrophy provides the most recent example [7–9].

Perhaps most surprising has been the finding that carbohydrates are also involved in the regulation of a number of signaling pathways. Fringe, for example, is a β1,3 *N*-acetylglucosaminyltransferase [10,11] whose action modulates the interaction of the Notch receptor with its ligands, and mutations in Brainiac, a glycolipid-specific β1,3 *N*-acetylglucosaminyltransferase [12,13], effect oogenesis. Genetic studies have also shown that proteoglycans/glycosaminylglycans play key roles in development, and, in *Drosophila* and *Caenorhabditis elegans*, they have been shown to be involved in regulating the fibroblast growth factor, Wnt, transforming growth factor-β, and Hedgehog signaling pathways [14].

Carbohydrate Structure and Diversity

The structural diversity characteristic of the oligosaccharides found in nature stem principally from three sources: (1) a large number of monosaccharide types, (2) the multiple ways in which the monosaccharides can be linked

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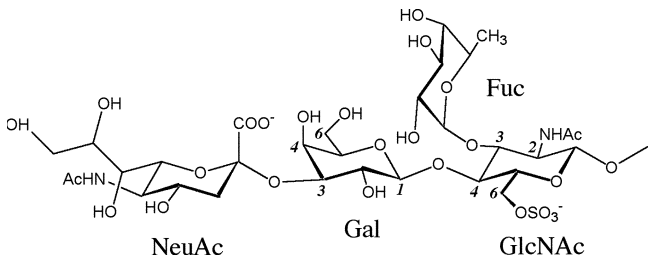


Figure 1 Structural representation of the sulfated sialyl Lewis x tetrasaccharide, NeuAcα2-3Galβ1-4[Fucα1-3](6–sulfo)GlcNAc. NeuAc, Gal, GlcNAc, and Fuc label the monosaccharide moieties *N*-acetylneuraminic acid, galactose, *N*-acetylglucosamine, and fucose, respectively.

together, and (3) the fact that oligosaccharides can be further modified chemically (e.g., sulfate, phosphate, and acetyl). The basic themes are illustrated in Fig. 1, which shows the structure of sulfated sialy Lewis x, a tetrasaccharide important in selectin-mediated recognition. Because most monosaccharides have more than one hydroxyl group available for glycosidic bond formation, oligosaccharides, unlike their peptide counterparts, can form branched structures. The oligosaccharide structures linked to lipid or to protein, through Ser/Thr (O-linked) or Asn (N-linked), typically contain between 1 and 20 monosaccharide moieties and may be branched or linear. The much longer linear glycosaminylglycans, either in isolation or as the oligosaccharide chains of proteoglycans, are found on cell surfaces and in the extracellular matrix.

In vivo, oligosaccharides are synthesized by glycosyltransferases, each of which typically has a unique donor, acceptor, and linkage specificity. As such, a very large number of glycosyltransferases and related enzymes are required to generate the oligosaccharide diversity seen in nature. Although the basis for this diversity is not fully understood, general themes are beginning to emerge. The so-called terminal elaborations (e.g., sialic acid, galactose, and sulfate) typical of the N-linked oligosaccharides of multicellular organisms, for example, seem to have appeared as part of the machinery required to mediate cell–cell and cell–matrix interactions [15]. In addition, it seems likely that oligosaccharide diversity has also been driven by evolutionary pressures arising from the need to differentiate self from nonself [16].

Lectins and Carbohydrate Recognition

Carbohydrate-binding proteins or lectins, like their saccharide counterparts, are also found in organisms ranging from microbes to humans [1]. The canonical carbohydrate recognition domain (CRD), characteristic of a given lectin type, can be found either in isolation or in conjunction with other protein domains, including coiled-coil domains and membrane-spanning motifs. Although many of the known CRD types are completely unrelated at the protein structural level [17–19], they can be grouped into two broad classes [20]. The type I CRDs are typified by the bacterial carbohydrate transporters and are characterized by deep carbohydrate-binding sites that essentially envelop their small saccharide ligands. In type II CRDs, the carbohydrate-binding sites are more shallow in nature and the saccharide remains relatively exposed to solvent, even when bound to the CRD. As a result, the dissociation constants (K_d) for small mono- or disaccharides can approach 0.1 μ*M* for the type I CRDs, while the type II CRDs tend to bind small saccharides with K_d in the range of 0.1 to 1.0 m*M*.

Despite their relatively weak affinities for small saccharides, type II CRDs often show a strict mono- or disaccharide binding specificity. From a structural standpoint, this is achieved by a complementarity of fit between the CRD and the saccharide moiety which includes both hydrogen bond and van der Waals interactions. The structural and thermodynamic basis for this specificity has, in fact, been well studied and reviewed in detail elsewhere [17–23].

Given that the type II CRDs bind small saccharides relatively weakly, most of these lectin types have employed multivalency as a means of conferring additional affinity and specificity on their binding interactions with larger oligosaccharides [17]. In addition to the monosaccharide in the primary site, the CRD may possess subsites for interaction with other monosaccharides of the oligosaccharide. Alternately, many lectins cluster their CRDs as a means of making multivalent interactions with larger oligosaccharides or other extended structures such as cell surfaces. Members of the C-type lectin family, for example, are known to form monomers, trimers, tetramers, pentamers, and hexamers, as well as higher order oligomers, and in some cases a single polypeptide chain will possess more than one canonical CRD.

Carbohydrate-Mediated Signaling

Lectins as Receptors

Most of the current evidence for the biological roles of complex carbohydrates comes from systems where they act as ligands for membrane-bound receptors that are lectins. Typically, these receptors have one or more extracellular CRDs, a single transmembrane-spanning region, and a relatively short cytosolic tail. In most cases, they are probably activated by receptor cross-linking mechanisms.

L-, P-, and E-selectin are cell-surface, C-type lectins responsible for leukocyte homing [24]. Unlike other members of the family, they do not possess a monosaccharide binding specificity. They require at least a tetrasaccharide, sialyl Lewis x (Fig. 1) for binding, and specific sulfation further enhances binding to L- and P-selectin [25]. The crystal structures of P- and E-selectin, in complex with oligosaccharide/glycopeptide ligands, have shown the importance of electrostatics in these interactions, a factor thought to be important in the rapid binding kinetics required for leukocyte rolling [26]. Moreover, the structures have provided a rationalization for the specificity differences that ensure that lymphocytes target to lymph nodes and neutrophils reach sites

of inflammation. Although the selectins are not known to form oligomers, E-selectin-mediated clustering at contact points between interacting cells has been shown to activate the ERK1/2 signaling pathway [27].

DC-SIGN and DC-SIGNR are also C-type lectins, but in this case they are involved in dendritic cell/T-cell interactions [28], as well as the promotion of HIV-1 infection [29]. These lectins possess a mannose-binding specificity, but in addition show a marked increase in affinity for high mannose oligosaccharides [30]. The crystal structures of their CRDs in complex with a mannopentasaccharide show that the increased affinity arises from a further set of interactions in addition to those made with the mannose in the primary binding site [31]. Because these lectins also possess α-helical tetramerization domains, it seems likely that they would be capable of making high-affinity interactions with ICAM-3 and HIV gp120, two of their natural ligands. In fact, it has been suggested that the cross-linking of DC-SIGN tetramers, by the highly multivalent high mannose oligosaccharide containing HIV virus, provides the signal required to promote transport of HIV from the periphery to the T-cell-containing lymph nodes [29].

The hepatic asialoglycoprotein receptor, a member of the C-type lectin family, provides a well-characterized example of the interplay between structure, specificity, and receptor cross-linking. Although an isolated CRD of this receptor binds galactose with a K_d in the millimolar concentration range, the cell-surface form of the receptor can bind the appropriate triantennary N-linked oligosaccharide with nanomolar affinity. Cross-linking studies have shown that the HL-1 subunit forms trimers on the cell surface and that recruitment of an additional HL-2 subunit(s) generates the high-affinity receptor. The galactose terminii of the triantennary oligosaccharides (separated by 15 to 25 Å) are found to interact with both the HL-1 and HL-2 subunits [32]. Linking receptor specificity to receptor cross-linking in this way may be important for both receptor uptake and signal transduction [33].

The targeting of lysosomal enzymes is also dependent on receptor-mediated endocytosis. In this case, the cation-dependent mannose 6-phosphate receptor (CD-MPR) and the insulin-like growth factor II/cation-independent mannose 6-phosphate receptor (IGF-II/CI-MPR) specifically recognize the mannose-6-phosphate moiety on acid hydrolases destined for lysosomes [34]. Again, multivalency is important; CD-MPR binds mannose 6-phosphate with a dissociation constant in the micromolar concentration range, while the dimeric receptor binds tetrameric β-glucuronidase with nanomolar affinity. Both dimeric and tetrameric forms of the receptor are found in the Golgi membrane, and, based on the crystal structure of the dimeric CD-MPR, a model for its high-affinity interaction with β-glucuronidase has been proposed [35]. The IGF-II/CI-MPR receptor contains two canonical CRDs presumably capable of promoting high-affinity interactions with multivalent lysosomal enzymes, and together with CD-MPR these receptors are responsible for targeting over 50 structurally distinct lysosomal enzymes. Dimerization of the IGF-II/CI-MPR receptor by β-glucuronidase binding increases receptor internalization at the cell surface [36].

The siglecs are a family of sialic acid binding lectins whose canonical CRD is a member of the immunoglobulin (Ig) superfamily. They are particularly important in the immune system, where they function in processes ranging from leukocyte adhesion to hemopoiesis [37]. Members of the family show specificity differences for α2,3- versus α2,6-linked sialic acids, as well as for sialic acids modified with respect to *O*-acetylation. The crystal structure of the CRD of sialoadhesin in complex with 3′ sialylactose shows that interactions with the bound oligosaccharide are mediated primarily with the terminal sialic acid moiety [38]. Of particular interest are the roles played by *cis* interactions. CD22 (Siglec-2), for example, is a B-cell-specific receptor which, through interaction with α2,6-linked sialic acid containing glycoproteins on its own cell surface, inhibits B-cell receptor signaling. This stable inhibition can be broken by the addition of external competing saccharide and *in vivo* may be controlled by the regulation of sialytransferases and/or sialidase expression levels [39]. The cloning of several CD33-related receptors expressed on myeloid cell progenitors suggests new insight into the significance of their sialic acid binding properties. In all cases, these receptors possess cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs), elements now known to be hallmarks of inhibitory receptors central to the initiation, amplification, and termination of immune responses [40]. Through interactions with sialic acid containing self determinants, these receptors may play roles in the control of innate immunity [41].

Serum mannose binding protein (MBP), a component of the vertebrate innate immune system, is also a C-type lectin. Although not membrane bound, it signals activation of the complement cascade though a conformational change initiated by binding the cell surface of a foreign pathogen [42]. Like the asialoglycoprotein receptor, the CRD of MBP also recognizes only a terminal monosaccharide moiety, in this case mannose. The CRDs are also found to form trimers; however, in MBP they are mediated by long, triple-helical, coiled-coil domains that in addition promote the formation of trimer clusters containing 18 CRDs in total [43]. The crystal structures of truncated forms of the trimer show that the mannose binding sites are separated by 45 and 53 Å, respectively, in human [44] and rat [45] MBP. Thus, unlike the asialoglycoprotein receptor, which is designed to recognized the closely spaced galactose determinants of a single N-linked oligosaccharide, MBP is designed to bind the widely spaced mannose determinants typical of the cell surfaces of pathogenic microorganisms [46].

Glycoproteins as Receptors

It has long been known that certain multivalent, soluble plant lectins (e.g., PHA and Con A) can induce mitosis in lymphocytes and oxidative burst in neutrophils. The mechanism for initiation of these signals has generally been assumed to result from the cross-linking of cell-surface glycoproteins.

More recently, soluble animal lectins of the galectin type have also been found to induce a variety of signals, including, among others, apoptosis, oxidative burst, cytokine release, and chemotaxis in immune cells [47]. In structural terms, the galectins are either dimeric or contain more than one CRD on a single polypeptide chain and as such they are capable of cross-linking receptors [48]. Recent studies aimed at understanding T-cell homeostasis have suggested that CD45, CD43, CD7 [49], and the TCR–CD3 complex [50] are physiologically relevant cell-surface receptors for galectins-1 and -3, respectively.

Glycolipids as Receptors

The role of glycolipids as receptors for microbial lectins has been well studied. Bacterial AB_5 toxins possess a pentameric arrangement of B-subunit lectins which, through multivalent interactions, promote high-affinity binding with host cell-surface gangliosides [51]. In the case of cholera toxin, binding to G_{M1} on the cell surface is followed by retrograde transport and translocation across the ER membrane [52]. Once in the cytosol, the A1 fragment of the A subunit catalyzes the ADP ribosylation of the heterotrimeric Gαs protein, leading to the characteristic chloride and water efflux. In what is a fundamentally different type of interaction, the lectin subunits of the *Escherichia coli* P-fimbriae bind glycolipids in uroepithelial cells leading to ceramide release, activation of ceramide signaling pathways, and ultimately cytokine release through a process that also appears to involve activation of the TLR-4 receptor pathway [53–55]. Although not yet fully characterized, the interactions of glycosphingolipids with various adhesion and signaling receptors found in cell-surface microdomains are being found to mediate signaling events important in cell–cell interactions [56].

Proteoglycans and Glycosaminoglycans

Proteoglycans contain long linear oligosaccharide chains (glycosaminoglycans) made up of disaccharide repeats containing acidic monosaccharides and variable degrees of sulfation. They are found at the cell surface and in the extracellular matrix, where they interact with a wide variety of molecules, including, among others, signaling receptors, growth factors, chemokines, and various enzymes [57–59]. In the well-characterized fibroblast growth factor (FGF)–fibroblast growth factor receptor (FGFR) interaction, heparin/heparan sulfate serves as coreceptor. Two recent crystal structures of ternary complexes have begun to shed light on how the intrinsically multivalent oligosaccharide serves to promote receptor cross-linking in this system [60,61]. Recent evidence from studies on hepatocyte growth factor/scatter factor suggests that heparan and dermatan sulfate binding serves to promote a conformational change in the growth factor that promotes receptor binding [62]. In some cases, specific sulfation patterns appear to be important determinants of specificity [58,63]. The syndecans are cell-surface proteoglycans whose core proteins contain cytoplasmic signaling motifs. They have been implicated in the formation of focal adhesions, where interactions with heparin binding domains and other receptors are proposed to lead to adhesion, cross-linking, and signal transduction [64].

Small Soluble Saccharides

Small nutrient saccharides are often sensed by the receiving cells after entry through a transporter. In mammals, for example, glucose is sensed by an alteration in the adenosine triphosphate (ATP)/adenosine diphosphate (ADP) ratio resulting from glucokinase-initiated glucose metabolism. In microbes, small saccharides are often sensed by specific, non-enzyme cytosolic binding proteins that in turn regulate gene expression (e.g., the Lac-repressor of *E. coli*). In plants, nutrient sugars are also known to be important mediators of signal transduction [65], and the recognition of small soluble oligosaccharides by membrane and cytoplasmic receptors is important in plant host defense [66]. Although these examples are beyond the scope of this review, it is worth noting that these carbohydrate-mediated signaling mechanisms may be operative in systems yet to be characterized.

Carbohydrates and Lectins in the Nucleocytosolic Compartment

The O-linked glycosylation of serine and threonine residues of nuclear and cytoplasmic proteins by *N*-acetylglucosamine (O-GlcNAc) is involved in signal transduction in multicellular organisms [67]. This dynamic modification occurs at sites of protein phosphorylation and may serve to transiently block sites of phosphorylation. Although its roles are not yet fully characterized, O-GlcNAc has been found to modulate a wide range of cellular functions, including transcription, translation, nuclear transport, and cytoskeletal assembly [68].

Galectins are also cytosolic and nuclear proteins, but they are not known to bind carbohydrates in these compartments; however, galectins 1 and 3 have been implicated in pre-mRNA splicing, a process inhibited by oligosaccharide binding [69]. The galectins are also secreted from the cytoplasm (by nonclassical pathways), and it is at the cell surface that they perform the carbohydrate-mediated processes discussed previously.

For the sake of completeness it is worth noting that well-known second messengers such as cyclic AMP, GDP, GTP, etc. are ribose-containing glycoconjugates and that even more complex saccharide second messengers may be operative in insulin signaling [70].

Conclusions

The interactions between lectins and carbohydrates are relatively weak in nature and, as such, carbohydrate-mediated interactions may play important roles where weak interactions are required—the leukocyte rolling phenomenon perhaps providing a good example. In many cases, however, type II lectins have employed multivalency as a means of conferring

increased affinity and specificity on their binding interactions. The structures of the asialglycoprotein receptor and MBP provide important examples of this principle. Because receptor cross-linking or clustering is a natural outcome of such multivalent interactions, it is clear that lectin–oligosaccharide interactions are inherently well suited to mediating signal transduction by the so-called horizontal mechanisms. In contrast, the higher affinity type-I lectins, typified by the bacterial transport/chemosensory receptors, appear to employ a mechanism more akin to vertical signaling where ligand-induced conformational changes in the receptor lead to signal transduction [71]. Interestingly, the affinity of these receptors for their carbohydrate ligands is close to the minimum affinity ($K_d \sim 10^{-8}$ *M*) thought to be required for vertical signaling though 7TM receptors.

Although similar in some ways, it is clear that protein–carbohydrate interactions differ from protein–protein interactions in ways that might confer on them unique signaling roles or properties. Because glycosylation is a posttranslation modification capable of modifying any molecule with the appropriate acceptor, the subsequent recognition of carbohydrate determinants differs fundamentally from that involving specific protein–protein interactions. The galectins and siglecs, for example, bind β-galactosides and sialic-acid-containing ligands, respectively, and either of them might be expected to interact with more than one receptor type. As such, carbohydrate-mediated interactions may enable the activation of multiple signaling pathways or networks, as described by Bhalla and Iyengar [72]. Alternately, if carbohydrate-mediated interactions lead to heterogeneous cross-linked receptor arrays, this might result in spatial/geometric associations, where the triggering of one receptor type leads to the activation of another [73,74]. Brewer and colleagues [75] have also provided evidence for the ability of multivalent lectins to form homogeneous cross-linked arrays or lattices, even in the presence of competing ligands. In fact, in recent *in vivo* studies they have shown that galectin-1-induced apoptosis is accompanied by the redistribution and segregation of CD45 and CD43 on T-cell surfaces. Galectin-3-mediated cross-linked arrays have also been recently invoked in a model for T-cell receptor activation [50]. In a similar vein, it seems likely that the highly multivalent proteoglycans provide scaffolds upon which interacting molecules can be assembled and organized.

In addition to the potential for triggering signaling events, the formation of carbohydrate-mediated cross-linked arrays may also be important in receptor turnover, one way in which signaling events are modulated [76]. In fact, evidence already exists for the ability of galectin-3 to both accelerate [77] and retard the turnover of cell surface receptors (J. Dennis, personal communication). In what might be a variation on this theme, the priming of neutrophil leukocytes with lipopolysaccharide (LPS) leads to galectin responsiveness by inducing the transfer of receptor containing vesicles to the cell surface [78,79].

Our knowledge of lectin and glycoprotein structures shows that multivalent interactions are a recurring theme. Many lectins are oligomeric and/or membrane bound, and many glycoproteins (and certainly proteoglycans) possess multiple glycosylation sites. Their inherent ability to mediate cross-links make it certain that new examples of signaling roles will follow from the study of these complex and diverse molecules.

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CHAPTER 16

Rhinovirus–Receptor Interactions

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The attachment of a virus to specific cell-surface receptors is a key event in the life cycle of animal viruses. It determines the host range and tropism of infection and initiates delivery of the genome into the cell. Once bound to a receptor, the non-enveloped viruses such as the rhinoviruses must then transfer their genome directly across a membrane into the cytoplasm for reproduction [1].

Human rhinoviruses (HRVs) are a major cause of the common cold. They are small, icosahedral viruses, 300 Å in diameter, and belong to the Picornaviridae family, which includes Rhinovirus, Aphthovirus, Enterovirus, Cardiovirus, etc. Their capsid is composed of 60 copies each of four viral coat proteins, VP1, VP2, VP3, and VP4, on a T = 1 (or pseudo T = 3) icosahedral lattice [2]. The three major capsid proteins VP1, VP2, and VP3 all have the same basic eight-stranded β-barrel fold and a molecular weight of around 30 kDa. VP4 is a small protein located inside the capsid. The capsid encloses a single positive RNA strand of about 7000 bases. The HRV capsid has a star-shaped dome on each of the five-fold axes surrounded by a shallow depression or "canyon" and a triangular plateau centered on each three-fold axes and around each five-fold axes (Fig. 1).

A distinctive feature of VP1 is that it has a "pocket" or hollow within the β-barrel that is accessible from the exterior of the capsid. This hydrophobic pocket located at the base of the canyon is frequently occupied by a natural *pocket factor*, a fatty-acid-like molecule. This pocket factor is believed to stabilize the virus during its spread from cell to cell [3].

With one exception, HRVs are classified into a major group and a minor group based on their specificity for cell receptors (Fig. 2). The major group HRVs bind to the intercellular adhesion molecule-1 (ICAM-1) [4], which belongs to the immunoglobulin superfamily. ICAM-1 plays an important role in cell–cell interactions and contains five immunoglobulin-like domains. The minor group HRVs bind to members of the low-density lipoprotein receptor (LDL-R) family [5,6], which internalize LDL particles but also mediate the transport of macromolecules into cells by receptor-mediated endocytosis (Fig. 3). The ligand-binding amino terminus of the LDL receptors all contain various numbers of imperfect repeats of approximately 40 amino acids. These rigid ligand-binding domains are linked by four to five amino acids which confer some flexibility. Both ICAM-1 and the LDL receptors appear to bind their ligands by electrostatic interactions. HRV87 alone uses an unidentified sialoprotein as receptor [7] for which the receptor site is unknown. The major group HRV89 has the capacity to evolve under the pressure of passage *in vitro* to use an alternative receptor and even to infect cells devoid of its normal ICAM-1 receptor [8].

There is a remarkable difference in the location and accessibility of the receptor sites of the two groups of HRV. The major group HRV receptor site lies at the base of a depression or canyon around each five-fold axis [9] (Figs. 1 and 4). In contrast, the minor group HRV receptor binds to the star-shaped dome on the five-fold axis [10] (Figs. 1 and 4). The canyon hypothesis [1] proposed that the major group HRVs protect their receptor sites from immune surveillance by effectively hiding their receptor sites at the base of the canyon. The antibodies, being much larger than the ICAM-1 receptor, were supposed to be unable to reach the base of the narrow canyon. However, it was later shown that key viral amino acid residues involved in binding ICAM-1 are also accessible to antibodies [11]. Effectively, the receptor binding site is indeed accessible to antibodies but is flanked by residues capable of

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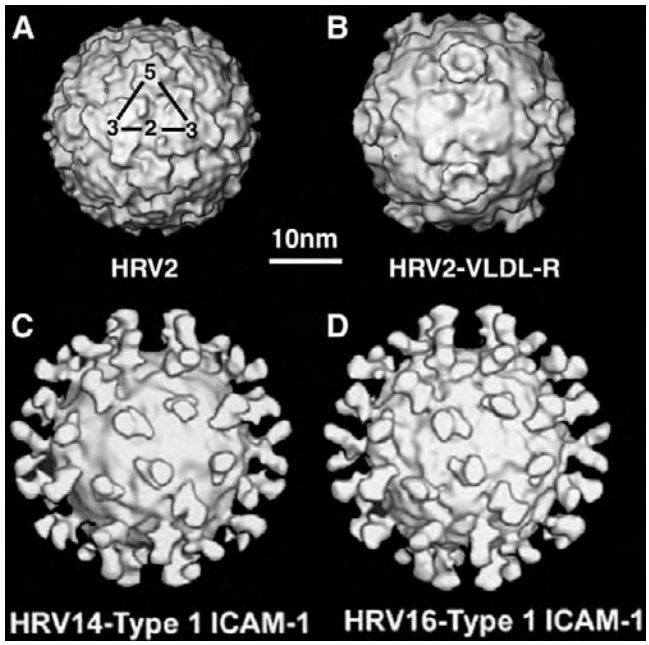


Figure 1 Surface views of the reconstructed cryo-electron microscopy maps of (A) the minor group HRV2 and (B) the complex of HRV2 and a soluble fragment of the VLDL receptor where a "crown" of receptor molecules is seen on each five-fold axis. The icosahedral axes of one asymmetric unit are indicated in (A). Similar views show the major group HRV14 (C) and HRV16 (D) complexed with a soluble fragment of ICAM-1. All reconstructions are viewed down a two-fold axis. (Figures 1A and B are adapted from Hewat, E. A. *et al.*, *EMBO J.*, **19**, 6317–6325, 2000; Figs. 1C and D are reproduced from Kolatkar, P. R. *et al.*, *EMBO J.* **18**, 6249–6259, 1999. With permission.)

mutating to give a viable virus that escapes immune surveillance [12]. This is an interesting example of a highly plausible hypothesis that is not quite correct.

Binding of ICAM-1 to major group HRVs, such asHRV14, initiates rapid uncoating at physiologic temperature without the need of any cellular machinery [13]. In contrast, binding of LDL receptors to minor group HRVs, such as HRV2, does not directly catalyze decapsidation [5], and the subsequent internalization into acidic endosomal compartments is required for the transfer of the viral RNA into the cytosol [14] (Fig. 3).

The difference in the stability of the virus–receptor complexes and in the receptor binding sites of the major and minor group HRVs correlate with differences in their uncoating mechanisms. Rossmann and colleagues [9] have

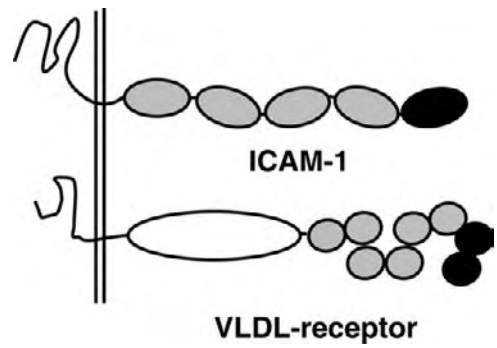


Figure 2 Schematic representation of the two proteins known to act as rhinovirus receptors. ICAM-1 contains five immunoglobulin-like domains and attaches to the major group HRVs by the N-terminal domain depicted in black. The ligand-binding amino terminus of the VLDL receptor (one of the LDL receptor family) contains eight imperfect repeats; two of these repeats bind to the minor group HRV2.

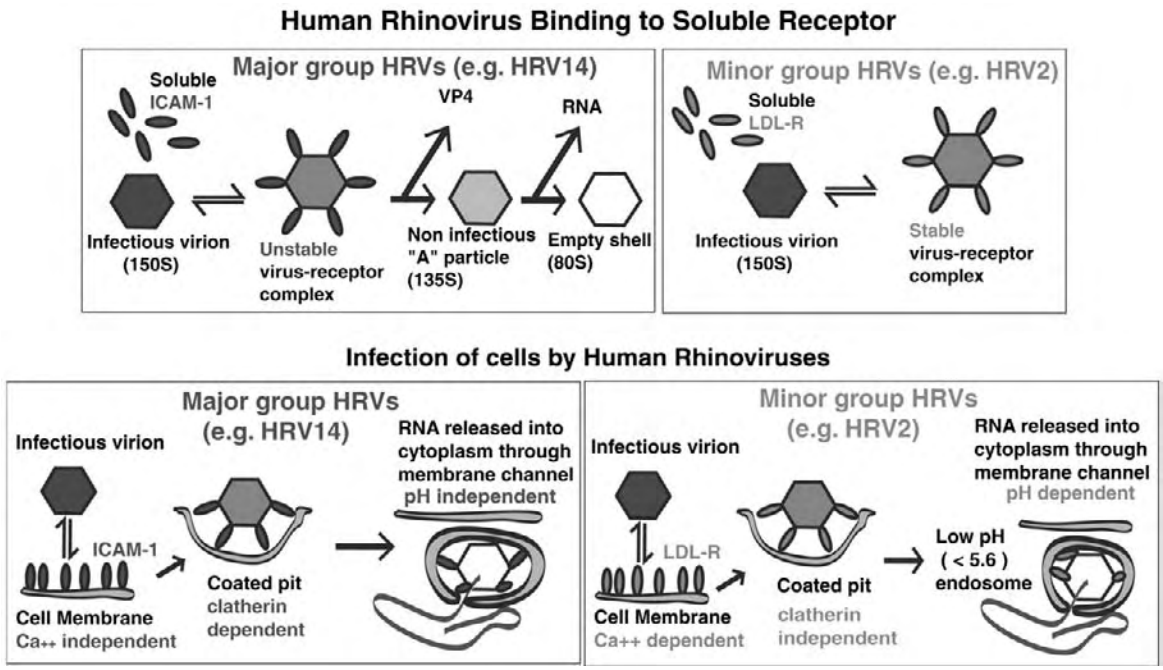


Figure 3 Schemas for attachment of soluble receptors to major and minor group HRVs and for the infection of cells by HRVs.

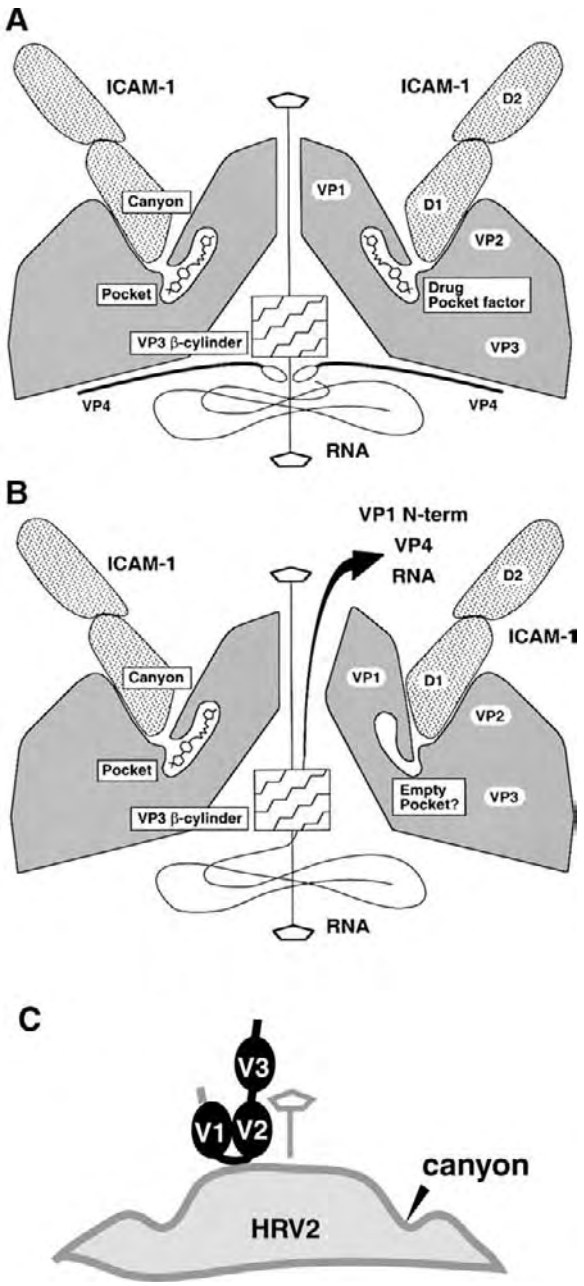


Figure 4 Schematic representation of the two step binding mechanism between ICAM-1 and the major group HRVs proposed by Kolatkar *et al.* [9]. See the text for a description of the first step shown in (A) and the second step in (B). Only two of the five ICAM-1 domains are shown. Part (C) shows how the VLDL receptor attaches to the minor group HRV2. Only three of the VLDL-R domains are shown. (Figures 4A and B are reproduced from Kolatkar, P. R. *et al.*, *EMBO J.*, **18**, 6249–6259, 1999; Fig. 4C is adapted from Hewat, E. A. *et al.*, *EMBO J.*, **19**, 6317–6325, 2000. With permission.)

proposed that ICAM-1 binds to the major group HRVs in a two-step process, as shown in Fig. 4. In the first step, ICAM-1 binds essentially to the base and one side of the canyon in the conformation as observed in cryo-electron microscopy reconstructions. The second step would then consist of expulsion of the natural pocket factor, as the ICAM-1 molecule binds to the other side of the canyon. This would induce the VP1 to flex at the canyon, moving away from the five-fold axis and thus opening the pentameric vertex. Because the binding site of the HRV2 receptor lies entirely on the dome on the five-fold axis and does not overlap the canyon or the pocket in the canyon at all, the mechanism must be quite different. The binding of VLDL-R to HRV2 as seen by cryo-electron microscopy is probably also the first step in a two step process [10]. The first step of receptor binding simply ensures that the HRVs are anchored to the membrane. The second step (i.e., expulsion of the pocket factor and flexing of VP1 to open a passage for the exit of the molecule of RNA) is then triggered by the low pH (5.6) in the endosome (Fig. 4). It is generally believed that the RNA exits along one of the five-fold axes. As the capsid opens, the VP4 and the N-terminus of VP1 are externalized. It has been hypothesized that both VP4 and the N-terminus of VP1 are inserted into the membrane in order to facilitate passage of the RNA across the membrane [1].

Antiviral compounds, such as the "WIN compounds" produced by the former Sterling Winthrop Research Institute, bind in the VP1 pocket. In many major group viruses, this induces a deformation of the canyon which causes a loss of receptor binding. It also stabilizes the capsid; however, in minor group viruses these antivirals do not affect receptor binding [15], and their antiviral effect is based on their stabilizing effect only. This behavior is in accord with the fact that the binding site of LDL-R on minor group viruses does not overlap the pocket at the base of the canyon.

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CHAPTER 17

HIV-1 Receptor Interactions

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Viral recognition of host receptors forms a special subset of molecular recognition. Unusual properties arise from the exceptional constraints that viruses encounter during infection. Although each virus is unique, examination of the human immunodeficiency virus type 1 (HIV-1) illustrates some of these special viral features.

Molecular Interactions

Human immunodeficiency virus type 1 is an enveloped retrovirus that infects $CD4^+$ T lymphocytes [1,2]. T lymphocytes are mobile and, once infected, live only a few days [3]. Thus, HIV must not only find the proper cell, but it must do so repeatedly over the hundreds of cycles of lymphocyte turnover that typify its persistent infection. The combined function of finding host cells and of properly initiating the viral fusion machinery is accomplished by the HIV-1 gp120 exterior envelope glycoprotein (reviewed by Wyatt and Sodroski [4]). The gp120 glycoprotein is initially synthesized as part of a trimeric gp160 glycoprotein, which is cleaved by cellular proteases into gp120 (N-terminal portion, roughly 500 amino acids, highly glycosylated) and gp41 (C-terminal portion, roughly 350 amino acids, transmembrane spanning) components. Noncovalent interactions keep this trimer of heterodimers associated as the biologically active viral spike.

The gp120 glycoprotein binds to the N-terminal membrane distal domain of the cellular CD4 receptor [5–8]. This interaction triggers conformational changes in gp120 that induce the formation of a binding site for the coreceptor, a member of the chemokine receptor family, either CCR5 or CXCR4 [9,10]. Binding by coreceptor initiates additional conformational changes that trigger the gp41 fusion machinery, leading to a fusion of the viral and cellular membranes and entry of the HIV-1 genome into the host cytoplasm (Fig. 1).

Although the small size of the virion (approximately 1000 Å in diameter) enhances diffusion, HIV virions are cleared rapidly from serum, and HIV gp120 employs several means to enhance receptor encounters. First, nonspecific electrostatic interactions generate binding to cell-surface polyanions such as heparin sulfate [11,12]. This electrostatic adhesion allows two-dimensional cell-surface scanning, enhancing the probability of gp120/cell-surface CD4 encounters. Second, it abducts innate immune responses on dendritic cells to promote infection in *trans* [13]. The gp120 glycoprotein displays high mannose N-linked glycans that bind to DC-SIGN and other dendritic cell receptors [14]. These receptors are used in innate immunity to scavenge for microbial invaders and to activate immune recognition, but binding to HIV gp120 results in the efficient presentation of the virus to suitable target cells (reviewed by Pohlmann *et al.* [15]).

These molecular interactions highlight several unique features of viral interactions. First, the ingenious manner by which the virus usurps host systems, with a redundancy of mechanisms to ensure viral propagation. Second, virions are not metabolically active, which has several diverse implications: highly specific recognition must occur without metabolic activation or proofreading; viral motion is propelled solely by Brownian forces; and large thermodynamic barriers (such as membrane fusion) must be overcome by using only energy stored in folded proteins. Third, HIV viral proteins function under severe constraints on genome size. The entire HIV genome is only 10 kilobase-pairs. These genome constraints are reflected at the DNA level by overlapping reading frames. On the protein level, they lead to a condensed multifunctionality. While eukaryotic recognition often involves a number of different proteins, each performing a specific task, the entire HIV recognition and entry procedure is accomplished with only two proteins. Multiple functionalities are encoded by different subunits as well as by different conformational states of the same polypeptide (Fig. 1).

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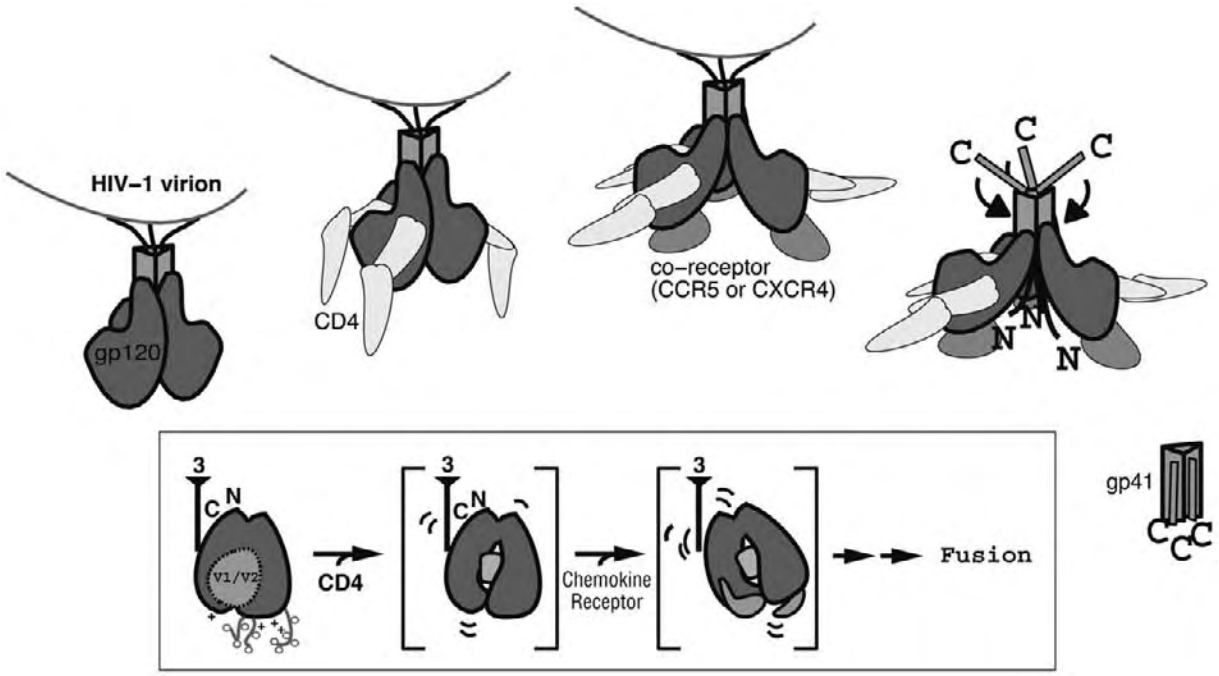


Figure 1 Molecular interactions and conformational states of the HIV-1 envelope glycoproteins. The top panel of figures illustrates the molecular interactions of the HIV-1 envelope glycoproteins. In the leftmost figure, a schematic of the biologically active viral spike is depicted, with gp120 molecules attached to the gp41 ectodomain. The subsequent figures diagram binding of CD4, followed by co-receptor (gray ovals), which initiates the gp41 fusion machinery: the N-terminal fusion peptide of gp41 is thrown into the target cell, and dramatic refolding of gp41 results in a final coiled-coil structure, with gp41 N- and C-ectodomain termini proximal. (For clarity, only the gp41 ectodomain is depicted. Thus the gp41 "C" corresponds to the membrane proximal portion of the gp41 ectodomain.) The boxed panel of figures illustrates these changes in the context of a single gp120 protomer. The leftmost figure shows the quiescent gp120. Basic surfaces (++) and high mannose N-linked glycan (o o) enhance cell-surface attachment and presentation to $CD4^+$ lymphocytes. In this quiescent state, the CD4 binding site is occluded by the V1/V2 variable loop, and the co-receptor binding site is not formed. Upon binding to CD4 (second figure), the inner and outer domains reorganize, forming both the Phe-43 cavity (at the center of gp120) and the bridging sheet and partially destabilizing quaternary interactions. Chemokine receptor binding (third figure) to the newly formed bridging sheet and V3 loop (light gray) trigger the gp41 fusion machinery. (Boxed panel adapted from Kwong, P. D. *et al*., *Nature*, **393**, 648–659, 1998, Fig. 5.)

Atomic Details

The X-ray crystal structure of core gp120 in complex with CD4 and a neutralizing antibody permitted one of these conformations to be examined at the atomic level (Fig. 2) [16,17]. The core gp120 construct used for crystallization contained deletions at the gp41-interactive region (at the gp120 N/C termini) as well as tripeptide substitutions for two loop regions. The crystal structure showed that core gp120 has two domains: an "inner" domain containing the N and C termini and a heavily glycosylated "outer" domain containing approximately 15 sites of N-linked glycosylation. Extensions emanating from β-hairpins of these two domains combine to form a four-stranded "bridging sheet" minidomain. This minidomain rests on hydrophobic residues contributed by the outer surfaces of the underlying inner and outer domains; thus, the integrity of the bridging sheet is intimately dependent on the precise alignment of the underlying domains.

The CD4 receptor binds at the nexus of the inner domain, outer domain, and bridging sheet. A total of ~1600 $Å^2$ of surface is buried in the interaction (~800 $Å^2$ from both CD4 and gp120), which is in the range typical for protein–protein interactions with nanomolar affinity. The interface itself is unusual. Two large interfacial cavities are present, and the gp120 component is contributed by mostly back-bone interactions from six separate sequence stretches. Thermodynamic studies indicate that gp120 undergoes significant conformational change upon binding to CD4. The gp120 glycoprotein appears to fold around CD4, with a coordinated alignment of the inner and outer domains and a reorganization of the bridging sheet [18].

The neutralizing antibody, 17b, captured in the ternary crystal complex binds to the gp120 bridging sheet, to a surface proximal but distinct from that bound by CD4. Sequence analysis shows that this relatively flat surface is highly conserved between different HIV-1 strains, although it appears to be conformationally masked prior to CD4 interaction.

The site of coreceptor binding overlaps with the 17b epitope. Mutational analysis shows that the coreceptor binding surface includes the bridging sheet and part of a variable surface loop, called the *V3 loop* [19]. Thus, the ternary structure provides a snapshot of the constant regions bound by both CD4 and coreceptor.

The bridging sheet is roughly 50 Å distal from the gp120 N and C termini, which interact with gp41. The manner in which

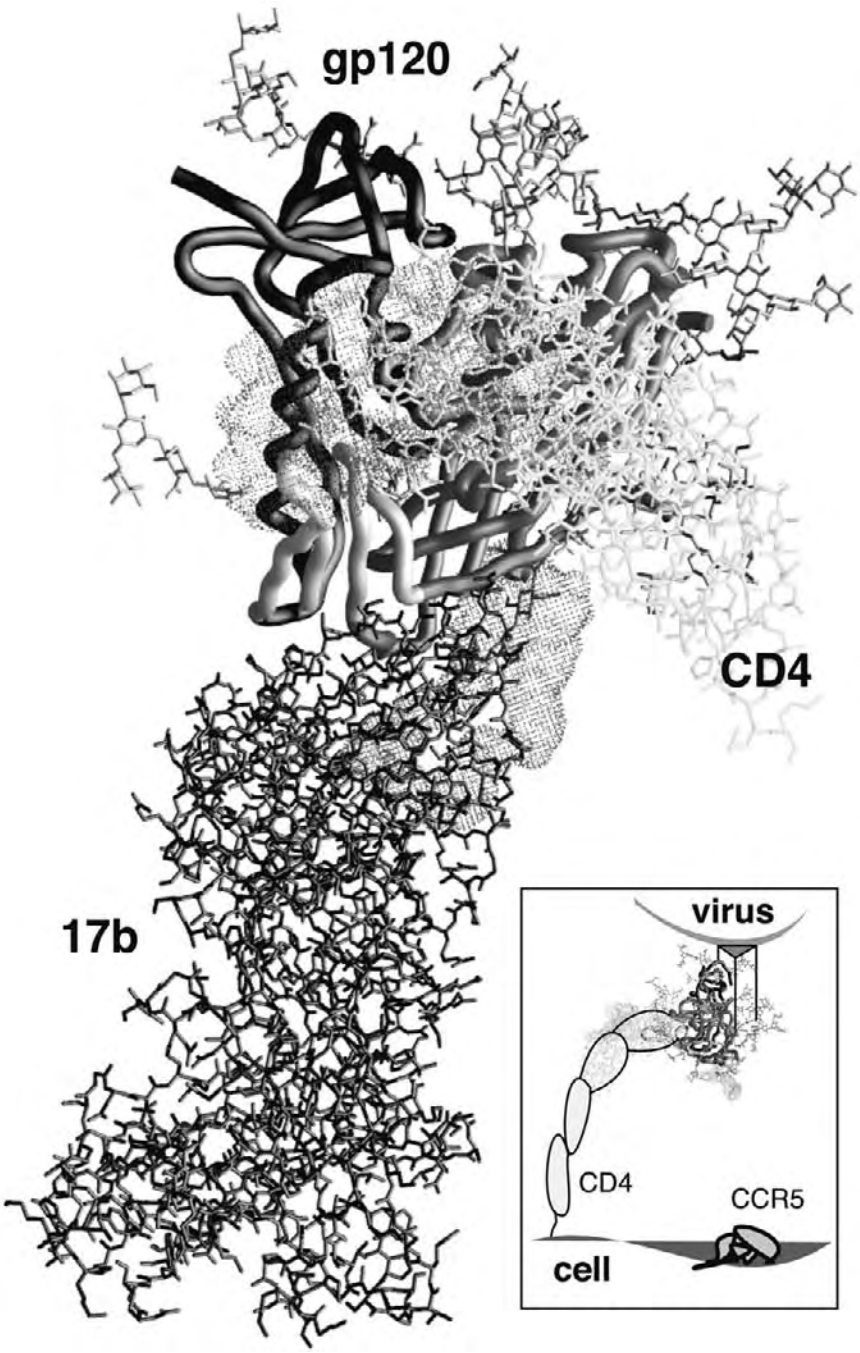


Figure 2 Atomic structure of the ternary complex of core gp120, CD4, and 17b neutralizing antibody. The N-terminal two domains of CD4 are shown in light gray, and the antigen-binding fragment of 17b in dark gray. For the gp120 core of the HXBc2 isolate, a carbon-alpha (Cα) worm representation is shown with inner domain in black, bridging sheet in light gray and outer domain in gray. The protein proximal pentasaccharide for each N-linked glycan is shown in gray all atom representation. The approximate positions of the V1/V2 and V3 variable loops are shown as semi-transparent surfaces. (To aid in orienting the viewer, a small boxed inset is shown which depicts gp120 and CD4 in the context of virus and cell surface, respectively. The orientation of gp120 and CD4 in this insert is related to the larger ribbon/atomic depiction by a 90° rotation about a vertical axis.)

a signal from coreceptor binding at the bridging sheet/V3 loop is transmitted to gp41 to trigger the fusion machinery is unclear. What is clear is that a number of intermediate conformational steps occur, differentiated antigenically and by accessibility of various neutralizing ligands. While these intermediate structures are currently under investigation, the final fusion-activated, coiled-coil structure of gp41 has been determined at the atomic level by a number of groups (Fig. 1) [20,21].

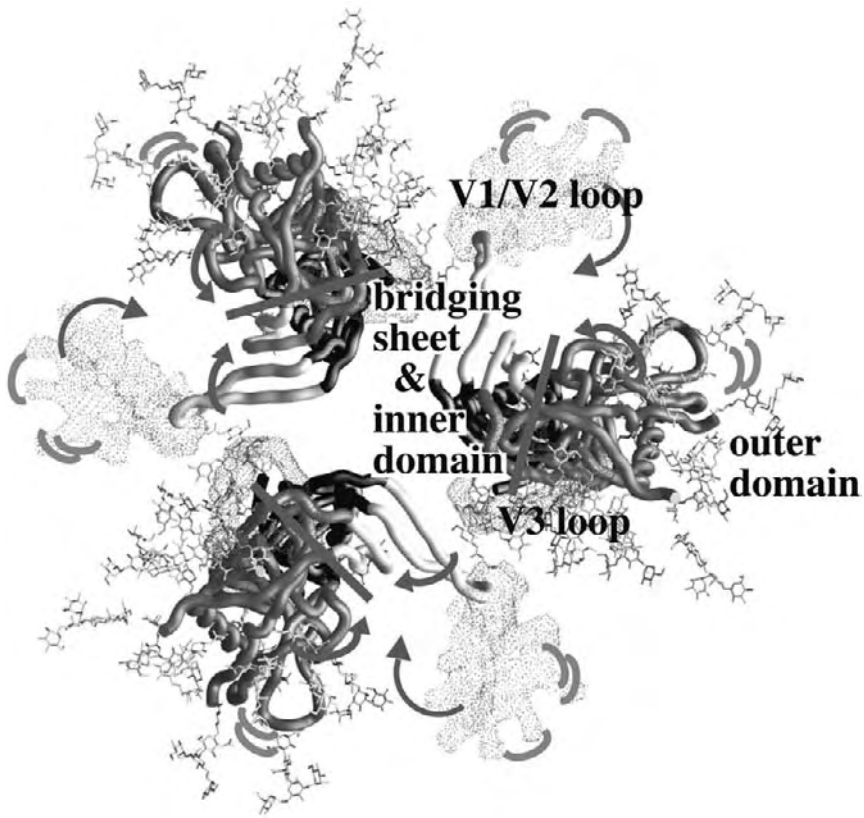


Figure 3 Mechanisms of humoral immune evasion. The trimeric structure of gp120 is depicted in the orientation obtained by optimization of quantifiable surface parameters [26]. The orientation of the right most protomer is related to the orientation of Fig. 2 by a ~90° rotation about a horizontal axis. This orientation depicts the trimer from the viewpoint of the target cell membrane. The shading scheme for the core gp120 is the same as in Fig. 2 (black Cα worm, inner domain; light gray Cα worm, bridging sheet; gray Cα worm, outer domain; all atom representation, carbohydrate; and semitransparent surfaces, variable loops). Oligomeric shielding of the inner domain by neighboring protomers is apparent, as is the extensive carbohydrate masking of the outer domain surface. The potential shielding of the CD4 binding site by the V1/V2 variable loop is shown with an arrow. The bridging sheet is not formed until CD4 binds; potential conformational alterations in outer domain and V1/V2 loop are highlighted.

Recognition in the Context of a Humoral Immune Response

An understanding of the parameters governing the HIV-1 receptor interactions would be incomplete without an understanding of the context in which this recognition occurs. While all recognitions pit specific versus non-specific interactions, HIV-1 receptor recognition occurs in the context of a persistent infection. In order to bind to receptor while simultaneously eluding neutralization by the humoral immune system, gp120 has evolved sophisticated strategies of evasion (Fig. 3) [17,22,27].

Three primary mechanisms protect the envelope protein surface not involved in receptor recognition: sequence variation, oligomerization, and carbohydrate masking. The small size of the HIV genome, coupled to high rates of replication error and recombination, facilitates rapid antigenic escape. Oligomerization uses protein–protein interfaces to sterically block access to conserved epitopes. This protects conserved epitopes that are involved in the gp120–gp120 interface as well as the gp120–gp41 interface. Antibodies directed against these epitopes are usually non-neutralizing and recognize only separate gp120 or gp41 components, not the oligomeric gp120/gp41 viral spike.

Carbohydrate masking involves covering exposed protein surfaces with a dense array of N-linked glycans. Because these glycans are derived from host biochemical pathways, they are interpreted as "self" by the immune system and do not elicit antibodies. In addition, the carbohydrate sterically inhibits access to the underlying protein surfaces. Epitopes protected by carbohydrate masking are thus immunologically "silent."

In terms of the potentially vulnerable receptor binding surfaces, the virus must recognize receptor, while at the same time eluding an ever-adapting immune response. The surfaces on gp120 that interact with cellular receptors are not only larger than the typical antibody footprint (600 $Å^2$), but they also must be functionally conserved and exposed.

HIV-1 receptor surfaces are partially protected by variable loops. These loops have little structural restraint, and sequence variation can occur at a rate roughly 1,000,000 times faster than the human genome [23]. The CD4 binding

site is protected by the V1/V2 variable loop. This loop emanates from the bridging sheet, is approximately 70 amino acids in length, and contains several sites of N-linked glycosylation. Both by steric occlusion and by antigenic variation, the loop shields the CD4 binding site from antibody recognition.

Another highly variable structure, the V3 loop, resides on the other side of the bridging sheet. This loop contains a conserved element at its tip that is required for chemokine receptor binding. The placement of a conserved functionally crucial element amidst a highly variable region allows protection to be conferred by the surrounding antigenic variation. A variation of this anti-"hot spot" mechanism of immune evasion is seen in the CD4 binding site itself. Analysis of a number of tight protein–protein interfaces shows that most have good complementary of fit, although only a small portion of the binding surface generates most of the binding energy (at an interaction hot spot) [24]. With CD4, the gp120 hot spot of interaction involves residues Phe-43 and Arg-59. The rest of the surface, however, does not show a nice complementarity of fit. A substantial portion of the interactive surface is buffered by a water-filled cavity. Residues on gp120 that contribute to this outer cavity are relatively variable in sequence. Such variation permits gp120 to escape from antibodies directed at the CD4 receptor binding surface. A similar cavity-filled interface is seen in the adenovirus interaction with its receptor, CAR, which like CD4 is a member of the immunoglobulin superfamily [25].

The most conserved exposed surface on gp120 is the bridging sheet, which mutational data show to be part of the chemokine receptor binding surface [19]. HIV hides this surface though another innovative means—conformational change [27]. Thus, this surface is not formed until cellular CD4 induces the appropriate conformational reorganization in gp120. Such conformational masking serves not only to reduce the elicitation of antibodies against the chemokine receptor binding site but also to prevent neutralization. Within the oligomeric viral spike, quaternary interactions oppose the conformational changes induced by CD4. Such opposition decreases the efficiency of both CD4 binding as well as of antibodies against the receptor binding region that require conformational change in order to bind. The degree of opposition is controlled by variable loop elements involved in quaternary contact [16]. Extensive variation within these loops allows this opposition to be modulated. With primary isolates, humoral pressures select the degree of opposition to permit only highly avid binding. Because such avidity is available for cell-surface receptors, but not for most antibodies, conformational masking allows HIV-1 to resist neutralization while simultaneously permitting receptor binding.

Analysis of the HIV-1 receptor interactions illustrates some of the unique features associated with viral receptor recognition. Not only is there the problem of specific binding to receptors, but there is also the complementary problem of avoiding specific recognition by the immune system. Compressed into the 500 amino acids of the HIV-1 gp120 are complex mechanisms of evasion and recognition. HIV-1 receptor recognition thus provides an example of a system driven to an extraordinary level of sophistication by the incredible evolutionary speed of HIV-1 opposed by the equally remarkable adaptive capabilities of the immune system.

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CHAPTER 18

Influenza Virus Neuraminidase Inhibitors

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Introduction

Two inhibitors of the influenza virus neuraminidase (NA) are currently licensed as drugs for the treatment of influenza: Relenza® (GlaxoSmithKline) and Tamiflu® (Roche). Several other companies are developing similar inhibitors. All were developed with the aid of structure-based drug design (SBDD), following elucidation of the X-ray structure of the influenza virus NA in 1983. Here, we will review the development of these compounds which represents one of the successes of SDBB.

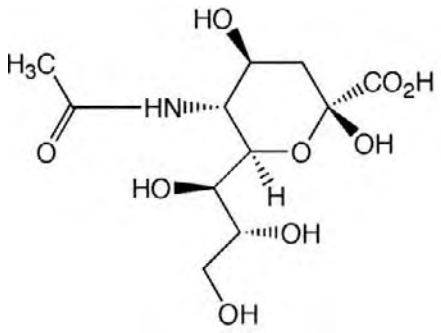
Flu Virus: Role of NA

Influenza remains a major cause of mortality and morbidity worldwide. Vaccination affords some protection but must be reformulated each year based on a prediction of the most likely strains circulating in the coming flu season. The antigenic drift and shift characteristic of the virus limits the effectiveness of the vaccine, and some warn of a re-emergence of a catastrophic pandemic strain such as occurred in 1918—the so called Spanish flu [1]. Two antiviral drugs (amantadine and rimantadine) have existed for some time that target the viral ion-channel protein M2 [2], but these are ineffective against the type B influenza virus and cause unwanted side effects. Of the several influenza virus proteins, the surface glycoprotein neuraminidase (NA) has emerged as the most successful target for antiviral development, although other work has been carried out on the hemagglutinin (HA) [3,4] and endonuclease [5].

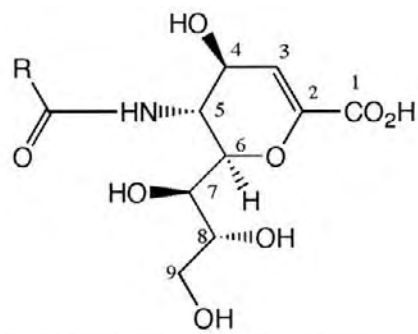
The influenza virus NA exists as a mushroom-shaped tetramer on the surface of the virus; a typical virus carries around 100 copies of NA and 400 copies of the other surface glycoprotein HA. HA contains domains that recognize sialic acid receptors (Neu5Ac, NANA) (Fig. 1, structure 1), the very sugar that NA hydrolyzes. NA catalyzes the cleavage of the α-ketosidic linkage between sialic acid and the adjacent sugar residue, which lowers membrane viscosity and permits entry of the virus into epithelial cells. NA also destroys the HA receptor on host cells, allowing the emergence of progeny virions from infected cells and presumably also removing sialic acid from the HA and NA of such virions to permit cell-to-cell spread of the virus [6,7]. Inhibitors of NA can therefore reduce this spread of the virus from the site of infection.

The first inhibitors were made in the 1960s through an attempt to understand the catalytic mechanism, which resulted in analogs of 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid (Neu5Ac2en, DANA) (Fig. 1, structure 2a) [8,9]. These compounds inhibited influenza virus NA with a $K_i \approx 4\ \mu M$, as they do most neuraminidases found in nature. Neuraminidases, or sialidases, are found in many pathogenic and nonpathogenic bacteria, where they are largely secreted and provide primarily a nutritional role, although in the case of *Vibrio cholerae*, for example, the enzyme plays a defined role in pathogenesis [10–12]. Animals possess neuraminidases (three are encoded in the human genome) identified by characteristic sequence fingerprints (the so-called bacterial neuraminidase repeats, or BNRs) not found in the viral enzyme [12]. Certain parasites possess the enzyme GPI-linked to their surface, and in the case of *Trypanosoma cruzi* the enzyme

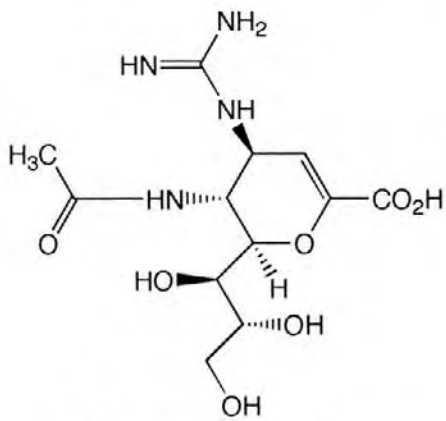
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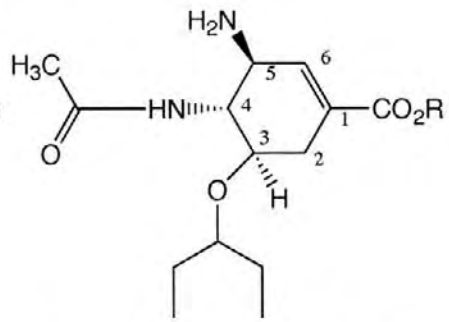
1 Sialic acid, NANA, Neu5Ac



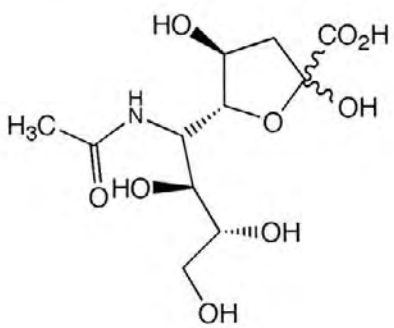
2a DANA, Neu5Ac2en (R=CH_3). **2b** FANA (R=CF_3)



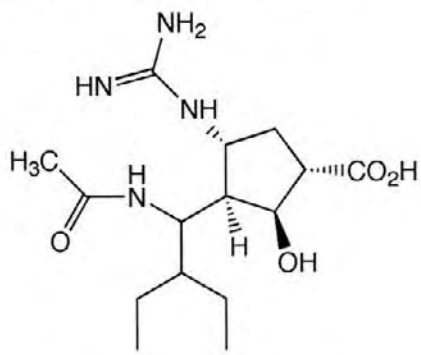
3 Relenza (GG167, Zanamivir)



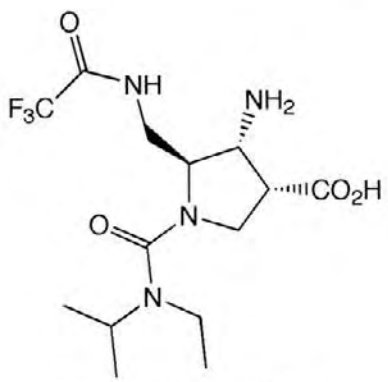
4a Tamiflu, Oseltamivir, GS4071 (R=CH_2CH_3).
4b Oseltamivir carboxylate, GS4104 (R=H).



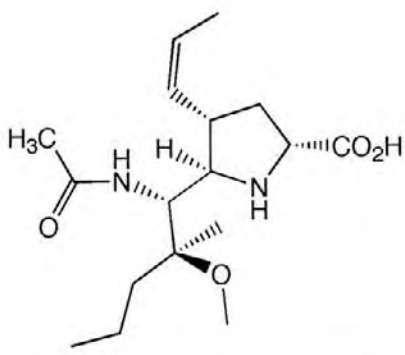
5



6 Biocryst compound (BCX-1812, RWJ-270201)



7 Abbott compound (A-192558)



8 Abbott compound (A-315675)

Figure 1 Influenza virus NA ligands.

serves as a more efficient *trans*-sialidase rather than a sialidase [13,14]. Finally, paramyxoviruses possess surface glycoproteins (HNs), which embody the functions of both hemagglutinin and neuraminidase and appear to have a combined, single sialic acid recognition site [15–17]. Any influenza virus inhibitors must therefore avoid inhibiting the endogenous human enzymes which play key roles in modulating cell-surface sialic acid in events from immune response to apoptosis [17,18].

Structure of NA

The tetrameric NA of the influenza virus contains a head of four roughly spherical catalytic domains and a stalk that is anchored in the viral membrane via a hydrophobic N-terminal tail. The crystal structure of the protease-released head region revealed a six-bladed β-propeller structure, and a complex with Neu5Ac2en identified the active site sitting roughly at the center of the propeller (Fig. 2a) [19,20]. There is extensive sequence variation among the various influenza virus NAs, for which nine immunologically distinct subtypes have so far been identified (N1 to N9) for the type A virus, with sequence identities as low as 40%. Even within a subtype, the variation is extensive, as illustrated in Fig. 2b. The residues within and surrounding the active site remain constant, however, and present an Achilles heel of the virus [21].

Active Site

The crystal structure of NA complexed with sialic acid, which is itself a weak inhibitor of influenza NA with a $K_i \approx 1$ m*M*, revealed sialic acid in a strained conformation with its hexose ring in a half-chair rather than a chair conformation (Fig. 3) [22]. Neu5Ac2en represents a transition-state analog, and its interactions with the active site are shown in Fig. 4a. A trifluoroacetyl derivative of Neu5Ac2en, FANA (Fig. 1, structure 2b), was the best inhibitor of the influenza NA for some years, with a $K_i = 0.8$ μ*M* [9]. Comparison of the several influenza NA structures now available reveal a relatively rigid active site, and so the challenge in inhibitor design has been to exploit the largely immobile features of this site [19,22–24]. The most effective inhibitors that have been developed to date include Relenza® (SKB) [25], Tamiflu® (Roche), BCX-1812 (Biocryst Pharmaceuticals) [26], and A-315675 (Abbott) [27]. Compound A-192558 from Abbott has been less successful [28], as have derivatives of benzoic acid [29]. Table 1 lists the K_i and IC_{50} of several ligands.

The sialic-acid-binding active site is a deep pocket, mainly acidic in nature, but with a basic side to it (Fig. 3). The four characteristic features of the site are:

1. A basic pocket formed by an arginine triad (Arg118, Arg292, Arg371) that interacts with the carboxylic acid of the ligand; this feature is a key determinant of the binding, and all inhibitors developed to date preserve the carboxyl moiety.
2. An acidic pocket formed by glutamates (Glu276, Glu277); Glu277 forms a strong H-bond with Tyr406, and together these residues are thought to stabilize an oxocarbonium ion intermediate in the reaction [30,31]. Glu276 interacts with O8 and O9 of the glycerol moiety of sialic acid and Neu5Ac2en, and Relenza preserves this interaction. Other inhibitors have placed a hydrophobic moiety at this position to improve the lipophilicity of the compounds, which

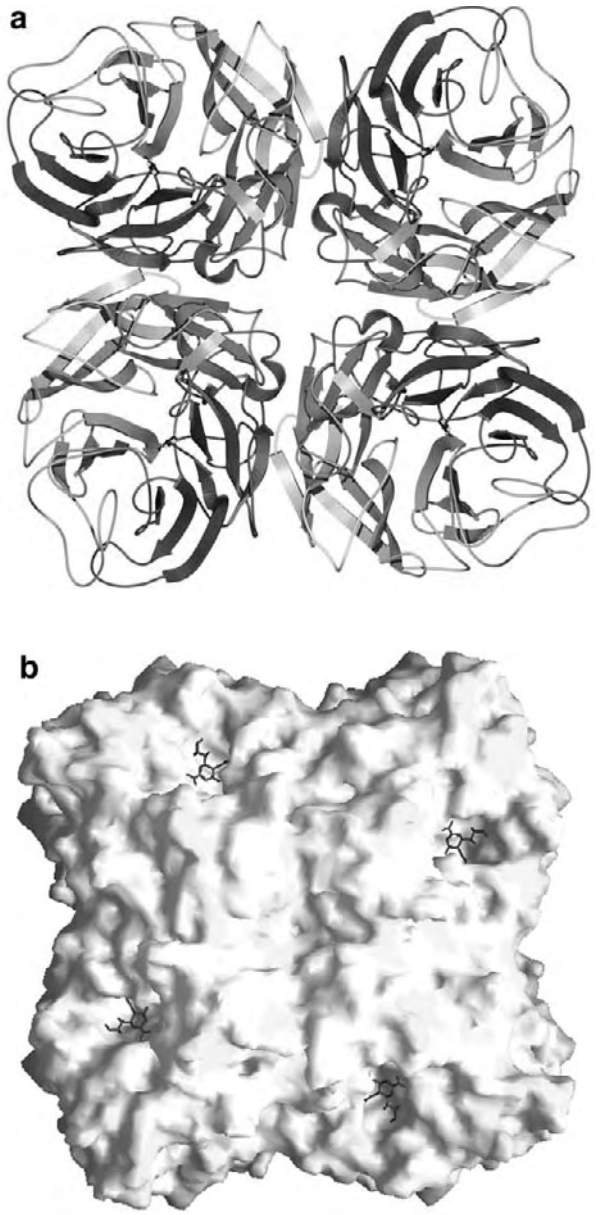


Figure 2 The influenza virus NA. (Top) Schematic drawing of the influenza virus NA tetramer, as if looking down onto the virus surface. Coloring is from blue at the N terminus to red at the C terminus. (Bottom) Surface representation of the same tetramer, showing the location of Neu5Ac2en bound in the active site. The yellow coloring shows amino acids that vary within the N8 subtype, revealing the antigenic drift that the virus undergoes.

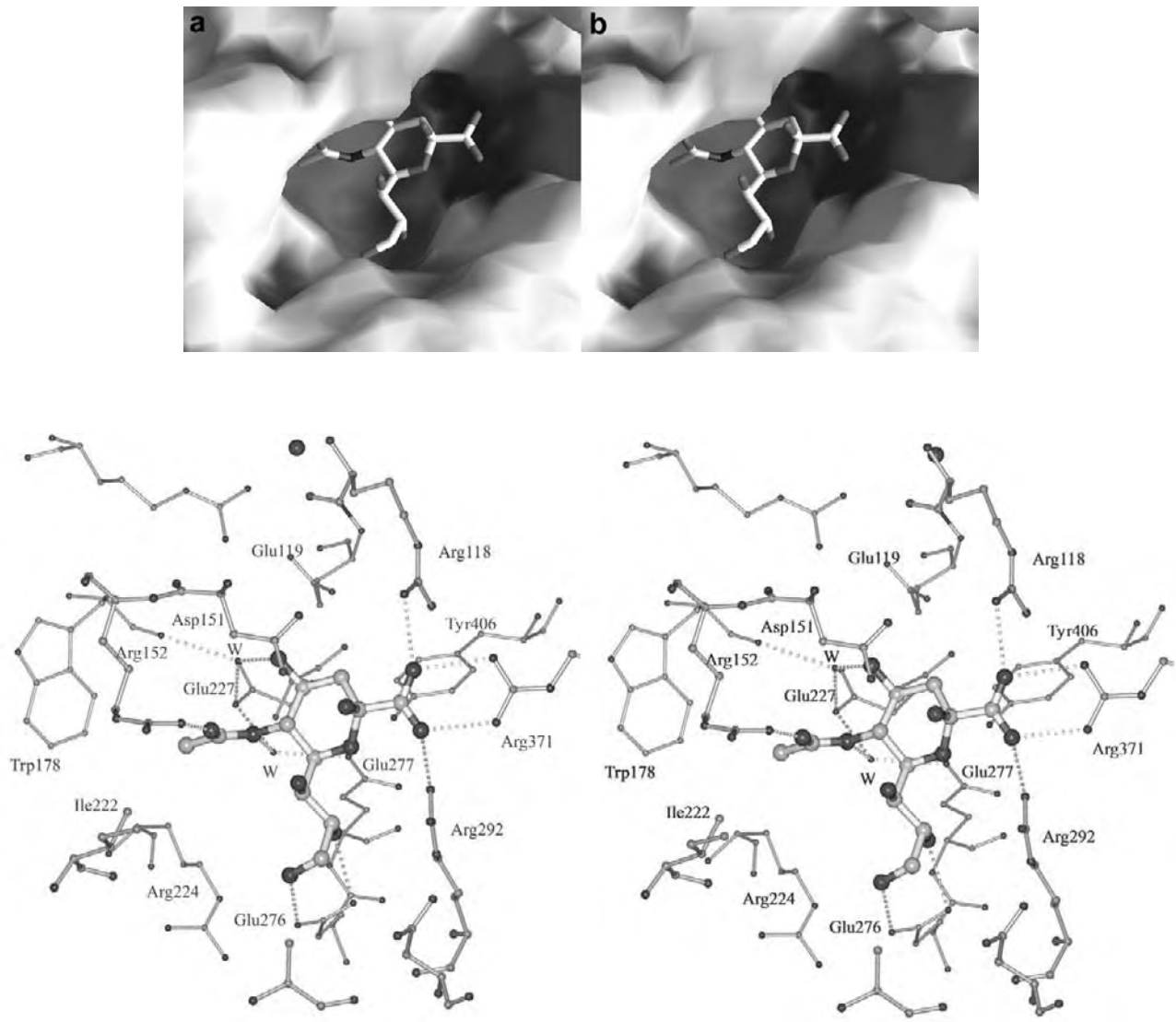


Figure 3 Stereo views of the active site with sialic acid bound (tern N9 influenza neuraminidase, PDB code 1MWE). (Top) Colored by electrostatic potential: blue positive, red negative. (Bottom) Hydrogen bonding interactions shown as dotted green lines; W denotes a water molecule.

Table I Inhibition Parameters for Ligands of Influenza Virus Neuraminidase

	Influenza A Virus		Influenza B Virus		
	K_i	IC_{50}	K_i	IC_{50}	Refs.
Sialic acid	1 m*M*	—	1 m*M*	—	9
DANA	4 μ*M*	0.015 m*M*	4 μ*M*	0.015 m*M*	9
FANA	0.8 μ*M*	—	20 μ*M*	—	9
Relenza	0.06–1.3 n*M*	0.3–2.3 n*M*	0.09–0.27 n*M*	1.5–17 n*M*	46
Tamiflu	0.10–1.3 n*M*	0.01–2.2 n*M*	1.1–2.1 n*M*	5.0–10.4 n*M*	49
BCX-1812	0.014–1.1 n*M*	0.1–1.4 n*M*	0.21–0.96 n*M*	0.6–11 n*M*	46
A-192558	—	0.28 μM	—	8 μ*M*	27
A-315675	0.024–0.21 n*M*	0.4–5.9 n*M*	0.14–0.31 n*M*	6.7–14.1 n*M*	49

subsequent crystal complexes have shown are accommodated by a movement of Glu276 to extend a hydrophobic pocket.

3. A hydrophobic pocket formed by Trp178, Ile222, and the methylene elements of the side chains of Arg152 and Arg224, all residues that are conserved across influenza viruses. This pocket accommodates the methyl group of the acetamido moiety of sialic acid and Neu5Ac2en. The oxygen of the acetamido group hyrodgen bonds to the guanidinium group of Arg152, and the acetamido nitrogen hydrogen bonds to a buried water molecule, which in turn interacts with Glu276 and Glu227. Most inhibitors have preserved the acetamido group or a triflouroacetamido group, such as in FANA and the Abbott A-192558 compound.
4. An acidic pocket formed from Glu119, Glu227, and Asp151, the latter residue being most likely to be involved in hydrolysis via a water molecule [30]. The two glutamates are conserved, yet play no obvious role in substrate binding or hydrolysis. The O4 hydroxyl of sialic acid and Neu5Ac2en does not hydrogen bond to any of these residues as there is a large cavity around this position. Most successful inhibitors have an amino or guanadino group substituted at this position, except for the Abbott A-315675 compound.

Inhibitor Development

Relenza

Relenza was developed through the use of the program GRID [32], which revealed a potentially strong binding site for an NH_3^+ group with a calculated binding energy of −16 kcal/mol in the vicinity of the position normally occupied by the O4 hydroxyl of sialic acid [33]. Using Neu5Ac2en as the scaffold, substitution of O4 with an amino group gained two orders of magnitude of binding over Neu5Ac2en, whereas substitution by a guanidino group (4-guanidino-Neu5Ac2en, Relenza) (Fig. 1, structure 3) gained five orders of magnitude of binding over Neu5Ac2en [33]. In complexes of Relenza with both influenza A [34] (Fig. 4b) and influenza B [35] virus NA (PDB codes 1NNC and 1A4G, respectively), the guanidino group interacts almost ideally with Asp151 and Glu227. Glu119 is also close enough to make a charge-charge interaction, although one study suggests that Glu119 may be neutral in the case of 4-amino-Neu5Ac2en binding [36]. Relenza-resistant mutants have been isolated *in vitro*, with mutations mainly in Glu119 [37–39], and, in one case, one of the catalytic arginines, Arg292 [40]. A resistant mutant has also been isolated *in vivo*, with the mutation of Arg152 → Lys [41]. Relenza is a successful inhibitor of influenza A and B virus NA, but its highly polar nature (calculated log *P* of −7) has necessitated administration as a powder, requiring an inhaler with all the inherent problems of such use. Replacement of the glycerol group of Relenza by a series of hydrophobic dihydropyrancaboxamides have provided inhibitors with a binding affinity similar to Relenza for influenza A NA, but with only micromolar inhibition of influenza B NA [35].

Tamiflu

The starting point for the development of Tamiflu was replacement of the dihydropyran ring with a cycohexene, which is chemically more stable and retains the ability to

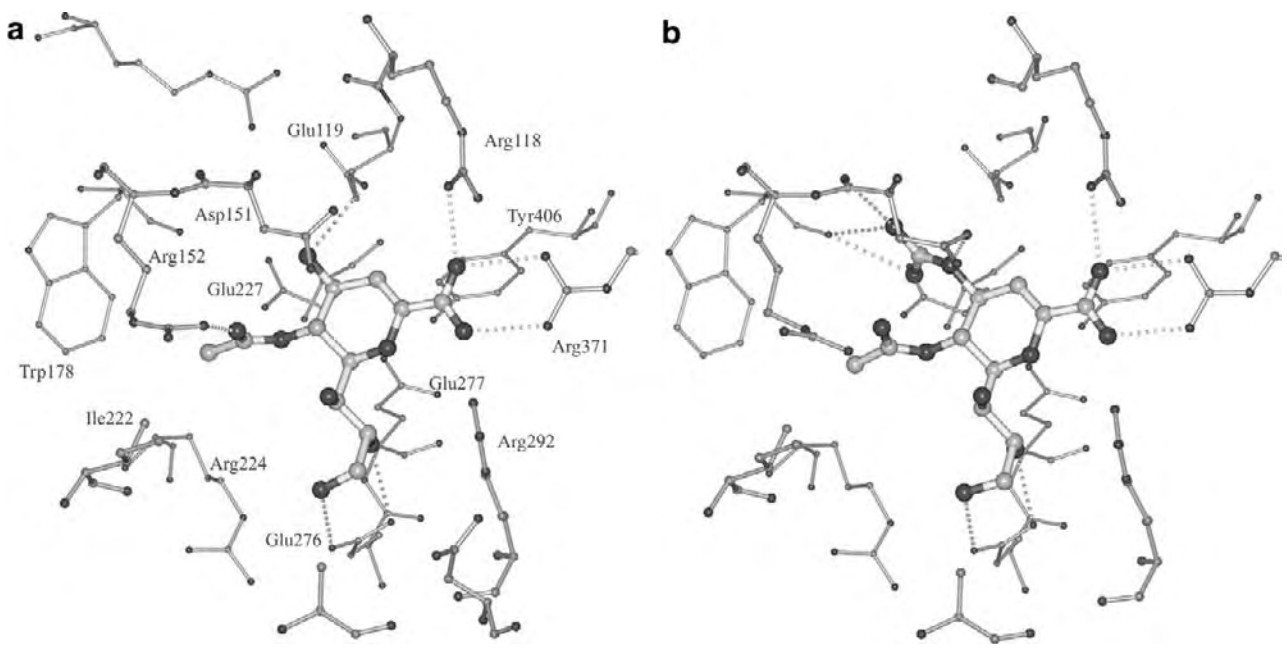


Figure 4 Interactions of the ligands with the active site of the tern N9 influenza virus NA: (a) Neu5Ac2en (PDB code 1F8B); (b) Relenza (PDB code 1NNC); (c) Tamiflu (PDB code 2QWK); and (d) BCX-1812. (e) Stereo view of a superposition of all four ligands reveals a rigid active site with only Glu276 altering position. Neu5Ac2en complex is yellow, Relenza complex is magenta, Tamiflu complex is cyan, and the BCX-1812 complex is green.

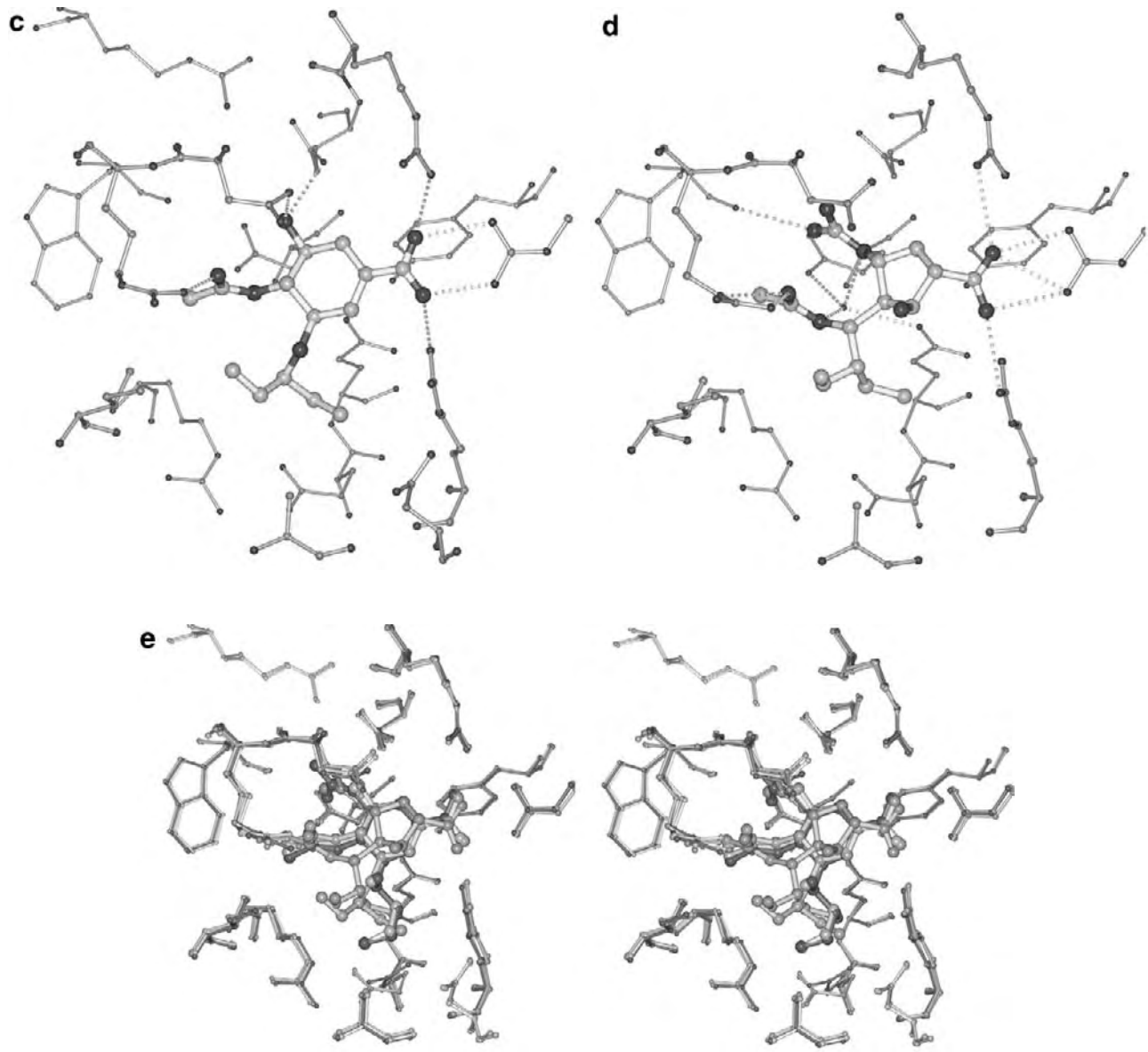


Figure 4 *(Continued)*

alter the stereochemistry of ring substituents [42]. The best inhibition was obtained with the double bond in the position equivalent to that in Neu5Ac2en, mimicking the carbonium cation intermediate. The carboxylate and acetamido groups were kept at C_1 and C_4, respectively, and an amino group at C_5 in light of the success of the Relenza development. In order to improve the lipophilicity, the glycerol group was substituted by a series of alkyl ethers. There is a remarkable correlation between the length, geometry, and rigidity of the alkyl chains and NA inhibitory activity, suggesting an incremental entropy gain. The crystal structure of the best inhibitor, with a 3-pentyl group (GS4071, later named Tamiflu carboxylate) (Fig. 1, structure 4a), showed that Glu276 is rotated away from the active site to extend the hydrophobic pocket (Fig. 4c). The prodrug of GS4071, an ethyl ester derivative (GS4104, Oseltamivir, Tamiflu) (Fig. 1, structure 2a; Fig. 4b) exhibits good oral efficacy [43].

Biocryst Compound (BCX-1812)

The starting point for the development of BCX-1812 was a furanose-based compound (Fig. 1, structure 5) that had the same ring substituents as sialic acid and Neu5Ac2en and inhibited influenza virus NA with a potency similar to Neu5Ac2en [44]. The structure of a complex of (Fig. 1, structure 5) with N9 influenza NA (Fig. 4d) showed that, although the furanose ring is significantly displaced compared to the pyranose ring of DANA, all of the ring substituents have very similar interactions with the enzyme. This reflects a feature of the active site, namely that there is little interaction with the ring itself. Consequently, a cyclopentane ring was chosen as the scaffold for chemical stability, with a carboxylic acid group placed at C_1. An interesting route in the development was the synthesis of racemic mixtures with a guanadino group at C_4 and an *n*-butyl at $C_{1'}$, followed by inspection of the high-resolution difference electron density maps to ascertain the

stereochemistry of the active isomer [26]. The *n*-butyl chain bound in two different modes in influenza A and B virus NA, reflecting the slightly different environments around the sialic acid glycerol binding pocket in the two enzymes. Again, Glu 276 moves, and in influenza A NA forms a salt bridge with Arg224 as had been observed in the binding of GS4071 [35]. In order to take advantage of both hydrophobic pockets, BCX-1812 (Fig. 1, structure 6) was developed, again as a racemic mixture, and the active isomer identified crystallographically. An interesting feature of BCX-1812 (Fig. 4d) is that the orientation of the guanidino group in the active site is different from that seen for Relenza (Fig. 4b). This may be why BCX-1812 remains effective against a Relenza-resistant mutant (Glu119 → Gly) [45], as is also true for Tamiflu, which has only an amino group at this position. BCX-1812 shows great promise as an oral treatment for influenza [46–48].

Abbott Compounds

Abbott published a series of inhibitors based on a pyrrolidine core, the best of which (A-192558) (Fig. 1, structure 7) had an IC50 of 0.28 μ*M* against influenza A NA [28,49]. One feature of the development of these inhibitors was the creation of focused combinatorial libraries by automated solid-phase synthesis, in one case containing 550 analogs [28]. Recently, a new compound with Ki of between 0.024 and 0.31 nM against a range of influenza virus NAs has been reported [27]. This compound, A-315675 (Fig. 1, structure 8), retains the carboxyl and acetamido groups, but does not have an amino or guanidino group. No details are available as to how this compound binds in the active site, but it is reported that only Glu276 moves in the active site upon binding, as in other complexes of other inhibitors with a hydrophobic moiety in place of the glycerol group [27].

Conclusion

The development of effective nanomolar-binding inhibitors of the influenza virus NA is one of the success stories of structure-based drug design. The active site is remarkably rigid (Fig. 4e), except for one conserved glutamic acid, Glu276, which is free to rotate 90° about χ_2. This creates an extensive hydrophobic pocket in a region normally occupied by the glycerol group of the natural substrate. The most successful inhibitors have exploited this pocket to provide molecules with greater lipophilicity and hence bioavailability so they can be given in tablet form. Analysis of successful influenza virus NA inhibitors reveals the following observations:

1. Interaction with all four sites is required.
2. A scaffold that allows stereoselectivity is essential.
3. The nature of the scaffold is less important, but carbocylic rings give greater chemical stability.
4. Replacement of the glycerol moiety with a hydrophobic group increases bioavailability.
5. Crystallography is a powerful tool for selecting active isomers for racemic mixtures, as was used in the development of BCX-1812.
6. Focused, diversity-oriented synthesis of side groups has been of some use, especially in conjunction with structural analysis.

An interesting spinoff has been a series of studies that have aimed at predicting binding affinities for inhibitors of the influenza virus NA [36,50,51]. Although these studies obtain good correlation between predicted and observed affinities, their predictive value in developing new inhibitors is unclear.

A disappointing postlude to the story is that, although we now have effective drugs for the control of influenza, their use so far has not been a great success in the clinic. A major problem is that, to be effective, the drugs must be taken within 48 hours of patients showing flu-like symptoms. Unlike Relenza, Tamiflu is also licensed as a prophylactic to stop the spread of the virus within families and close communities and it appears to be preferentially prescribed at this time.

Acknowledgments

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CHAPTER 19

Signal Transduction and Integral Membrane Proteins

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Introduction

Cells need to adapt their behavior continuously in response to a barrage of external stimuli. Integral membrane proteins are the best positioned to interact with outside stimuli and are, therefore, critical components of signal transduction. Ion channels, transporters, and receptors are integral membrane proteins that can mediate signaling across the cellular membrane. The availability of detailed structural information on these proteins has been limited by technical challenges unique to the high-resolution structure determination of membrane proteins by X-ray crystallography. In recent years, however, the X-ray structures of a few of these important proteins have been solved, providing insight into the molecular structural basis of signal transduction across the cell membrane.

Electrophysiology: Rapid Signal Transduction

Ion channels are the fundamental electrical signaling units of neurobiology. As molecular transducers, ion channels are highly sensitive (detectable gating thresholds as low as thermal noise), extremely efficient (ion transport rates up to 10^{-7} ions/sec), and very responsive (microsecond turn-on times). They are essential components of the cellular response to external stimuli and are directly responsible for the transmission of all electrical signaling events for multicellular organisms. For more than 50 years, biophysicists have used sophisticated patch clamping experiments and site-directed mutagenesis to understand the function of ion channels with exquisite detail. A major breakthrough in understanding the wealth of ion channel biochemical data began with the recent X-ray crystal structures of the K^+ ion channel KcsA from *Streptomyces lividans* [2,10] and a pair of CLC ion channel homologs from *Escherichia coli* (EcCLC) and *Salmonella typhimurium* (StCLC) [3]. These X-ray structures have provided the first clues for the molecular structural basis for the transport of ions involved in the transmission of electrical signals.

The KcsA structure reveals a pore region that is similar in protein sequence to all known K^+ ion channels. The KcsA is arranged as a tetramer of identical subunits creating a cone-shaped structure with the pore selectivity filter on the outer membrane leaflet side. A large water-filled cavity with helix dipoles located on the outer membrane side is uniquely positioned to overcome the electrostatic destabilization energy of a K^+ ion at the center of the bilayer. Selectivity is accomplished by main-chain carbonyl oxygen atoms of the K^+ ion channel signature sequence. The general architecture of KcsA establishes the structural basis underlying the selectivity of K^+ conduction. In contrast to KcsA, the CLC chloride channel overcomes the energetic barrier of ion transport by a different mechanism. The bacterial chloride ion channel is homodimeric in structure, with the selectivity filter formed within each monomer by two opposed membrane-spanning subunits. This anti-parallel arrangement defines a selectivity filter in which chloride ions are stabilized by electrostatic interactions with α-helix dipoles and coordination bonding.

Mechanosensation: How Do We Feel?

What is the molecular basis by which we sense touch? Mechanosensitive (MS) ion channels present an elegant

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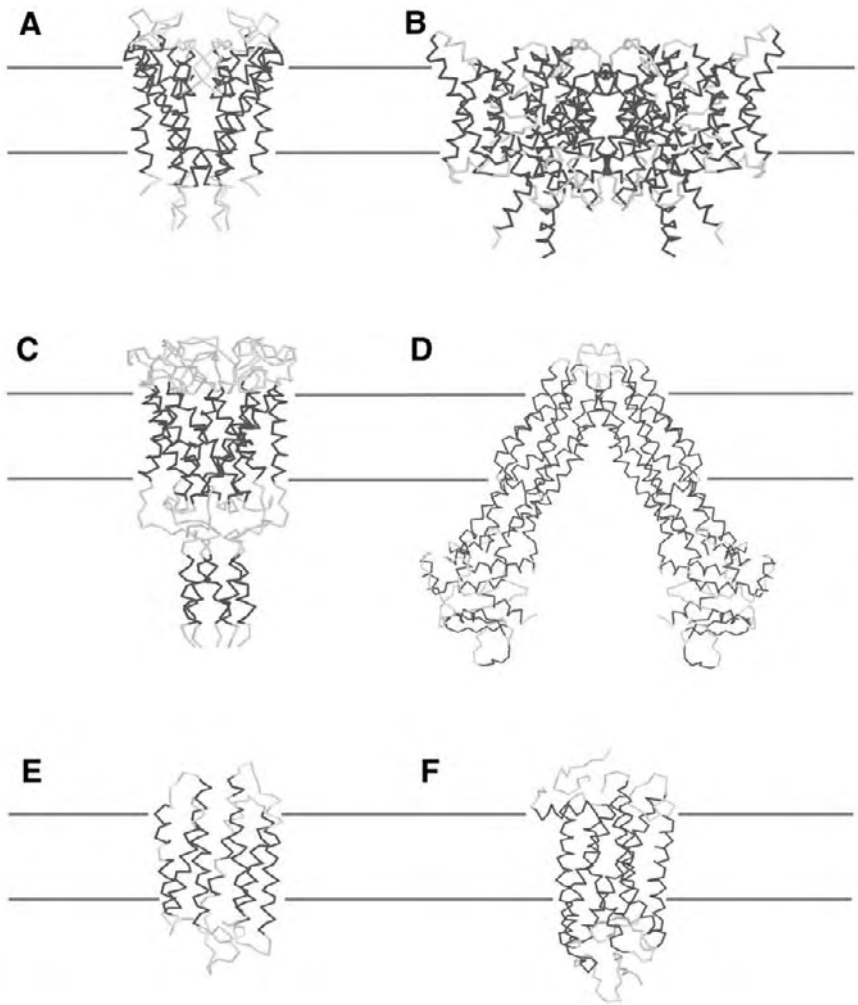


Figure 1 Structure of integral membrane proteins involved in signal transduction. (A) KcsA potassium ion channel structure [2]; (B) chloride CLC ion channel [3]; (C) mechanosensitive ion channel of large conductance (MscL [1]); (D) MsbA multidrug ABC transporter homolog [1], (E) bacteriorhodopsin [6]; and (F) bovine rhodopsin [5]. The α-helices and β-sheets are colored in red and yellow, respectively. The membrane-spanning portion of the molecule is indicated with green lines.

solution to the need for a rapid signaling response to external physical stimuli. MS channels are classified by their ability to alter their opening probability in response to lateral tension in the lipid bilayer. In bacteria, mechanosensitive ion channels help microbes react to hypoosmotic stress by allowing them to expel cytoplasmic solutes such as ions and small molecules into the surrounding medium. The crystal structure of the mechanosensitive ion channel from *Mycobacterium tuberculosis* (TB-MscL) gives some clues as to the structural basis of the cellular response to lateral tension in the lipid bilayer resulting from increased osmotic pressure [1]. TB-MscL is arranged as a homopentamer of 15-kDa subunits. The membrane-spanning domain of the channel consists of ten transmembrane α-helices that are significantly tilted relative to the normal of the cell membrane. The cytoplasmic domain consists of a helix bundle and is likely to be disrupted upon channel opening. MS channels of the MscL family have large conductances on the order of approximately 2.5 nS [8]. Upon channel opening, MscL is thought to form a large pore through the cell membrane with a opening of at least 10 Å.

Some unique features of the TB-MscL structure suggest a general mechanism for channel gating in response to lateral tension in the bilayer. First, the gate or "plug" of TB-MscL is located on the inner membrane leaflet side of the cell membrane. Second, there is a cluster of bulky hydrophobic residues positioned to interact directly with neighboring lipid molecules of the inner membrane leaflet. And, finally, the experimentally determined electron density maps have revealed highly ordered lipid/detergent near these bulky residues, suggesting a strong interaction with lipids of the inner membrane leaflet. These features of TB-MscL suggest that structural changes due to lateral tension could be directly transmitted to the transmembrane α-helices via bound lipid. Rearrangement of the transmembrane α-helices causes the plug to pull apart, allowing ion conduction and enabling bacteria to respond rapidly to changing tonicity.

Active Transporters: Rapid Response and Energy Management

When a cell encounters a new environment, a rapid response is required to take advantage of nutrients in the surrounding media. If useful substrates such as sugars, amino acids, and ions are in relatively low concentration, then highly regulated and efficient transport systems must be activated to uptake substrates through their cell membranes. Likewise, if cells encounter toxins such as antibiotics or anti-cancer drugs, they must be quickly transported out of the cell. Both types of transport are accomplished by a diverse array of energy-dependent pumps located in the cell membrane. The active transport systems of the cell membrane are critical for a rapid cellular response, and cells devote a significant portion of their resources to the maintenance of transporters on the cell surface. For example, transport proteins comprise nearly 5% of the genes encoded in the *E. coli* genome, and nearly half of these transporters belong to the ATP-binding cassette (ABC) transporter super family. ABC transporters are highly conserved from bacteria to human and contain a highly conserved nucleotide binding domain (NBD) that binds and catalyzes the hydrolysis of ATP. ABC transporters are thought to translocate substrate by coupling the energy derived from ATP hydrolysis to structural rearrangements in the portion of the molecule spanning the cell membrane. ABC transporters are involved in the import and export of a wide variety of substrates, including amino acids, peptides, sugars, ions, lipids, and hydrophobic drug molecules. Several ABC members of the multidrug resistance ABC (MDR–ABC) transporter group severely reduce the effectiveness of chemotherapeutics and antibiotics, leading to a failure of treatments for cancer and infectious diseases. The X-ray structure of the MDR–ABC transporter homolog MsbA from *E. coli* sheds light on this type of substrate transport [1].

MsbA is a dimer (≈129 kDa) of two identical peptides, each containing a transmembrane domain of six membrane-spanning α-helices and a nucleotide binding domain that hydrolyzes ATP. In the course of the X-ray structure determination, a third domain has been identified that bridges the transmembrane domain and the NBD. This domain is well positioned to scan the head groups of various lipids and could serve as a trigger for initiating the ATP hydrolysis. A prominent feature of Eco-MsbA is a large opening facing the inner membrane leaflet side of the cell membrane. This opening leads into a large chamber that has a polar interior. Structural changes caused by the recruitment of lipid A molecules into the chamber triggers ATP hydrolysis by the NBD. Energy derived from this process closes the chamber, producing a microenvironment that is unfavorable for hydrophobic substrates in the inner membrane leaflet side. At this point, the substrate flips to the energetically more favorable position in the outer leaflet side of the chamber and is then expelled into the outer leaflet of the bilayer. The structure of Eco-MsbA provides a structural basis for the transport of lipids and a wide variety of hydrophobic cytotoxins across the cell membrane.

Although the structure of MsbA provided the first glimpse of an ABC exporter, the structural basis of transport in the opposite direction across the membrane has, until recently, remained a mystery. Impressive work done by Rees and colleagues [4] has resulted in the first structure of an ABC importer, the vitamin B_{12} transporter BtuCD. The structure reveals for the first time an ABC transporter with direct contact between the nucleotide-binding domains and in a manner reminiscent of the popular Rad50 ABC dimer. Contact between the nucleotide binding domains could explain the observed cooperative kinetics of ATP hydrolysis during the transport cycle. In addition, the precise point of contact between the ABC cassette and the membrane-spanning domain is seen, providing insight into the coupling of ATP hydrolysis to substrate transport. Generally, most members of the ABC transporter family have been predicted to contain 12 transmembrane segments, as is the case with MsbA. BtuCD is unusual in that it was found to have an astonishing 20 transmembrane helices. One explanation offered for such a dramatic departure from the canonical view could be that a common core structure of membrane-spanning subunits exists, while the surrounding helices vary according to function. The BtuCD structure represents yet another milestone in the understanding of ABC transporter mechanics and provides the first structural evidence that supports the idea of catalytic cooperatively between the ABC cassettes during the transport cycle.

Receptors: Gate Keepers for Cell Signaling

The classic paradigms for signal transduction across the cell membrane are the membrane-bound receptors. Crystal structures of a bacterial ion channels and transporters have been elucidated, and the next frontiers of membrane protein structural biology will likely focus on smaller mammalian targets. One of the most widely studied families of receptors is the G-protein-coupled receptors (GPCRs). GPCRs are not only fascinating from a scientific standpoint but are also pharmaceutically important drug targets. Nearly 60% of all the drugs on the market target GPCRs. All GPCRs are predicted to have seven membrane-spanning α-helices with a NH_2-terminal domain that binds ligand (such as a hormone) and a c-terminal cytoplasmic domain that binds and activates a specific G protein. How does a ligand/drug molecule on the outside of the cell membrane transmit a signal across the cell membrane to initiate a cascade of signals via a cytoplasmic G protein? The answer to this question will surely require a detailed molecular structure. Some initial clues about GPCR function might be derived from the structures of the light-transducing bacteriorhodopsin [6] and bovine rhodopsin [5].

Bacteriorhodopsin is a photon-driven ion pump that shares the same putative 7-TM membrane-spanning topology

as GPCRs. In the bacterium *Halobacterium salinarium*, bacteriorhodopsin converts light energy to a proton gradient that is, in turn, used by the membrane-bound ATP synthase. The process of converting light to useful energy is remarkably efficient, with quantum yields of greater than 60%. Similarly, bovine rhodopsin, which is a member of the GPCR family, is a photoreceptor protein found in the rod cells of vertebrates and is responsible for vision in low light. In both molecules, the conversion of light energy is accomplished by a retinal chromaphore called 11-*cis*-retinal, which is a derivative of vitamin A and is covalently linked via a Schiff base bound to the membrane-spanning portion of the molecule. Upon photoisomerization of the chromophore, several intermediates are formed, and an all-*trans* chromophore is generated. This conversion leads to the activation of phosphodiesterase and the closing of cyclic-GMP-gated cationic channels. The resulting hyperpolarization of the channels is transmitted through the photoreceptor cell to the synapsed nerve cells of the optic fiber.

Most classes of GPCRs, although similar to rhodopsin, have a more extensive NH_2-terminal domain that binds ligand. The structures of the NH_2 domains of the Methuselah GPCR from *Drosophila* [9] and the Cholecystokinin-8 receptor have been determined [7]. However, the molecular structural basis of how these NH_2 domains interact with the membrane-spanning portion of the molecule to achieve signal transduction signal is unknown. In addition, the binding and activation interactions between receptors and the G proteins still remains a mystery. The answer to these quetions will require molecular detail that can only be provided by a high-resolution structure of a complete GPCR/G-protein complex.

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CHAPTER 20

Structural Basis of Signaling Events Involving Fibrinogen and Fibrin

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Fibrinogen is an extracellular protein found in significant concentrations in the blood plasmas of all vertebrate animals. It is a large, multi-domained protein, some portions of which share common ancestry with lectins and other cytotactic proteins found throughout the animal kingdom [1]. Although the principal role of fibrinogen has to do with its polymerization into fibrin clots, the protein also interacts with a number of other extracellular proteins, blood platelets, and a variety of cells. Directly or indirectly, the fibrinogen–fibrin system is involved in hemostasis, inflammation, wound healing, and angiogenesis. Fibrinogen also interacts with various bacteria, especially certain strains of *Staphylococcus*.

Fibrinogen is a covalent dimer composed of two sets of three nonidentical chains ($\alpha 2\beta 2\gamma 2$). The β and γ chains are homologous over their full lengths, but the α chain homology is limited to its amino-terminal third. The molecular weights of vertebrate fibrinogens range from 320,000 to 400,000, the variation invariably being due to differences in the α chains, the carboxyl terminal two-thirds of which are extremely variable from species to species. In contrast, the carboxyl-terminal halves of the β and γ chains are globular and conserved. Together, they constitute bi-lobed macro domains at the extremeties of the extended molecule, being connected to a small central domain by three-strands coiled coils made up of all three chains (Fig. 1).

Several regions of the fibrinogen molecule are highly mobile and are not resolvable in crystallographic electron density maps, even at moderately high resolution [2]. The flexible parts include the entire α-chain carboxyl region, which can contain from 300 to 500 amino acid residues, depending on the species (region I in Fig. 1). Additionally, the last 15 residues at the carboxyl terminus of the γ chain have not been pinned down with any precision [3,4], nor have the amino-terminal segments corresponding to approximately the first 30 residues of the α chain and the first 60 of the β chain (numbering varies slightly from species to species; the numbering here is based on human fibrinogen). Several of these mobile regions figure prominently in interactions with other proteins and with cells.

Apart from the mobile and highly variable carboxyl-terminal domains of the α chains, the general framework of all vertebrate fibrinogens is highly conserved, as evidenced by the ready superposition of the chicken fibrinogen crystal structure on that of a modified bovine fibrinogen [5,6]. The length of the protein is about 45 nm.

The conversion of fibrinogen to fibrin is initiated by thrombin-removing short peptide regions, called *fibrinopeptides*, from the amino-terminal ends of the α and β chains. The consequence of these narrowly specific proteolytic events is the exposure of sets of A and B "knobs" on the α and β chains, respectively, that fit into holes on the terminal globular domains of neighboring fibrinogen molecules. The initial knob-hole interactions position a pair of A knobs (Gly–Pro–Arg is the sequence at the newly exposed α-chain site, residues 17–19) so as to pin together two neighboring molecules by fitting into holes on their γ-chain carboxyl domains. Further propagation results in a noncovalently associated, two-molecule-thick, half-staggered protofibril. Interactions involving the B knob (Gly–His–Arg is the sequence at the newly exposed β-chain site) can fill holes in the β-chain carboxyl domains and, directly or indirectly, lead to lateral growth of the fibrin network. Meanwhile, thrombin-activated factor XIII reinforces the fibrin polymer by introducing γ-glutamyl-ε-amino crosslinks, initially between the

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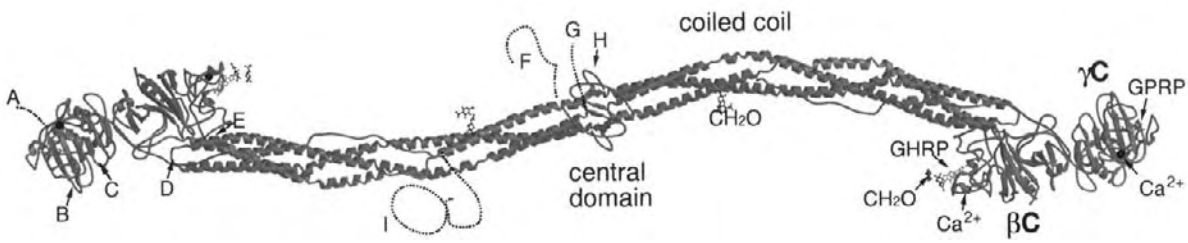


Figure 1 Ribbon model of those portions of fibrinogen for which high-resolution X-ray structures are available. Highly mobile regions of the molecule are not shown fully (broken lines), including the carboxyl-terminal domain of α chains (I) and the last 15 residues of γ chains (A), as well as the amino-terminal segments of α and β chains (G, F). The molecule is a covalent dimer with a pseudo-axis of symmetry running through the central domain. Key structural features are labeled on the right half, and some reported recognition sites are designated on the left half. A, γ-chain carboxyl terminal (platelets, fibroblasts, staphylococcal clumping factors); B, γ-chain 383–395 (αMβ2); C, γ-chain 195–202 (αMβ2); D, γ-chain 117–133 (ICAM-1); E, α-chain 151–158 (t-PA stimulator); F, β-chain 15–42 (angiogenesis, heparin-binding); G, α-chain 17–20 (αMβ2); H, α-chain 15–44 and β-chain 61–72 (thrombin); I, α-chain 240–610 (αMβ2).(Adapted from Yang, Z. *et al.*, *Biochemistry*, 40, 12515–12523, 2001.)

carboxyl-terminal segments of abutting γ chains, but eventually also between carboxyl domains of α chains.

Fibrin can be distinguished from fibrinogen by many recognition systems. Quite apart from sites lost with the removal of the fibrinopeptides and the coincident appearance of the A and B knobs, the mere act of polymerization can mask certain sites. Additionally, conformational changes occur, some of which have been observed in crystal structures of fibrin(ogen) fragments complexed with synthetic knobs [7]. Other more subtle changes may occur during the later stages of polymerization. For example, there is a region of the α chain that has been implicated in the stimulation of tissue plasminogen activator [8] that is wholly inaccessible to solvent in fibrinogen but which somehow becomes accessible as a result of the polymerization process.

Over the years, there have been numerous reports describing regions of fibrinogen or fibrin responsible for binding various macromolecules or cells. The availability of X-ray structures now provides a backdrop for visualizing some of these at atomic resolution (Fig. 1). Among the cells and particles known to bind fibrin(ogen) are platelets, endothelial cells, monocytes, lymphocytes, neutrophils, and fibroblasts, all of which are actively involved in hemostasis, wound healing, inflammation, or angiogenesis. For the most part, studies have utilized fragments of fibrin(ogen), antibodies directed against localized features, site-directed mutagenesis of recombinant fibrinogens, or synthetic peptides corresponding to specific regions.

Some parts of fibrinogen have been implicated in several different events. The carboxyl-terminal segments of γ chains bind platelets [9] and fibroblasts [10]; the same sites bind to certain strains of *Staphylococcus aureus* [11]. The locations of these sites at the tips of the dimeric fibrinogen molecule are well disposed for bridging and clumping cells or platelets. The crosslinking of these segments in fibrin by factor XIII must render the sites inaccessible. Similarly, certain regions of the α-chain carboxyl domain have been implicated in binding to platelets and various leucocytes, and these must also be compromised by becoming crosslinked in the final stages of clot formation.

The flexible amino-terminal segment of the β chain is another targeted region. The bacterium *Staphylococcal epidermis* binds to a peptide segment that includes a bond cleaved by thrombin [12]. Other entities bind in the region of β-chain residues 15 to 42, exposed after thrombin attack, including certain cadherins [13] and heparin [14]. Angiogenesis is also stimulated by this general region [15].

In the main, two kinds of cell surface proteins have been associated with fibrinogen binding: (a) members of the immunoglobulin family such as cadherins [13] and ICAM-1 [16], and (b) heterodimeric integrins. The most commonly implicated integrins are αMβ2 and αχβ2 [17]. Some findings about the sites of interaction remain uncertain in that the same integrins have been reported to interact with widely differing regions of the fibrinogen molecule for which there are no apparent structural similarities. Final resolution may have to await crystal structures of complexes of fibrin(ogen) fragments with specific integrins or other interactants.

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CHAPTER 21

Structural Basis of Integrin Signaling

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Introduction

The integrins are a family of proteins that reside in the plasma membrane of most cells of multicellular organisms [1]. They are the primary receptors that recognize the protein components of the extracellular matrix (ECM). Binding to the ECM triggers intracellular signaling pathways that regulate adhesion, migration, growth, and survival [2]. These pathways often intersect with those generated by receptors for soluble factors [3]. However, integrins differ from "classical" signaling receptors in a number of ways. First, because the ECM is static and polyvalent, integrins cluster at the sites of attachment. Second, ligand-bound integrins form connections with the cytoskeleton that regulate cell shape and rigidity, as well as providing platforms for signaling complexes. Third, integrins can also transmit signals from the inside of the cell to the outside. Thus, integrin signaling is a bidirectional process that evolves rapidly in time and space as the cell adapts to its environment, allowing integrins to be sensors and messengers of the surroundings and shape of the cell, as well as the mechanical forces acting upon it [2].

Integrin signaling typically involves conformational changes within the integrin molecule that are propagated across the plasma membrane. Under some circumstances, lateral self-association of integrins ("clustering") is sufficient for signaling [4,5], and a number of molecules that associate laterally with integrins have also been identified that contribute to signaling [6]. However, the focus of this section is on the conformational changes within individual molecules that control the recognition of extracellular and intracellular binding partners.

Structure

Integrins are αβ heterodimers, consisting of a head domain from which emerge two legs, one from each subunit, ending in a pair of single-pass transmembrane helices and short cytoplasmic tails (Fig. 1). In the absence of ligand, bonds between the legs and tails are believed to hold the head in an inactive or resting conformation that has low affinity for ligand [7,8]. During outside-in signaling, ECM binding to the head triggers conformational changes that are propagated down the legs and through the plasma membrane, leading to a reorganization of the C-terminal tails that allows them to bind intracellular proteins [3]. During inside-out signaling, cytosolic proteins bind and sequester one or both of the cytoplasmic tails, triggering conformational changes in the head that lead to a high-affinity active integrin.

The integrin "head" is composed of a seven-bladed propeller from the α-subunit that makes an intimate contact with a GTP-ase-like domain of the β-subunit (called either an A or I domain by different authors, and I domain here), in a manner that strongly resembles the heterotrimeric G proteins [9]. Instead of a catalytic center, the I domain contains an invariant ligand binding site called MIDAS (metal ion-dependent adhesion site), in which a metal ion is coordinated by three loops from the I domain, and a glutamic or aspartic acid from the ligand completes an octahedral coordination sphere around the metal. Specificity is provided by ligand contacts to the surface surrounding the MIDAS, which is highly variable among integrin family members, and in some cases by additional contact to the α-subunit propeller. A helix that emanates from one of the MIDAS loops packs against the central axis of the propeller,

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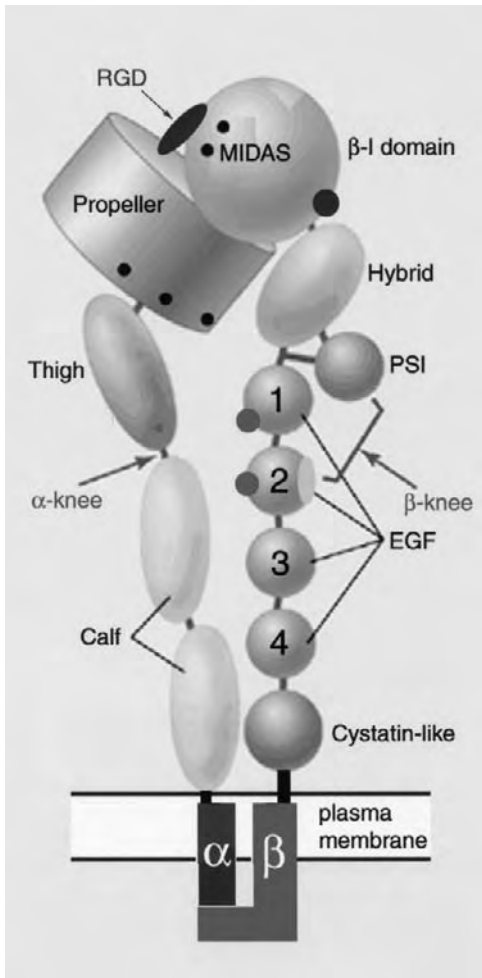


Figure 1 Integrin domain organization. Activation epitopes [13,33] are shown as red, blue, and cyan disks.

thus providing a potential link between ligand binding and the quaternary structure. In certain integrins, an additional I domain (α-I) is inserted into the α-subunit, between two loops on the upper surface of the propeller, where it forms the major ligand binding site. Modeling studies indicate that this domain will form contacts with both the propeller and the β-I domain that regulate the conformation and ligand affinity of the domain, and indeed mutations to the outer surface of the domain can lead to loss- or gain-of-function [10]. The remaining domains of the two subunits form a pair of legs that contact each other along their length, ending at their closely apposed C termini. The legs are followed by a pair of single-pass transmembrane helices and short (except for β4) cytoplasmic tails, typically 20 to 50 residues in length. These tails lack catalytic activity and transduce signals by binding to intracellular structural and signaling proteins.

Quaternary Changes

Early biophysical and immunochemical studies demonstrated that integrin signaling is associated with large changes in quaternary structure [11]; however, there remains much controversy over the structure of the resting integrin, as well as the nature of the conformational changes underlying signal transduction [12]. The overall structure of the integrin, based on electromagnetic (EM) images, was expected to have straight legs. However, in the first crystal structure of the entire extracellular portion of an integrin (αVβ3), it is severely bent at the knees [9]. The authors proposed that this bending was likely to be a crystal artifact; they further suggested that the crystallized fragment represents the activated, high-affinity state of the integrin, as it binds ligand with high affinity in solution and is able to bind peptide ligand mimetics in the crystal. However, based on the NMR structure of two domains that were poorly ordered in the crystal structure and on the location of epitopes for activating antibodies within these domains, Springer, Blacklow, and colleagues have suggested that the "knees-bent" or genuflected integrin represents the inactive conformation *in vivo* and that a "switchblade" opening of the integrin is associated with activation [13]. In a third model of quaternary changes in integrins, Hantgan and colleagues [14] have provided evidence, using EM and hydrodynamic studies of peptide-bound integrin, that the α- and β-head segments separate on activation. Such a model would extend the analogy with the heterotrimeric G proteins [15]. In the G proteins, the GTP-ase domain locks onto the propeller domain, regulating ligand binding in two ways: steric blockade of the propeller and allosteric control of the GTP-ase domain. On binding GTP, the GTP-ase domain dissociates from the propeller, enabling both domains to bind their respective ligands. Binding of an RGD-style ligand could play an role analogous to GTP. The G protein model is also consistent with the observations of Mould *et al.* [16], who mapped two distinct binding sites, one on the propeller and the other at the MIDAS motif, for two different regions of fibronectin, separated by ≈40 Å. In the crystal structure of αVβ3, the fibronectin binding sites are much closer together, suggesting that the head must separate in order to engage both sites on fibronectin and supporting the notion that the head separates on activation.

Curiously, none of these integrin models is consistent with the assignment of a long-range disulfide in the β-subunit [17], although Yan and Smith [18] have provided evidence that disulfide shuffling occurs in integrin αIIbβ3 and modulates activation, raising the possibility of further, thus far uncharacterized, large-scale quaternary changes underlying activation and signaling.

Tertiary Changes

Crystal structures of recombinant α-I domains with and without ligand have demonstrated a dramatic conformational switch between closed and open states involving a change in the details of metal coordination at the MIDAS motif that is mechanically linked to a 10-Å downward shift of the C-terminal helix (α7) [19]. Mutational studies of the I domain in the context of the intact integrin have confirmed

that these conformational changes underlie affinity control and that the conformational state of the I domain is regulated by the quaternary organization of the integrin [10,20,21].

The conformation of the β-I domain in the crystal structure is much more similar to the closed (i.e., inactive) conformation of the α-I domain, although Xiong *et al.* have proposed the opposite [9]. Furthermore, the same group recently soaked a short circular RGD peptide that acts as a ligand mimetic into the same crystals [22], and the conformational changes are consistent with those expected for a liganded domain within the context of a closed quaternary structure, in which tertiary changes are in the direction of those observed in the α-I domain but are frustrated by the closed quaternary structure [12]. Indeed, three mutations that suppress activation map to the loops that link one of the MIDAS loops to the C-terminal (α7) helix [23]. It is conceivable that in the activated integrin, a large shift of α7, comparable to that observed in the α-I domain, occurs in concert with a hinge-like motion of α-I, allowing it to roll around its N-terminal connector and freeing it from the propeller. The crystal structure of an authentic active integrin–ligand complex is required to test this proposal.

Tail Interactions

Abundant biochemical and genetic data support the notion that interactions between integrin α and β cytoplasmic tails hold the resting integrin in a low-affinity conformation [7,24,25]. For example, a classic study by Ginsberg and colleagues [7] showed that a salt bridge between the αIIb Arg^{995} and β3 Asp^{723} was necessary and sufficient to hold the integrin in its resting state. It is puzzling, therefore, that several nuclear magnetic resonance (NMR) analyses have failed to demonstrate such an interaction directly [26,27]. A recent paper by Vogel and coworkers, however, provides the first direct structural evidence for extensive interactions, albeit with truncated tail fragments [28].

Given this confusion, it has been difficult to develop a definitive model of how reorganization of the cytoplasmic tails propagate through the transmembrane domain to the ligand-binding head. Two simple models are supported by data. The first is a *scissors model*, in which the integrin pivots about some point between its legs, leading to a separation of both the head and tail domains [2,29]. Such a movement is consistent with the EM studies of Hantgan *et al.* [14]. Inside-out signaling is simple to envisage in such a model and would simply require that a cytosolic protein is bound tightly to one or both tails, pulling them apart. An example of such a protein is talin, which activates integrins by binding to an NPxY motif near the center of most β tails via a phosphotyrosine binding (PTB)-like domain in the head region [30]. A second possibility is a *piston model*, in which one or both tails move up and down with respect to the plasma membrane, changing the border of the transmembrane and cytoplasmic domains. Typically, an R or K is positioned 23 hydrophobic amino acid residues carboxy terminal to the predicted start of the transmembrane domain, followed by four to six hydrophobic residues [24] that also appear to be membrane-imbedded in the resting integrin [31]. It has been proposed that changes in the localization or orientation of the integrin transmembrane domain could occur during physiological integrin activation. For example, the binding site of the β2 integrin regulatory protein, cytohesin-1, is in the hydrophobic membrane proximal region [32], so that sequestering this region could "pull" on the β-subunit, altering the packing between the α-subunit propeller and the β-I domain.

Concluding Remarks

In spite of major advances in the past 12 months, understanding the structural basis of integrin signaling is far from complete. The major missing data include the structure of a true ligand-bound, active integrin and definitive structural data on the interactions between the cytoplasmic tails and how these are affected by complex formation with cytoplasmic binding partners.

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CHAPTER 22

Structures of Heterotrimeric G Proteins and Their Complexes

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Introduction

The alpha subunits of heterotrimeric G proteins belong to the superfamily of intracellular GTP hydrolases that use the energy derived from the binding of guanosine triphosphate (GTP) to effect signal transduction. The energy derived from GTP binding is used to stabilize an activated state of the G protein that is able to bind and regulate certain molecules, called *effectors*, in the cell. This capability is diminished or lost when the G protein hydrolyzes GTP. It is regained when a new molecule of GTP is bound, a process that is catalyzed by ligand-activated, seven-transmembrane helical G protein-coupled receptors (GPCRs). Intracellular targets of G protein regulation include a small group of second-message-generating molecules such as potassium and calcium ion channels, phospholipase Cβ isoforms (PLCβ), adenylyl cyclases (ACs), cyclic GMP phosphodiesterase, and regulators of other signaling pathways, such as the p115 Rho guanine nucleotide exchange factor (p115RhoGEF). The sequence of GTP binding, hydrolysis, product release, and reformation of the G protein–GTP complex constitutes a signaling cycle. Steps within this cycle are subject to regulation that shapes the temporal characteristics of the signal, from ligand–receptor recognition to G-protein–effector interaction. The three-dimensional structures of many of the components of this cycle have been described in several functionally relevant states (Table 1). These structures provide insight into the molecular mechanics of G-protein-mediated signal transduction. Here, we briefly describe the three-dimensional structures of G proteins and the molecular processes that constitute the signaling pathway. This area of research has been extensively reviewed [1–4] and the reader is directed to the primary literature for details.

Heterotrimeric G proteins have two functional components. The alpha subunits (Gα) are GTP binding proteins which, when bound to GTP, preferentially interact with effectors. Dimers composed of tightly bound β (Gβ) and γ (Gγ) chains constitute the second functional unit. Gβγ dimers act both as inhibitors of nucleotide release from Gα and as regulators of effector proteins, either independently or coordinately with Gα.

Gα Subunits

Gα subunits are members of the Ras superfamily, which also includes translation elongation factors and the components of the signal recognition apparatus. In mammals, the family of Gα isoforms is encoded by 16 genes; these can be sorted into four closely related homology groups or classes named for representative members of each class: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$ (Fig. 1). Two variants of $G\alpha_s$ are generated by alternative mRNA splicing. Each member of the Gα family interacts specifically with one effector or effector isoform, although certain effectors are regulated by more than one species of Gα. Known effectors include all isoforms of AC: $G\alpha_s$, $G\alpha_{olf}$, and $G\alpha_i$ (a negative regulator of types I and V AC) [5]; PDE: $G\alpha_t$; PLCβ isoforms: $G\alpha_q$ class members [6]; and p115RhoGEF: $G\alpha_{13}$ [7]. Effectors of certain Gα proteins, $G\alpha_o$, and $G\alpha_z$, remain in question.

Ras superfamily proteins are built upon a scaffold of six parallel β-strands, layered on each side by a set of five α-helices (Fig. 2). Unique to the heterotrimeric Gα family is an α-helical bundle domain inserted into the loop between

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Table I Selected Structures of Heterotrimeric G Proteins and Their Complexes

Protein	Nucleotide	Ref. and PDB code
$G\alpha_t$	Mg^{2+}•GTPγS[a]	[15] 1TND
$G\alpha_{i1}$	Mg^{2+}•GTPγS	[13] 1GIA
$G\alpha_{i1}$	Mg^{2+}•GppNHp[b]	[65] 1CIP
$G\alpha_s$	Mg^{2+}•GTPγS	[17] 1AZT
$G\alpha_{i1}$	Mg^{2+}•GDP•AlF_4^-	[13] 1GFI
$G\alpha_t$	Ca^{2+}•GDP•AlF_4^-	[25] 1TAD
$G\alpha_{i1}$ (G203A)	GDP•Pi	[66] 1GIT
$G\alpha_{i1}$	Mg^{2+}•GDP•SO_4^{2-}	[67] 1BOF
$G\alpha_t$	Mg^{2+}•GDP	[16] 1TAG
$G\alpha_{i1}$	GDP	[14] 1GDD
$G\alpha_{i1}$•$G\beta_1$•$G\gamma_2$	GDP	[45,49] 1GG2
$G\alpha_t$•$G\beta_1$•$G\gamma_1$[c]	GDP	[48] 1GOT
$G\alpha_t$•GoLoco[d]	GDP	[68] 1KJY
$G\beta_1$•$G\gamma_1$	—	[47] 1TBG
$G\beta_1$•$G\gamma_1$•Phosducin	—	[69,70] 1AOR,1B9X
$G\alpha_{i1}$•RGS4	Mg^{2+}•GDP•AlF_4^-	[35] 1AGR
$G\alpha_t$•RGS9	Mg^{2+}•GDP•AlF_4^-	[20] 1FQK
$G\alpha_t$•RGS9•PDEγ[e]	Mg^{2+}•GDP•AlF_4^-	[20] 1FQJ
$G\alpha_s$•AC[f]	Mg^{2+}•GTPγS	[19] 1AZS
$G\alpha_s$•AC[g]	Mg^{2+}•GTPγS	[71] 1CJU

[a]GTPγS–guanosine 5′-[γ-thio]triphosphate.
[b]GppNHp–guanosine-5′-(βγ-methylene)triphosphate.
[c]Gα subunit is a chimera comprising residues 26 to 215 of bovine $G\alpha_t$, residues 220 to 298 of rat $G\alpha_{i1}$, and residues 295 to 350 of bovine $G\alpha_t$.
[d]GoLoco motif peptide from RGS14.
[e]PDEγ–cyclic GMP phosphodiesterase γ subunit.
[f]AC: a complex between the C1 domain of adenylyl cyclase type V and the C2 domain of adenylyl cyclase type II. These domains comprise the catalytic unit. A soluble forskolin derivative is bound at the regulatory site of AC. The domains adopt the open conformation.
[g]This complex contains the ATP analog β-L, 2′,5′, dideoxy adenosine triphosphate, and two magnesium ions. The domains adopt a closed conformation.

the first and second β strands of the Ras-like domain. Gα subunits are modified by N-terminal myristoylation [8] ($G\alpha_t$) and thioester-linked palmitoylation ($G\alpha_s$, $G\alpha_q$, $G\alpha_{13}$), or both ($G\alpha_i$, $G\alpha_o$, $G\alpha_z$) [9,10]. The latter confers plasma membrane localization upon $G\alpha_s$ and $G\alpha_q$ but may be reversed upon activation [11]. Myristoylation is required in some cases for activity, for example, efficient inhibition of adenylyl cyclase by $G\alpha_{i1}$ [12].

GTP is bound between the helical and Ras-like domains but interacts primarily with conserved sequence motifs within the Ras domain [13–17]. The P-loop, which enfolds the alpha and beta phosphates of the nucleotide, contains a characteristic Walker A sequence motif, $G^A/_TGESGKST$ [18], which is permissive for the tight turn required to encompass the phosphate. The lysine residue is a critical β-phosphate ligand and the following serine residue binds the catalytic Mg^{2+} ligand. The connector leading from the helical domain to the Ras-like domain contains a series of residues called Switch I. The arginine residue (178 in $G\alpha_{i1}$) within this sequence (…RVXTTG…) is an important catalytic ligand, and the succeeding threonine is the second Mg^{2+} ligand (Fig. 3). The gamma phosphate group of GTP is cradled by a tight turn (…DVGGQ…) which precedes Switch II, an irregular and conformationally mobile helix (α2). The glutamine residue in this series (204 in $G\alpha_{i1}$) plays a critical catalytic role in GTP hydrolysis. However, in the structures of Gα subunits bound to slowly hydrolyzable GTP analogs, the catalytic glutamine and, in $G\alpha_{i1}$, the catalytic arginine as well are either poorly ordered or adopt conformations in which they would be incapable of providing catalytic assistance (Fig. 3). A key role of GTP, in league with Mg^{2+}, is to maintain the conformational state and structural integrity of the helical Switch II via a set of hydrogen bonds and oxygen-metal interactions that link Mg^{2+}•GTP with the P-loop, Switch I, and Switch II. Structural studies of $G\alpha_{i1}$ and $G\alpha_t$ show that, in the guanosine diphosphate (GDP) state, Switch II is either wholly disordered or adopts an alternate conformation. The well-ordered state induced by GTP also

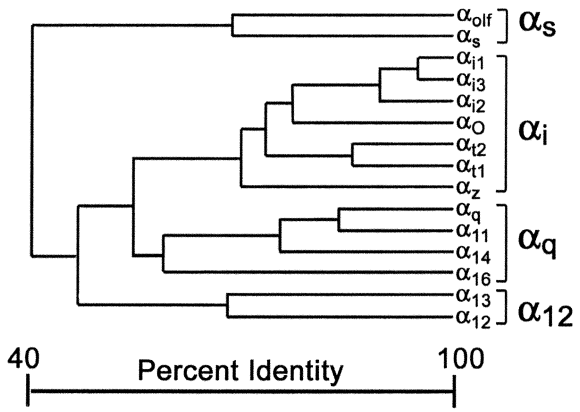


Figure 1 A phylogenetic tree, using CLUSTAL_W(81) of the mammalian family of human Gα subunits.

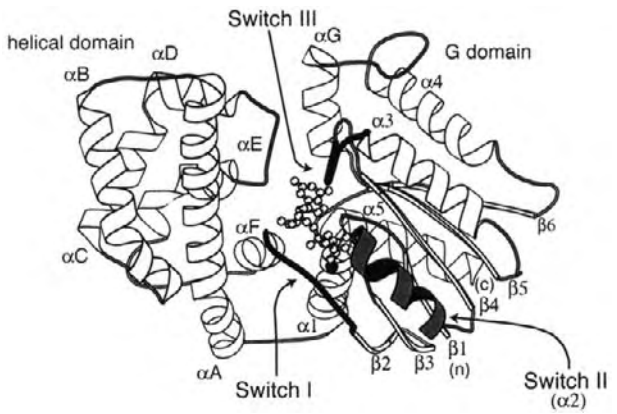


Figure 2 Schematic of the GTP-bound complex of $G\alpha_{i1}$ with secondary structure elements (arrows: β-strands, coils: α-helices). GTP is shown as a ball-and-stick model. (From Sprang, S. R., *Annu. Rev. Biochem.*, 66, 639–678, 1997. With permission.)

promotes a set of ionic contacts between Switch II and the β4–α3 loop called Switch III. Upon GTP hydrolysis, the network of interactions between the three switch regions is altered or lost.

The purine ring of the guanine nucleotide is cradled by two conserved loops, β5–αG and β6–α5 (Fig. 2). The aspartate residue within the first sequence (…FLNKKD…) confers specificity towards guanine nucleotides. The second loop acts in a supporting role. A variety of α mutations have been described, some of physiological relevance, that directly affect GTP hydrolysis, nucleotide specificity exchange, and effector coupling. Such mutants are useful for probing or controlling the action of G proteins in cell culture or *in vivo* (Table 2).

Ga–Effector Interactions

In the two Gα–effector complexes for which structures are known, Gα binds the effector in the same manner, even though the structures of the effectors themselves are quite different. The interactions are dependent on the "activated" state of Gα that is stabilized by GTP. In the complex

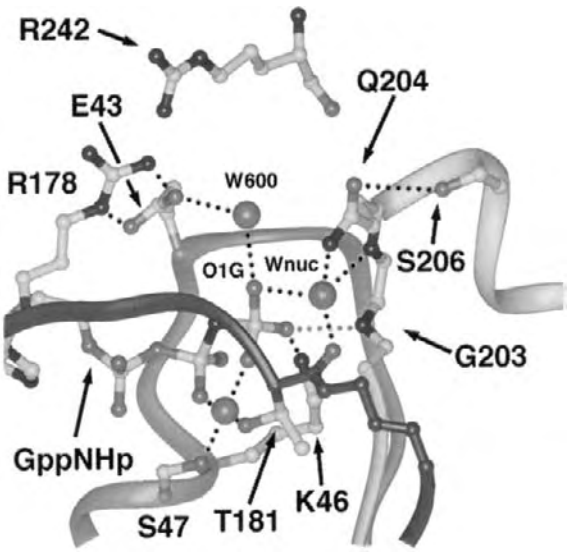


Figure 3 Close-up of the catalytic site of the $G\alpha_{i1}\bullet Mg^{2+}\bullet$GppNHp complex, showing the side chains involved in catalysis and Mg^{2+} binding. Residues of Switch I are blue, the P-loop is green, and Switch II is pale yellow. (Adapted from Coleman, D. E. and Sprang, S. R., *J. Biol. Chem.*, 274, 16669–16672, 1999.)

between $G\alpha_s\bullet$GTPγS and the catalytic domains of adenylyl cyclase [19] and that between $G\alpha_t\bullet$GTPγS and the γ subunit of PDE [20], the effector is bound at the cleft between Switch II and the α3–β5 loop of Gα (Fig. 4). The effector specificity of Gα is conferred both by side chains in the effector binding segments and the conformation of the polypeptide chain within them. For example, $G\alpha_{i1}$ inhibits the $G\alpha_s$-stimulated activity of adenylyl cyclase isoforms I and V, but does not bind to the $G\alpha_a$ activation site [21], possibly interacting at a dyad-related site in the C1 domain instead. The ability of $G\alpha_{i1}$ to discriminate its own from the $G\alpha_s$ binding site is unlikely to be entirely due to the amino acid sequence of the Switch II and α3–β5 loops, because all but two amino acids in the adenylyl cyclase contact region are conserved between the two Gα subunits. The failure of $G\alpha_{i1}$ to act as an activator (or $G\alpha_s$ as an inhibitor) may stem from differences between the two proteins in the spacing and orientation of the α3–β5 loop and the α4–β6 loop that buttresses it [17]. Indeed, the α4–β6 loop had been proposed, on the basis of mutagenesis experiments, to direct the specificity of $G\alpha_{i2}$ and $G\alpha_s$ toward their respective binding sites on AC [22], even though the structure of the complex revealed no direct contact with effector. In its interaction with PDEγ, $G\alpha_t$ uses the same structural elements that $G\alpha_s$ employs in contacting AC. Although the chemical basis of certain of the Gα–effector interactions are conserved, the amino acid sequence differences between $G\alpha_t$ and other Gα subunits are sufficient to ensure specificity. There is structural and biochemical evidence to suggest that $G\alpha_s$ stimulates adenylyl cyclase by controlling the relative orientation of its catalytic domains. $G\alpha_t$ (transducin), on the other hand, sequesters an inhibitory subunit of cyclic GMP phosphodiesterase.

Table II Selected Mutations in Gα Subunits

Mutation	Gα	Structural element	Effect	Structure	Ref.
G49A	$G\alpha_s$	P-loop	Reduced GTPase rate	—	[72]
G42V	$G\alpha_{i1}$	P-loop	Reduced GTPase rate	$G\alpha_{i1}$, 1ASO, 1AS2, 1AS3	[73]
S54N	$G\alpha_s$	P-loop; Mg^{2+} ligand	Weak Mg^{2+} binding; reduced receptor activation; negative dominant	—	[74]
R201X	$G\alpha_{i1}$, $G\alpha_s$	Switch I Catalytic R	Reduced GTPase rate; activating mutant	—	[75,76]
G202T	$G\alpha_o$	Switch II	Dominant negative	—	
G226L	$G\alpha_s$	Switch II Catalytic Q	Reduced rate of Gβγ release (prevents activation), loss of Mg^{2+} affinity	$G\alpha_{i1}$(G203A), 1GIT, $G\alpha_{i1}$(G203A)$\beta_1\gamma_2$ 1GG2	[77,78]
Q227L	$G\alpha_s$, $G\alpha_{i1}$	Switch II	Abolishes GTPase activity	$G\alpha_{i1}$(Q204L), 1GIL	[72,79]
D273N	$G\alpha_o$	Switch II NKXD motif	Switches nucleotide specificity to XTP (in background of Q205L)	—	[80]
A366S	$G\alpha_s$ $G\alpha_{i1}$	β6-α5 loop	Increases GDP release rate, decreases thermostability	$G\alpha_{i1}$(A326S), 1BH2	[63,64]
T325A V328A F332A	$G\alpha_t$	α5 (C-terminal helix)	Increases GDP release	—	[62]

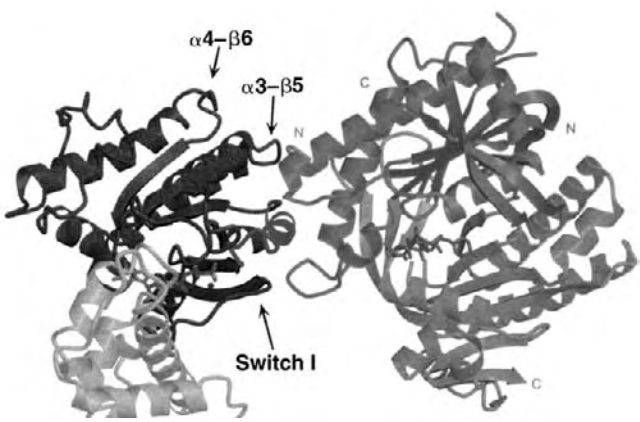


Figure 4 Schematic of $G\alpha_s$•Mg^{2+}•GTPγS bound to the catalytic domains of adenylyl cyclase. The Ras-like domain of $G\alpha_s$ is rendered in charcoal and Switch II is red. The C1 and C2 domains of type V and type II AC, respectively, are tan and magenta. (Adapted from Tesmer, J. J. G. *et al.*, *Science*, 278, 1907–1916, 1997.)

GTP Hydrolysis by Gα and Its Regulation by RGS Proteins

Gα proteins hydrolyse GTP with a slow catalytic rate of approximately 0.05 s^{-1} at physiological temperature. Because Gα•GDP binds effectors with less affinity than Gα•Mg•GTP, the rate of hydrolysis determines the lifetime of the signal, as measured by the output of activated effector. The origin of the kinetic barrier may be deduced from the structure of the complex between Gα bound to GDP and magnesium fluoroaluminate (Mg^{2+}•AlF_4^{-1}) [13]. Fluroaluminate (AlF_3) and its hydrates mimic the γ phosphate of GTP [23] and in the presence of GDP promote the activated state of Gα [24]. Structural studies of $G\alpha_{i1}$ and the $G\alpha_t$•GDP•Mg•AlF_4^{-1} complexes demonstrate that AlF_4^{-1} forms a hexacoordinate complex with a β phosphate oxygen of GDP and a water molecule (the presumptive nucleophile) as axial ligands, thereby approximating the pentacoordinate transition state for phosphorolysis [13,25]. The structures show that, relative to the ground state (Fig. 2) the Switch I arginine and Switch II glutamine must be substantially reoriented in order to stabilize the transition state. It is therefore possible that a conformational rearrangement within the active site corresponds to the kinetic barrier to GTP hydrolysis.

In some cellular contexts (for example, regulation of adenylyl cyclase by $G\alpha_s$), the rate of signal termination may indeed correspond to the intrinsic GTPase rate of the regulatory Gα. However, it is clear that other physiological responses decay much more rapidly following agonist withdrawal (for example, visual recovery after a light flash or deactivation of G protein-regulated K channels.) Rapid signal termination is achieved by RGS (regulator of G protein signaling) proteins, a family of proteins that have in common a homologous stretch of ≈120 amino acids termed the RGS-box [26]. RGS domains function as GTPase-activating proteins (GAPs) for Gα subunits [27,28]. Rate enhancement conferred by RGS ranges from 5- to 10-fold (for RGS9 regulation of $G\alpha_t$ [29]) to well over 50-fold (for RGS4 stimulation of$G\alpha_{i1}$ [30]). RGS proteins show varying degrees of specificity towards their Gα substrates [26], and most

Gα subunits including $G\alpha_s$ [31] are known to be subject to the action of one or more RGS proteins. Biochemical and crystallographic analysis indicate that RGS domains stabilize a conformation of Gα that promotes binding of GDP•Mg•AlF_4^{-1} [32–34]. GAP activity and Gα recognition is achieved by specific interactions between the surface of the RGS domain and the three switch segments of Gα (Fig. 5). In contrast to certain Ras-family GAPs, RGS proteins do not supply catalytic residues to the catalytic site of Gα, but rather stabilize the catalytically competent conformation [35].

Effectors may contain domains that exhibit GAP activation toward Gα. A C-terminal dimerization and binding region within phospholipase Cβ isoforms comprises part of a domain that expresses this activity [36–38] but is evidently not an RGS domain. A recently discovered effector of $G\alpha_{13}$ [7], p115RhoGEF, contains a domain with remote sequence and strong structural homology to RGS domains [39,40] but requires structural elements outside of the RGS box for GAP activity [41]. Such domains, unique to the p115RhoGEF family, have been termed rgRGS modules.

Although RGS domains bind to Gα Switch regions, they do not in general compete with the binding of effectors. PDEγ binding is positively cooperative with that of RGS9 to $G\alpha_t$ [42], whereas it inhibits binding of RGS7 [43]. In their interactions with $G\alpha_{i1}$ and $G\alpha_t$, RGS domains interact with Switch I and the N-terminal half of Switch II; these are adjacent to but do not overlap effector contact surfaces [17]. Small structural changes that accompany formation of the ternary complex between $G\alpha_{i1}$, PGEγ, and the RGS9 suggest a structural basis for cooperative binding [20]. RGS proteins are therefore able to terminate signaling without the requirement for Gα-effector dissociation. *In vivo*, tight spatial coupling among Gα, RGS, effector, and receptor could generate a high steady-state rate of GTPase activity and continual effector activation as long as GPCR agonist is present [26].

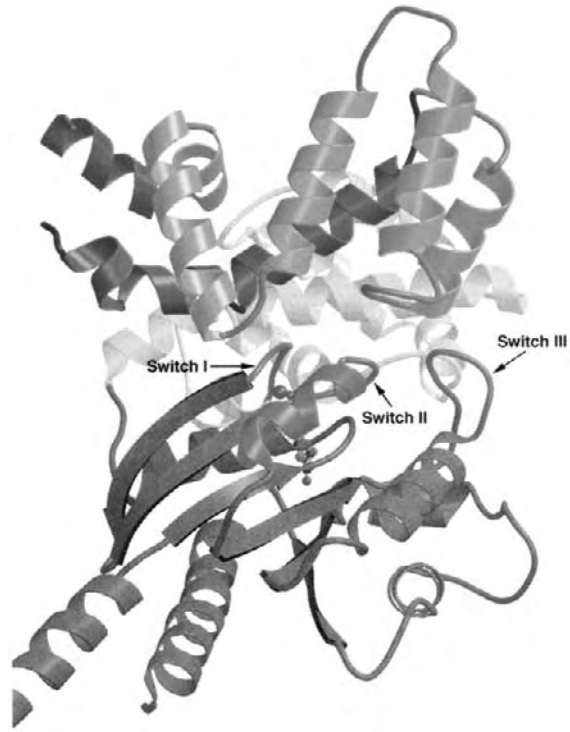


Figure 5 The complex of the RGS domain from RGS4 bound to the Ras-like domain of GDP•Mg^{2+}•AlF_4^{-1} activated $G\alpha_{i1}$. (Adapted from Tesmer, J. J. G. *et al.*, *Cell*, 89, 251–261, 1997.)

Gβγ Dimers

Upon GTP hydrolysis, the affinity of $G\alpha_s$ for AC is reduced about 10-fold [44]. The most potent factor in signal termination may be the high affinity of Gβγ for Gα•GDP. Gβγ binds to the effector-binding surface of Gα but requires a conformation of Switch II that cannot be attained in the GTP-bound state. This nonsignaling state of Gα is stable in the presence of GDP and exhibits high affinity for Gβγ [45]. Receptor-catalyzed exchange of GDP for GTP also causes full or partial dissociation from Gβγ. When not bound to Gα, Gβγ subunits are able to regulate other effectors such as inward-rectifying potassium channels [46] and phospholipase Cβ isoforms [6]. Thus, receptor-activation of a G protein heterotrimer releases two regulatory species that can act independently or coordinately on downstream effectors. Five closely related Gβ subunits have been described, together with 12 isoforms of Gγ.

Gβ subunits are toroidal structures, consisting of seven four-stranded antiparallel β-sheets, each projecting like the blades of a propeller from the central axis of the molecule (Fig. 6) [45,47]. The seven-fold symmetry is reflected in the amino acid sequence of Gβ, which is composed of seven so-called WD (or WD40) repeats, represented by the consensus sequence $[GHX_{3-5}\Phi_2X\Phi X\Phi X_{5-6}\Phi(S/T)(G/A)X_3DX_4WD]$, where X is any residue, Φ denotes a hydrophobic residue, and parentheses enclose alternate possibilities [2]. The sequence repeat is staggered with respect to the structural repeat (one propeller "blade") such that the first β-strand within the WD motif corresponds to the last β strand of the *n−1*th blade, and the following three β strands of the WD constitute the first three strands of the *n*th blade. This construction ensures a lap-joint in which the N- and C-terminal strands of the propeller are hydrogen-bonded to each other. The first of the two Asp residues in the motif is invariant and participates in a hydrogen-bonded network with the His, Ser/Thr, and Trp residues in most of the blades. The first ≈40 residues of Gβ, preceding the seven-bladed propeller, are folded into an α helix. The Gγ subunit is an extended molecule consisting of three α-helical segments that do not contact each other. Gγ subunits are farnesylated ($G\gamma_1$, $G\gamma_{11}$) or geranylgeranylated (all others) at their C termini, thereby tethering them to the plasma membrane and promoting high-affinity interactions between Gβγ and Gα subunits and with effectors (see references in [2]). The N-terminal helix of Gγ forms a parallel coiled-coil with that of Gγ. The second and third helices lie over the surface of the Gβ torus that is formed by the AB and CD β-strands of each propeller. This surface contains two hydrophobic pockets, located

between successive propellers, that accept nonpolar residues from the end of Gγ helix 3 and the succeeding loop region [48,49]. The Gγ binding surface is highly conserved among the five isoforms of Gβ. The limited selectivity between Gβ and Gγ isoforms seems to involve the interaction of hydrophobic residues in helix 2 of Gγ with its binding surface on Gβ. Gβ5 is the most divergent among the five isoforms of Gβ. It has recently been shown to interact most strongly with the G-gamma-like domains (GGLs) present in a variety of proteins, most notably the members of the RGS11 family [50,51].

Gα binds to the surface of Gβ opposite that to which Gγ is bound (Fig. 7). Gα interacts with Gβ at two distinct and separate surfaces, both of which are required for high-affinity binding. The N-terminal helix of Gα contacts the side of the Gβ torus at blade 1. The Ras-like domain of Gα binds Gβ at Switch I and Switch II. All of the residues of Gβ that contact Gα are conserved among Gβ isoforms. Although the Gβ-binding residues within the Ras domain of Gα are well conserved, the N-terminal helix of Gα is more variable, and this might confer some degree of conformational specificity to Gα–Gβ interactions. The orientation of this helix with respect to Gβ1 differs in complexes with $G\alpha_{i1}$ and $G\alpha_t$. A series of mutagenesis studies have demonstrated that several effectors of Gβγ, including PLCβ2, β-adreneric receptor kinase, type II adenylyl cyclase, G-protein-regulated inward rectifying potassium channels (GIRK), and the calcium channel α1B subunit, all contact Gβγ at the same molecular surface to which Gα subunits binds [52].

GPR/GoLoco Motifs

Recently, a diverse group of proteins containing 25-30 residue GPR (G-protein regulatory) [53,54], or GoLoco [55] motifs were shown to inhibit, like Gβγ, the dissociation of GDP from $G\alpha_{i1}$ and $G\alpha_o$. GPR/GoLoco motifs bind only to the GDP-bound Gα isoforms and also inhibit binding of Gγβ [53,56,57]. The GPR/GoLoco repeat from RGS14 adopts an extended conformation when bound to $G\alpha_{i1}$•GDP, forming contacts with Switch II and crossing the gap between the Ras-like and helical domains. A conserved arginine residue from GoLoco engages the GDP β-phosphate, thus stabilizing the nucleotide within the catalytic site. GPR-containing molecules provide a mechanism for receptor-independent activation of Gβγ signaling pathways, while inhibiting reactivation of $G\alpha_{i1}$.

Gα–GPCR Interactions

Although the mechanism by which receptors activate G proteins is beyond the scope of this review, some mention of Gα–GPCR recognition is in order. The structure of one GPCR, rhodopsin, has been reported, in an inverse-agonist-bound (i.e., resting) state [58]. The structural basis of the heterotrimer–receptor interaction is known only from extensive mutagenesis and cross-linking studies [3,59–61], particularly of the rhodopsin–$G\alpha_t$ interface. These studies point to the α4–β6 loop and the C-terminal helix, α5, of $G\alpha_t$. Mutations of residues located at the inward face of the α5 helix of $G\alpha_t$ dramatically increase receptor-independent rates of nucleotide release [62]. Mutation of a conserved alanine residue within the purine-contacting α4–β6 loop



Figure 6 The complex of $G\beta\bullet\gamma_2G\beta_1$ is colored yellow, except for the second (from the N-terminus) WD repeat, which is rendered in orange. The four-stranded antiparallel β "blades" that comprise the propeller fold are numbered. Individual strands in one repeat are lettered (a) through (d), in order of sequence. The Gγ subunit is green. The amino termini of both subunits are labeled.



Figure 7 The complex of $G\alpha_{i1}$•GDP with $G\beta_1\gamma_2$. $G\beta_1$ and $G\gamma_2$ are colored as in Figure 6. The sidechain of tryptophan 99 in $G\beta_1$, shown as a stick model, is prominent in the interface with $G\alpha_{i1}$, which is rendered in charcoal. The switch regions of $G\alpha_{i1}$ are red, and GDP is shown as a ball-and-stick model.

preceding α5 increases the intrinsic nucleotide exchange rate and also reduces the thermostability of $G\alpha_{i1}$ [63,64]. Some residues that affect receptor coupling are located in the Gα–Gβ interface but distant from the putative receptor binding surface, suggesting that Gβγ plays a direct role in GPCR-mediated nucleotide exchange.

The mechanism by which GPCRs catalyze nucleotide exchange from Gα remains one of the more puzzling mysteries in the structural biology of signaling. Crystal structures of receptors bound to heterotrimeric G proteins, in a spectrum of functional states, will eventually be determined and will provide some, but probably not complete, insight into receptor function. Equally important is the need to understand the organization and dynamic behavior of G protein signaling complexes at the cell membrane. The tools required for such investigations are still being developed (see http://www.cellularsignaling.org/).

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SECTION B

Vertical Receptors

Henry Bourne, Editor

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CHAPTER 23

Structure and Function of G-Protein-Coupled Receptors: Lessons from the Crystal Structure of Rhodopsin

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Introduction

The crystal structure of rhodopsin was recently solved at 2.8-Å resolution. As a prototypical seven-helical G-protein-coupled receptor (GPCR), rhodopsin has provided significant insights toward defining structure–activity relationships among other related receptors. In particular, many advances in understanding the molecular mechanism of receptor activation and how an active receptor catalyzes the exchange of guanine nucleotides on heterotrimeric G proteins have been suggested from biochemical and biophysical studies of rhodopsin and expressed rhodopsin mutants. The report of a high-resolution crystal structure of rhodopsin now provides new opportunities to understand how GPCRs work. For example, the ligand-binding pocket of rhodopsin is remarkably compact, and several apparent chromophore–protein interactions were not predicted from extensive mutagenesis or spectroscopic studies. The transmembrane helices are interrupted or kinked at multiple sites. An extensive network of interhelical interactions stabilizes the ground state of the receptor. The helix movement model of receptor activation, which might apply to all GPCRs in the rhodopsin family, is supported by several structural elements that suggest how light-induced conformational changes in the ligand-binding pocket are transmitted to the cytoplasmic surface. Future high-resolution structural studies of rhodopsin and other GPCRs will form a basis to elucidate the detailed molecular mechanism of GPCR-mediated signal transduction.

Introduction to Rhodopsin: a Prototypical G-Protein-Coupled Receptor

Rhodopsin (Rho) is a highly specialized G-protein-coupled receptor (GPCR) that detects photons in the rod photoreceptor cell. Within the superfamily of GPCRs that couple to heterotrimeric G proteins, Rho defines the so-called family A GPCRs, which share primary structural homology [1–3]. Rho shares a number of structural features with other GPCRs, including seven transmembrane segments (H1 to H7) (Fig. 1). In visual pigments, a Lys residue that acts as the linkage site for the chromophore is conserved within H7 in all pigments, and a carboxylic acid residue that serves as the counterion to the protonated, positively charged Schiff base is conserved within H3. The position analogous to the Schiff base counterion is one helix turn away from the position of an Asp residue conserved in biogenic amine receptors that serves as

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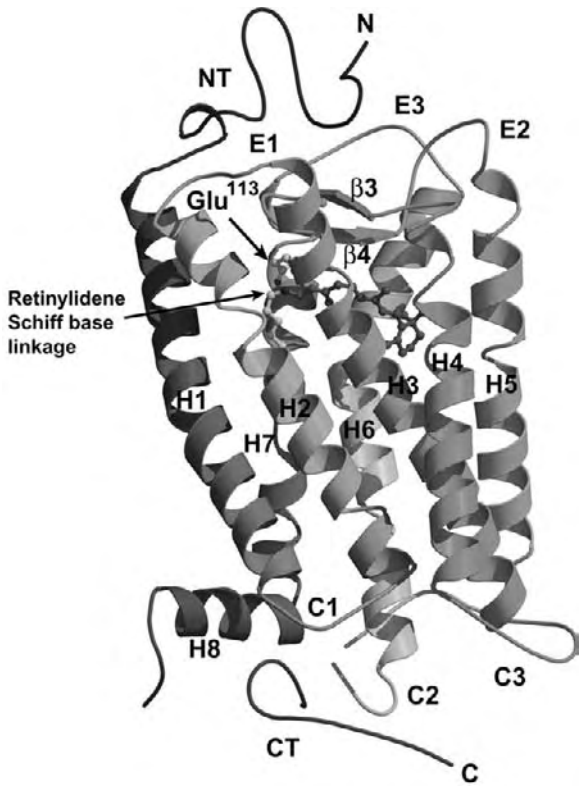


Figure 1 A molecular graphics ribbon diagram of Rho prepared from the 2.8-Å crystal structure coordinates (PDB 1f88). The amino terminus (N) and extracellular surface is toward the top of the figure and the carboxyl terminus (C) and intracellular surface is toward the bottom. Seven transmembrane segments (H1 to H7), which are characteristic of GPCRs, are labeled. The RET chromophore is shown in magenta, and the side chain of Glu-113 and the retinylidene Schiff base linkage are shown to highlight the orientation of the chromophore in the binding pocket. The Schiff base imine nitrogen is labeled. The Rho crystal structure does not resolve a small segment of the C3 loop linking H5 and H6 or a longer segment of the carboxyl-terminal tail distal to H8. The α-helical transmembrane segments are tilted with respect to the presumed plane of the membrane bilayer, and they contain significant kinks and irregularities.

the counterion to the cationic amine ligands. A pair of highly conserved Cys residues is found on the extracellular surface of the receptor and forms a disulfide bond. A Glu(Asp)/Arg/Tyr(Trp) tripeptide sequence is found at the cytoplasmic border of H3. This sequence is conserved in family A GPCRs and has been shown to be involved in G-protein interaction [4,5].

Molecular Structure of Rhodopsin

The extracellular surface domain of Rho is comprised of the amino-terminal tail (NT) and three interhelical loops (E1, E2, and E3) (Fig. 1) [6]. There is significant secondary structure in the extracellular domain and several intra- and inter-domain interactions. The E2 loop is extremely interesting in that it is folded deeply into the core of the membrane-embedded region of Rho. In addition to contacts with the chromophore (11-*cis*-retinol), E2 forms extensive contacts with other extracellular regions. The β3 and β4 strands, which arise from E2, run anti-parallel. The β4 strand is situated more deeply within the membrane-embedded region of Rho than the β3 strand. The β4 strand is adjacent to the chromophore and forms the extracellular boundary, or roof, of the ligand-binding pocket. A disulfide bond between Cys-110 and Cys-187, which forms the extracellular end of H3, is highly conserved among all class A GPCRs.

More than one-half of the 348 amino acid residues in Rho make up the seven transmembrane segments (H1 to H7) included in the membrane-embedded domain. The crystal structure of this domain is remarkable for a number of kinks and distortions of the individual transmembrane segments, which are otherwise generally α-helical in secondary structure. Many of these distortions from idealized secondary structure were not accounted for in molecular graphics models of Rho based on projection density maps obtained from cryoelectron microscopy [7]. H7 is the most highly distorted of the seven transmembrane helical segments. There are kinks at two Pro residues, Pro-291 and Pro-303. In addition, the helix is irregular around the region of residue Lys-296, which is the chromophore attachment site. Pro-303 is a part of the highly conserved Asn/Pro/X/X/Tyr motif (Asn-302/Pro-303/Val-304/Ile-305/Tyr-306 in Rho).

The membrane-embedded domain of Rho is also characterized by the presence of several intramolecular interactions that may be important in stabilizing the ground state structure of the receptor. One of the hallmarks of the molecular physiology of Rho is that it is essentially silent biochemically in the dark. The bound chromophore serves as a potent pharmacological inverse agonist to minimize activity. The Rho structure reveals numerous potentially stabilizing intramolecular interactions, some mediated by the chromophore and others arising mainly from interhelical interactions that do not involve the chromophore-binding pocket directly. For example, a complex H-bond network appears to link H6 and H7. The key interaction here is between Met-257 and Asn-302. The precise functional importance of the highly conserved Asn/Pro/X/X/Tyr motif (Asn-302/Pro-303/Val-304/Ile-305/Tyr-306 in Rho) is unclear. However, one key structural role is to mediate several interhelical interactions. The side chains of Asn-302 and Tyr-306 project toward the center of the helical bundle. The hydroxyl group of Tyr-306 is close to Asn-73 (cytoplasmic border of H2), which is also highly conserved. A key structural water molecule may facilitate an H-bond interaction between Asn-302 and Asp-83 (H2). A recent mutagenesis study of the human platelet-activating factor receptor showed that replacement of amino acids at the positions equivalent to Asp-78 and Asn-302 in Rho with residues that could not H bond prevented agonist-dependent receptor internalization and G-protein activation [8].

The 11-*cis*-retinol chromophore is a derivative of vitamin A_1, with a total of 20 carbon atoms (Fig. 2). The binding site of the chromophore lies within the membrane-embedded

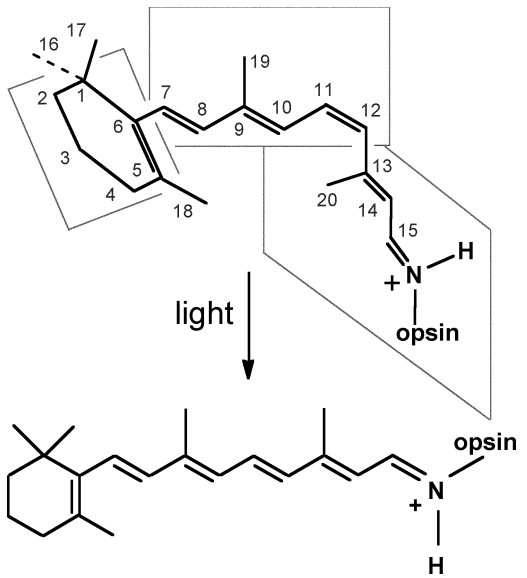


Figure 2 Photoisomerization of the 11-*cis*-retinylidene chromophore (RET) to its 11-*trans* form is the only light-dependent event in vertebrate vision. The RET chromophore is a derivative of vitamin A_1 with a total of 20 carbon atoms. The structure of the chromophore in rhodopsin appears to be 6s-*cis* 11-*cis* 12s-*trans* 15-*anti*-retinylidene protonated Schiff base. The planar surfaces are meant to depict the twists about the C-6–C-7 and C-12–C-13 bonds. Photoisomerization in Rho occurs on an ultrafast time scale, with photorhodopsin as the photoproduct formed on a femtosecond time scale [23]. The photolyzed pigment then proceeds through a number of well-characterized spectral intermediates. As the protein gradually relaxes around 11-*trans* RET, protein–chromophore interactions change and distinct λ_{max} values are observed. Important photochemical properties of Rho in the rod cell disc membrane include a very high quantum efficiency (≈ 0.67 for Rho versus ≈ 0.20 for RET in solution) and an extremely low rate of thermal isomerization.

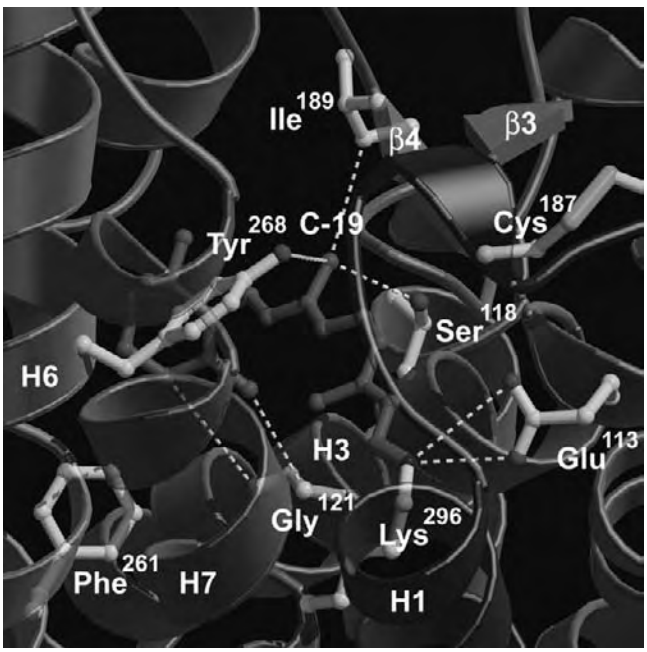


Figure 3 The RET chromophore-binding pocket of bovine Rho. The RET chromophore-binding pocket is shown from slightly above the plane of the membrane bilayer looking between transmembrane segments H1 and H7. Several amino acid residues are labeled, including the Schiff base counterion Glu-113. At least three residues appear to interact with the C-19 methyl group of the chromophore: Ser-118, Ile-189, and Tyr-268. The C-19 methyl group might provide a key ligand anchor that couples chromophore isomerization to protein conformational changes. Some additional key amino acid residues are labeled, including the Cys-187, which forms a highly conserved disulfide bond with Cys-110.

domain of the receptor (Fig. 3). All seven transmembrane segments and part of the extracellular domain contribute interactions with the bound chromophore. The chromophore is located closer to the extracellular side of the transmembrane domain of the receptor than to the cytoplasmic side. Glu-113 serves as the counterion for the Schiff base attraction of the chromophore to Lys-296. In all, at least 16 amino acid residues are within 4.5 Å of the chromophore: Glu-113, Ala-117, Thr-118, Gly-121, Glu-122, Glu-181, Ser-186, Tyr-191, Met-207, His-211, Phe-212, Phe-261, Trp-265, Tyr-268, Ala-269, and Ala-292. The most striking feature of the binding pocket is the presence of many polar or polarizable groups to coordinate an essentially hydrophobic ligand.

The cytoplasmic domain of Rho is comprised of three cytoplasmic loops and the carboxyl-terminal tail: C1, C2, C3, and CT. Loops C1 and C2 are resolved in the crystal structure, but only residues 226 to 235 and 240 to 246 are resolved in C3. CT is divided into two structural domains. C4 extends from the cytoplasmic end of H7 at Ile-307 to Gly-324, just beyond two vicinal Cys residues (Cys-322 and Cys-323), which are posttranslationally palmitoylated. The remainder of CT extends from Lys-325 to the carboxyl terminus of Rho at Ala-348. The crystal structure does not resolve residues 328 to 333 in CT.

A number of cytoplasmic proteins are known to interact exclusively with the active state of the receptor (R*). Because the crystal structure depicts the inactive Rho structure that does not interact significantly with cytoplasmic proteins, the structure can provide only indirect information about the relevant R* state. Perhaps the most extensively studied receptor–G-protein interaction is that of bovine Rho with G_t. Detailed biochemical and biophysical analysis of the R*–G_t interaction has been aided by mutagenesis of the cytoplasmic domain of bovine Rho. Numerous Rho mutants defective in the ability to activate G_t have been identified. Several of these mutant receptors were studied by flash photolysis [9], light-scattering [10], or proton-uptake assays [11]. The key overall result of these studies is that C2, C3, and H8 are involved in the R*–G_t interaction.

H8 is a cationic amphipathic helix that may bind a phospholipid molecule, especially a negatively charged phospholipid such as phosphatidylserine. In fact, spectroscopic evidence has been reported to show an interaction between Rho and a lipid molecule that is altered in the transition of Rho to metarhodopsin II, the spectrally defined form of R* [12]. H8 points away from the center of Rho, and the area of the membrane surface covered by the entire cytoplasmic surface domain appears to be roughly large enough to accommodate G_t in a one-to-one complex.

Molecular Mechanism of Receptor Activation

Although the crystal structure of Rho does not provide direct information about the structure of R* or about the dynamics of the Rho to R* transition, it does provide a wealth of information that should help to design experiments using existing methods to address specific questions regarding the molecular mechanism of Rho activation. An inactive receptor conformation must be capable of changing to an active conformation which catalyzes nucleotide exchange by a G protein. In Rho, the chromophore is in its "off" state, but switches to the "on" state 11-*trans* geometry by photoisomerization, which leads to the R* conformation of the receptor. Recent studies have suggested that steric and/or electrostatic changes in the ligand-binding pocket of Rho may cause changes in the relative disposition of transmembrane (TM) helices within the core of the receptor. These changes may be responsible for transmitting a "signal" from the membrane-embedded binding site to the cytoplasmic surface of the receptor. Trp mutagenesis [13], mutagenesis of conserved amino acid residues on H3 and H6 [14,15], and the introduction of pairs of His residues at the cytoplasmic borders of TM helices to create sites for metal chelation [16] have recently provided insights regarding the functional role of specific helix–helix interactions in Rho. These results indicated a direct coupling of receptor activation to a change in the spatial disposition of H3 and H6. This could occur if movements of H3 and H6 were coupled to changes in the conformation of the connected intracellular loops, which are known to contribute to binding surfaces and tertiary contacts of Rho with G_t.

More direct evidence for changes in interhelical interactions upon receptor activation were provided by extensive site-directed spin labeling and electron paramagnetic resonance (EPR) spectroscopy studies of the transition of Rho to R* in modified, or expressed, mutant pigments. The results suggested a requirement for rigid body motion of transmembrane helices, especially H3 and H6, in the activation of Rho [17]. A slight reorientation of helical segments upon receptor activation is also supported by experiments using polarized attenuated total reflectance infrared difference spectroscopy [18]. Finally, movement of H6 was also detected by site-specific chemical labeling and fluorescence spectroscopy [19]. The structural rearrangement of helices upon activation might not result in an R* structure that is drastically different from that of Rho as an engineered receptor with four disulfide bonds (between the cytoplasmic ends of H1 and H7, and H3 and H5, and the extracellular ends of H3 and H4, and H5 and H6) was still able to activate G_t [20].

Because the arrangement of the seven transmembrane segments is likely to be evolutionarily conserved among the family of GPCRs, the proposed motions of H3 and H6 may be a part of a conserved activation mechanism shared among all receptor subtypes [21,22]. In other class A GPCRs, agonist ligand binding would be coupled to a change in the orientations of H3 and H6.

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CHAPTER 24

Human Olfactory Receptors

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Olfaction, the sense of smell, is a versatile and sensitive mechanism for detecting volatile odorous molecules [1,2]. It is mediated by hundreds of olfactory receptor (OR) proteins in the membrane of the chemosensory neurons, extended by the formation of long cilia. Odorant binding initiates a cascade of signal transduction events that involve a G-protein-dependent elevation of cAMP second messenger and opening of cAMP-gated ion channels [3]. The olfactory system utilizes a combinatorial receptor-coding scheme to discriminate different odorants [4–7]. A specific odor is recognized as a pattern of saturation values, which may be viewed as an "activity vector" generated across an array of ORs. As each sensory neuron expresses only one OR gene (in fact, a single allele thereof) [8], the pattern of receptor activation is faithfully represented in an array of cellular activities, as conveyed to the central nervous system.

In humans, a repertoire of more than 1000 OR genes has been elucidated by cloning and genomic data mining [9–12]. Genes of this "olfactory subgenome", the largest subgroup within the G-protein-coupled receptor (GPCR) hyperfamily, are disposed in dozens of clusters on most human chromosomes. This genomic disposition is accounted for by an elaborate process of gene and cluster duplication, as well as gene conversion events [13,14]. Approximately two-thirds of all human ORs are pseudogenes that have accumulated up to 27 frame-disrupting mutations [9,15]. Such an observation is consistent with the diminished importance of the sense of smell in primates, including humans. The OR subgenome consists of 17 gene families [16,17], which belong to either class I (fish-like) or class II (tetrapod-specific) receptors. Interestingly, the proportion of intact genes is greater among class I receptors, suggesting they have greater functional importance.

The genetics of ORs has only begun to be elucidated. In humans, it is commonly believed that OR polymorphisms underlie the widespread inter-individual variations in odorant threshold [18]. This is in line with genetic models developed in other species [19–21]. Providing direct evidence for this notion by genotype–phenotype correlations in humans constitutes an important future challenge that may involve the identification of pseudogenes present only in certain individuals [22].

As is the case for most integral membrane proteins, the three-dimensional structure of ORs has not yet been determined. Therefore, scientists have resorted to alternative methods of structure prediction. These include homology modeling [23,24] based on the structure of bovine rhodopsin, the only GPCR for which crystallographic structural information is available (see Chapter 22). This approach is rendered more valid because both ORs and rhodopsin belong to class A GPCRs [25]. Homology modeling was performed despite the marginal sequence similarity between ORs and rhodopsin ($\approx$21% amino acid identity over most of the protein sequence) and was greatly aided by the occurrence of sequence motifs common to the two sets of proteins (Fig. 1). Such shared features include the overall seven-helix structure, the conserved extracellular cysteine bridge between the first and second extracellular loops, the (D/E)RY motif in the transition zone between the third transmembrane helix and second intracellular loop, the SY motif in transmembrane 5 (TM5), and the NP motif in TM7 [10,26].

Variability analysis identified 17 hypervariable residues which point to a putative odorant binding pocket formed by TM3 to TM6; these residues were proposed to form the complementarity-determining regions (CDRs). The use of enhanced variability as a criterion for functional importance is analogous to an approach originally proposed for immunoglobulins [26]. Additional information on potential functional residues is provided by correlated mutation analysis [27] and by comparisons of variability patterns in orthologous and paralogous sequences ([28]; Man, Gilad, and Lancet, unpublished work).

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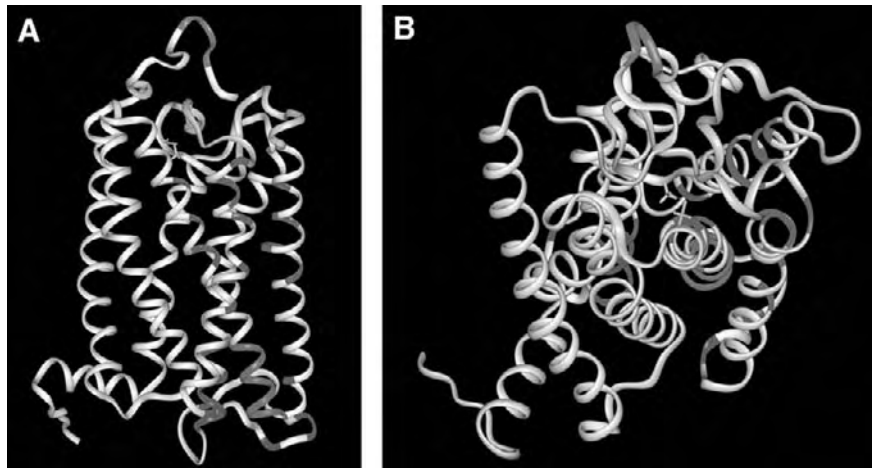


Figure 1 A three-dimensional homology model of an OR protein (OR1E1) using the structure of bovine rhodopsin ([39]; PDB code 1F88). As a template, in side view (A, extracellular at the top), and in a frontal view into the putative binding site from the extracellular side (B). Functionally important features are shaded and include the following: the conserved MAYDRYVAIC motif in the end of TM 3; exceptionally conserved residues in the second and third intracellular loops; the putative disulfide bond between the first and second extracellular loops; the *N*-glycosylation site near the amino terminus; the second extracellular loop that covers the binding site; the 17 putative CDRs [26].

Functionally important residues were also highlighted using sequence conservation analysis (Fig. 1). In particular, ten sequence motifs concentrated in either the extracellular-most or intracellular-most parts of the receptors have been identified [26,29]. It was suggested that these motifs are involved in the interactions of the receptors with their signaling partners upstream (e.g., odorant-binding protein, OBP, [30]) or downstream (olfactory GTP-binding protein [31]). While many GPCRs contain conserved palmitoylation sites in their carboxy-terminal region (such as rhodopsin; see Chapter 22), which anchor them to the membrane, only 26% of intact human ORs contain such sites [10].

The second extracellular loop of ORs has attracted particular attention. It is comparatively long, and, in addition to the cysteine residue conserved in all GPCRs, it contains two conserved cysteines, perhaps forming an internal disulfide bond [10,32]. This loop also has a relatively high variability, and a correlation was found between residues within it and those in the putative binding pocket [27]. This might suggest that the loop acts as an auxiliary recognition domain, reflecting the unique specificity of the odorant-binding pocket [33]. The finding of OR mRNA in the axon terminals of olfactory receptor neurons within their target glomerular synaptic complex [34,35] led to the hypothesis [33] that the second extracellular loop participates in the guidance of olfactory neuronal axons [36].

Functional expression studies have the potential to identify the ligands that activate each receptor. Such studies have been hindered by the apparent failure of the transfected OR proteins to translocate efficiently to the plasma membrane [19]. Solutions to this problem have included *in vivo* infection with OR-containing viral vectors in rat olfactory epithelium [37]; expression of chimeric receptors containing rhodopsin sequences in a heterologous cell system, in conjunction with a promiscuous G protein [19]; and expression in oocytes [19]. A combination of calcium imaging and single-cell reverse transcription–polymerase chain reaction (RT-PCR) analysis has also been used to identify receptors that recognize specific odorant molecules and to elucidate a combinatorial code [5]. One study led to a relatively comprehensive elucidation of the odorant-binding characteristics of one OR protein, I7 [38]. Comparison of the highly homologous mouse and rat I7 receptors [19] resulted in the identification of a one-residue substitution, V206I, responsible for a shift in ligand binding preference from octanal in rat to heptanal in mouse. This residue resides in the extracellular region of transmembrane segment five in the vicinity of residues previously predicted to confer specificity, but points away from the homology-modeling-based proposed binding site, a point that will require additional scrutiny.

Future studies should involve a considerable augmentation of ligand–receptor relationships in the olfactory system in mammals. This should include improved protein expression methodologies, as well as genetic studies that would link olfactory sensitivity phenotypes to OR genotypes.

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CHAPTER 25

Chemokines and Chemokine Receptors: Structure and Function

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Introduction

The name *chemokine* is derived from "chemotactic cytokine" and the hallmark activity of chemokines is chemotaxis, the ability to induce directed cell movement. Chemokines are encoded by a large gene family with at least 45 members. The receptors for chemokines also belong to a gene family with at least 18 members and all are G-protein-coupled receptors. The sequence similarities found in the chemokine gene family are reflected in their similar three-dimensional structures; however, chemokines display a diverse range of activities. Chemokines were originally identified as potent leukocyte attractants involved in inflammatory disease. More recently, they have been found to play critical roles in the natural development and regulation of the immune system. In addition, chemokines and their receptors have been utilized by pathogens to subvert the host immune system. This review will explore the many functions of this important family of immune modulators.

Chemokine Structure and Function

Although their discovery has been relatively recent (mostly within the past 15 years), chemokines and their receptors have rapidly become appreciated for their impact on health and disease. They are involved in a broad variety of natural biological processes, including development, inflammation, immunity, and angiogenesis. In addition, these sequences have been corrupted by pathogens to subvert the innate and adaptive immune responses. This review provides a brief introduction to chemokine structure and activities. More detailed reviews can be found in the reference section [1–4].

Over 45 human chemokines have been characterized. The first chemokines to be identified were associated with inflammatory disease, but their discrete biologic activities were not known. Once interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), and others were shown to attract leukocytes, other proteins with similar structure were also identified with leukocyte chemotactic activity [5–8]. The earliest identified chemokines were isolated by standard protein purification techniques, including heparin affinity chromatography. Others were soon identified as induced sequences in cDNA libraries that encoded similar protein structures [9–11]. Most of the newer chemokines were found in expressed sequence tag (EST) cDNA libraries by sequence similarity, while others have been discovered in the course of sequencing the human genome. Because many laboratories and many methods have been responsible for chemokine discovery, the original chemokine nomenclature is confusing. Recently a more comprehensive nomenclature has been developed [12]. This is presented in Table 1, along with the receptors and cell types with which they interact.

Chemokines are 8- to 10-kDa proteins that share significant homology in their amino acid sequences. Chemokines share between 20 and 80% identity and are found as gene families in all species of vertebrates. The four families of chemokines have been distinguished on the basis of the relative position of their cysteine residues. The α and β chemokines contain four cysteines (sometimes six) and are the largest families. One amino acid separates the first two cysteine residues (cysteine–X amino acid–cysteine, or CXC) in the α chemokines. In the β chemokines, the first two cysteine residues are adjacent to each other (cysteine–cysteine, or CC). The other two families of chemokines contain a single member each: lymphotactin, with only two cysteines [13],

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Table I Chemokine Receptors and Ligands

Receptor	Expression	Ligands
CXCR1	Neutrophils	CXCL8 (IL-8)
CXCR2	Neutrophils	CXCL8 (IL-8), CXCL1–3 (gro-α/β/γ), CXCL5 (ENA-78), CXCL6 (GCP-2), CXCL7 (NAP-2)
CXCR3	Activated T cells (Th1)	CXCL9 (mig), CXCL10 (IP-10), CXCL11 (ITAC)
CXCR4	T cells and other leukocytes	CXCL12 (SDF-1)
CXCR5	B cells	CXCL13 (BLC, BCA-1)
CXCR6	Activated T cells	CXCL16
CCR1	Monocytes, activated T cells	CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), CCL 14–16 (HCC 1, 2, 4), CCL23 (MPIF-1)
CCR2	Monocytes, activated T cells	CCL2 (MCP-1), CCL8 (MCP-2), CCL7 (MCP-3), CCL13 (MCP-4)
CCR3	Eosinophils, basophils	CCL11 (Eotaxin), CCL24 (Eotaxin-2), CCL26 (Eotaxin-3), CCL5 (RANTES)
CCR4	T cells (Th2)	CCL22 (MDC), CCL17 (TARC)
CCR5	Macrophages, Th1 cells	CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES)
CCR6	Activated T cells, dendritic cells	CCL20 (LARC, MIP-3a)
CCR7	Naïve lymphocytes, mature dendritic cells	CCL19 (ELC, MIP-3b), CCL21 (SLC, 6Ckine)
CCR8	T cells (Th2)	CCL1 (I-309)
CCR9	Gut homing α4β7+ T cells	CCL25 (TECK)
CCR10	Skin homing CLA+ T cells	CCL27(CTACK), CCL28 (MEC)
CX_3CR1	Monocytes, microglia, T cells	CX3CL1 (Fractalkine)
XCR1	Lymphocytes	XCL1 (Lymphotactin)

and fractalkine, in which the first two cysteine residues are separated by three amino acids (CXXXC) and the chemokine domain is at the amino terminus of a membrane-bound glycoprotein (fractalkine is also unusual in its size, 95 kDa) [14].

The three-dimensional structures of many chemokines have been determined by either X-ray crystallography or nuclear magnetic resonance (NMR) (reviewed in Clore and Gronenborn [15] and Rojo *et al.* [16]). Because of their multiple cysteine residues, the structures are confined by disulfide bridges. The first amino terminal cysteine forms a disulfide bond with the third, and the second cysteine with the fourth. Because of the disulfide constraints and relatively high sequence similarity, different chemokines share quite similar structural features. As also shown by structural studies, chemokines are isolated as dimers; however, dimerization does not appear to be necessary for receptor binding and may occur most frequently at the high concentrations used for the structural studies.

The structures of chemokines are critical for function. Alteration of a single residue, especially near the amino terminus, can greatly affect activity. For example, amino-terminal modification of RANTES results in a potent receptor antagonist [17]. Several chemokines undergo natural amino-terminal proteolytic processing after secretion which can alter their activity. Platelet basic protein is inactive until processed at its amino-terminal end by monocyte proteases to form neutrophil-activating peptide 2, a potent neutrophil chemoattractant [18]. The protease CD26 removes two amino acids from macrophage-derived chemokine (MDC) [19]; this destroys its activity on CCR4 but enhances its binding to CCR5, enabling it to inhibit HIV entry. HCC-1 is found in serum at relatively high concentrations, but it is inactive until its amino-terminal end is cleaved off [20]. Limited proteolysis may be a general mechanism that allows local factors to regulate chemokine activity.

The α chemokines (with 16 human members thus far) can be divided into two functional groups. The CXC chemokines that contain the sequence glutamic acid–leucine–arginine (ELR) preceding the CXC sequence are chemotactic for neutrophils [21]. Such chemokines play an important role in acute inflammatory diseases. The other (non-ELR) group of CXC chemokines tend to act on lymphocytes. For example, IP-10 and MIG (monokine induced by interferon-γ) attract activated T cells [22], and stromal-cell-derived factor 1 (SDF-1) acts on resting lymphocytes [23]; SDF-1 also plays a critical role in cardiac and neuronal development [24,25].

There are at least 28 human β chemokines. In general, these CC chemokines attract monocytes, eosinophils, basophils, and lymphocytes with variable selectivity, but they do not attract neutrophils. The four monocyte chemoa tractant proteins and eotaxin form a subfamily for which the members are approximately 65% identical to each other [26]. As with the CXC family, the amino-terminal amino acids preceding the CC residues of β chemokines are critical for biologic activity and leukocyte selectivity [27].

Chemokines can be divided into two general classes based on whether they are induced by pro-inflammatory cytokines

or are constitutively expressed [4]. The induced chemokines are upregulated very quickly at sites of infection or trauma and control the recruitment of leukocytes to the affected area. The constitutive chemokines are generally more involved in controlling migration of leukocytes through various tissues. This allows for naïve T and B cells to encounter antigen in secondary lymphoid organs and results in their subsequent activation and differentiation. In addition, the migration of T cells in the thymus is regulated by chemokines as thymocytes proceed through development [28].

Chemokine Receptors

Chemokines induce cell migration and activation through interactions with a family of cell-surface receptors containing seven transmembrane regions [2]. Typical of all heptahelical receptors, ligand binding induces a cascade of intracellular events mediated by activation of G proteins. Early studies showed that chemokine responses were sensitive to inhibition by pertussis toxin, confirming coupling of these receptors through the G_i family of G proteins [29]. A key result of chemokine binding is the activation of phospholipase C, producing inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol [30]. Elevation of these products leads to release of Ca^{++} from intracellular stores and activation of protein kinase C. Chemokine receptor engagement can also lead to stimulation of other intracellular enzymes such as phosphatidylinositol 3-kinase [31,32], mitogen-activated protein kinase, and the ras family of GTP binding proteins [33,34,35]. These signaling events are key mediators of the ultimate cellular responses to chemokines, most notably cell migration. Different chemokines may participate in alternative downstream signaling pathways. For example, chemokines that have acute inflammatory properties ultimately activate nuclear factor κB (NF-κB) and modulate transcription of inflammatory cytokines. Other chemokines are involved in alternative signaling cascades responsible for cellular differentiation or angiogenesis [36].

The first chemokine receptors identified were the IL-8 receptors in 1991 [37,38]. Since then, a total of 18 chemokine receptors have been identified. They are divided into several classes, depending on the type of chemokine they bind (see Table 1). Six CXC receptors (CXCRs) that interact with one or more of the CXC chemokines have been identified. In addition, ten CCRs bind only CC chemokines. There are also single members in the CX3CR and XCR classes. Most of the CXCRs and CCRs interact with multiple chemokines, resulting in considerable apparent redundancy of chemokine function [1,2].

Chemokine receptors contain conserved motifs found in all members of the family of chemoattractant receptors. Certain regions in the extracellular domains have been implicated in chemokine ligand binding. The amino terminus of the receptors was found to be necessary and to confer specificity for binding to their cognate ligands. However, a region of the third extracellular loop, extending into the transmembrane domain, was also determined to be involved in high-affinity ligand binding and receptor signaling [39,40]. Most chemokine receptors have two disulfide bonds in their extracellular domains that are necessary for chemokine binding, while the majority of GPCRs have a single disulfide bridge [41]. Regions in the third intracellular loop and carboxy terminus have been shown to interact with G proteins, similar to other G-protein-coupled receptors [42]. The carboxy terminus is also rich in serine and threonine residues, which are thought to be phosphorylated, leading to interaction with arrestin to turn off the receptor signal [43]. Posttranslational modifications of the chemokine receptors include glycosylation (residues in the amino terminus and third extracellular domain) and sulfation (tyrosine residues in the amino terminus), which are necessary for high-affinity interactions with chemokine ligands [44].

Each chemokine receptor is expressed on a subset of cells and confers responsiveness of those cells to particular chemokines. Some receptors are restricted to a particular leukocyte. For example, CXCR1 is found almost exclusively on neutrophils [38]. Others are more broadly expressed, such as CXCR4. Other chemokine receptors are expressed only by a subset of cells in a certain activation state. CXCR3 for example is expressed only on activated T cells of the Th1 subtype. T cells especially seem to regulate their expression of many chemokine receptors in response to activating cytokines and other external stimuli [22]. Receptor regulation with cell activation allows for a selective amplified response to a particular antigen.

In addition to their role in cell migration, chemokine receptors are utilized by various pathogens to gain access to host cells [45]. The most striking example is HIV, which can interact with CXCR4 and CCR5, along with CD4, to infect T cells and macrophages (reviewed in Clapham and McKnight [46]). This discovery, made in the mid-1990s, has inspired great efforts to produce inhibitors of the HIV/receptor interaction as a treatment for AIDS.

While chemokines are necessary for coordinating leukocyte defense against external invaders, inflammation often occurs inappropriately and can lead to a variety of diseases [47]. Over-expression of chemokines and chemokine receptors has been reported in many conditions, leading to leukocyte accumulations in tissues. These include rheumatoid arthritis, multiple sclerosis, psoriasis, ulcerative colitis, asthma, and arteriosclerosis. In animal models for many of these diseases, chemokine inhibitors (generally blocking antibodies) have been found to prevent development of inflammatory lesions. Chemokines and their receptors have also been implicated in carcinogenesis [48].

Small molecule inhibitors of chemokine/receptor interactions are being developed for treatment of many human inflammatory diseases [49,50]. GPCRs have historically been very good targets for drug development and hold promise for success in the chemokine area. Some chemokine inhibitors that have reached human clinical trials target CCR1, CCR5, and CXCR4. Others that are in preclinical development include inhibitors of CCR2, CCR3, CCR4, CCR6, CXCR2,

and CXCR3. By targeting specific receptors, the hope is that only subsets of leukocytes involved in disease will be affected while general immune functions can still occur. The future of chemokine inhibitors looks bright, and we should soon have clinical data to confirm the potential for utilizing these inhibitors for treating disease.

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CHAPTER 26

The Binding Pocket of G-Protein-Coupled Receptors for Biogenic Amines, Retinal, and Other Ligands

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Introduction

G-protein-coupled receptors (GPCRs) represent a very large superfamily of receptors that are critical for signaling of a diverse group of ligands to heterotrimeric G proteins [1]. At least 30% of predicted potential drug targets are GPCRs [2]. Ligands for these receptors include light, odorants, tastes, small-molecule neurotransmitters, peptides, glycoprotein hormones, proteases, and others. GPCRs are composed of seven transmembrane segments, with an extracellular amino terminus and a cytoplasmic carboxy terminus. In the class A, rhodopsin-like GPCRs, the binding pocket is formed among the transmembrane segments and/or extracellular loops. The role of these receptors, as for all GPCRs, is to transmit a conformational change induced by extracellular signaling molecules from the ligand-binding pocket via the transmembrane segments to the cytoplasmic surface of the receptor and subsequently to the G protein. The conformational change produced by agonist binding facilitates the interaction of the receptor with G protein and the subsequent exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP), which results in dissociation of the G-protein heterotrimer to form activated $G\alpha$ and $G\beta\gamma$.

Despite an enormous amount of research on the structure and function of these receptors, until very recently no high-resolution structure of any GPCR was available. In 2000, the crystal structure of bovine rhodopsin was determined to 2.8 Å [3]. Based on an analysis of the consistency of previous data from a number of different GPCRs with the structure of rhodopsin, we have inferred that the overall structures of rhodopsin and of class A receptors are very similar, although we also identified localized regions where the structure of these receptors may diverge [4]. We further proposed that several of the unusual bends and twists in the transmembrane segments (TMs) of rhodopsin are also present in other GPCRs, despite the absence of amino acids that might have been thought to be critical to the adoption of these features. Thus, different amino acids or alternative microdomains can support similar deviations from regular α-helical structure, thereby resulting in similar tertiary structure. Such structural mimicry may be a mechanism by which a common ancestor could diverge sufficiently to develop the selectivity necessary to interact with diverse signals, while still maintaining a similar overall fold. The shared three-dimensional architecture of different class A GPCRs suggests that they may also share the basic mechanisms of ligand-induced conformational change. Thus, although there is no logical necessity

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that all GPCRs use the same cognate amino acid positions to bind ligand [5], as discussed below, it seems likely that many indeed do so.

With hundreds of class A receptors and more than 700 GPCRs in the human genome alone, it is important to develop a language and nomenclature that can allow scientists studying different receptors to communicate. To facilitate the comparison of aligned residues in different GPCRs, we use and advocate the indexing method introduced by Ballesteros and Weinstein [6], in which the most conserved residue in each transmembrane segment (TM) is given the index number 50. Thus, for example, the Arg in the highly conserved (E/D)RY sequence at the cytoplasmic end of TM3 is $Arg^{3.50}$, and the other residues in TM3 are indexed relative to this position, with the preceding $Asp^{3.49}$ and the subsequent $Tyr^{3.51}$. This method, which counts from the most conserved position rather than from inexact inferences of the beginning of the TMs, facilitates comparison among different GPCRs. $Arg^{3.50}$ now takes on significant meaning in any GPCR, and $Arg131^{3.50}$ in the β2-adrenergic receptor (AR) identifies not only the absolute residue number in the β2-AR sequence but also the position of the aligned residue in other GPCRs. In contrast, unless one's research focuses on these receptors, for example, Arg131 in the human β2-AR or Arg135 in bovine rhodopsin or Arg519 in the human thyrotropin receptor are meaningless residue numbers without reference to a multiple alignment and much counting of residues. The index residues in each of the TMs of bovine rhodopsin are $Asn55^{1.50}$, $Asp83^{2.50}$, $Arg135^{3.50}$, $Trp161^{4.50}$, $Pro215^{5.50}$, $Pro267^{6.50}$, and $Pro303^{7.50}$ (Fig. 1). All of these are highly conserved in aminergic receptors and rhodopsins and therefore allow unambiguous alignment of the TMs of these receptors.

The Binding Pocket of GPCRs

In this chapter, we compare the binding sites of aminergic GPCRs and the retinal-contact residues in rhodopsin. Other than rhodopsin itself, the aminergic GPCRs have been the most exhaustively studied GPCRs, although the comparison is probably relevant to many other class A receptors as well. The results of a solvent-accessible surface area (SASA) analysis of the high-resolution structure of rhodopsin in the presence and absence of retinal are shown in Table 1. The difference between these two calculated surfaces represents the surface of the binding-site crevice in rhodopsin that is protected from water by retinal. It is important to

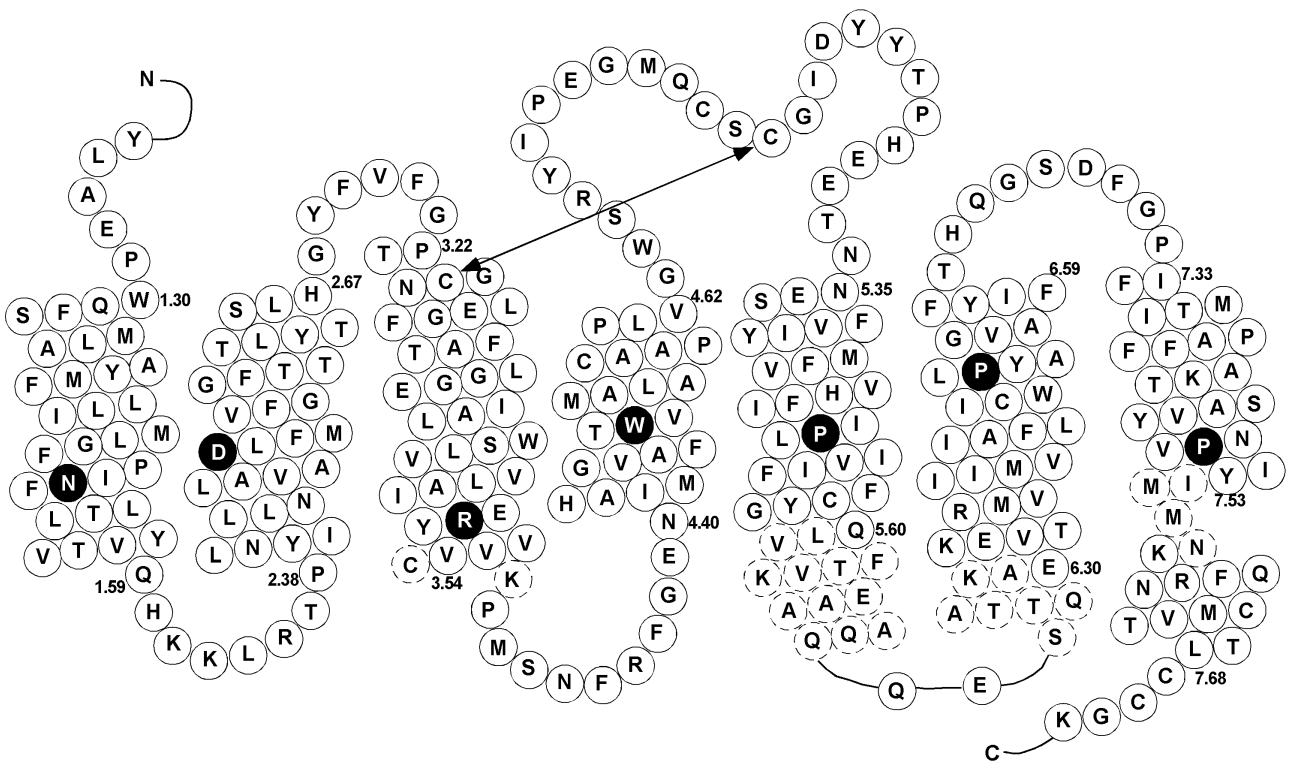


Figure 1 Helical net representation depicting the relative positions of the residues in the transmembrane domain of rhodopsin and the indexing system. The helix ends are shown as per the rhodopsin structure. TM1 is from index 1.30 (rhodopsin residue 35) to 1.59 (64), TM2 is from 2.38 (71) to 2.67 (100), TM3 is from 3.22 (107) to 3.54 (139), TM4 is from 4.38 (149) to 4.62 (173), TM5 is from 5.35 (200) to 5.60 (225), TM6 is from 6.30 (247) to 6.60 (277), and TM7 is from 7.33 (286) to 7.54 (307). The most conserved residue in each TM is $Asn55^{1.50}$, $Asp83^{2.50}$, $Arg135^{3.50}$, $Trp161^{4.50}$, $Pro215^{5.50}$, $Pro267^{6.50}$, and $Pro303^{7.50}$. Their single letter codes are shown in white on filled black circles. Residues at the cytoplasmic ends of TM5, TM6, and TM7 predicted by spin-labeling studies to be in an α-helical conformation but which are not in an α-helical conformation in the rhodopsin structure are shown with dotted circles (4).

Table I Residues in Bovine Rhodopsin Found in SASA Calculations To Be Protected by Retinal and Representative Receptors in Which the Aligned Residue Has Been Implicated in Ligand Binding

	SASA(Å^2)[a]			
Residue	NR	WR	Δ	Representative receptors
Glu113$^{3.28}$ [b]	0.5	0.0	0.5	ACM1, D2DR, NK2R
Gly114$^{3.29}$ [b]	0.2	0.0	0.2	ACTR, D2DR, GASR, MSHR, P2YR
Ala117$^{3.32}$ [b]	14.2	2.8	11.3	5H1A, A2AA, ACM1, B2AR, D2DR, ETBR, HH2R
Thr118$^{3.33}$ [b]	27.6	0.0	27.6	AA1R, ACM3, D2DR
Gly121$^{3.36}$ [b]	6.6	0.6	6.0	5H2A
Glu122$^{3.37}$	20.3	0.0	20.3	AA1R, AA2A, P2YR
Leu125$^{3.40}$ [b]	1.6	0.8	0.8	ACM1, HH3R
Cys167$^{4.56}$ [b]	0.3	0.0	0.3	B2AR, HH1R
Glu181	3.6	0.6	3.0	—
Ser186	3.2	0.0	3.2	—
Cys187	8.4	0.0	8.4	(disulfide-bonded)
Gly188	1.4	0.0	1.4	5H1D, A1AA
Ile189	3.5	0.0	3.5	A1AA
Tyr191	3.0	0.2	2.8	—
Met207$^{5.42}$ [b]	24.2	0.4	23.8	5H1A, A2AA, B2AR
Phe208$^{5.43}$ [b]	16.1	11.3	4.8	B2AR, D2DR
His211$^{5.46}$ [b]	1.4	0.7	0.7	A2AA, B2AR, D2DR
Phe212$^{5.47}$ [b]	55.0	36.6	18.4	5H2A, A1AA, D2DR
Phe261$^{6.44}$ [b]	11.9	1.5	10.4	ET1R
Trp265$^{6.48}$ [b]	42.3	2.6	39.7	5H1B, 5H2A, D2DR
Tyr268$^{6.51}$ [b]	34.6	0.0	34.6	A1AB, D2DR
Ala269$^{6.52}$ [b]	18.7	2.5	16.2	5H1B, 5H2A, B2AR
Ala272$^{6.55}$ [b]	1.0	0.2	0.8	B2AR, D2DR
Ala292$^{7.39}$ [b]	8.0	0.0	8.0	5H1A, 5H1B, A2AA, ACM1, B2AR, GASR, P2UR, P2YR
Ala295$^{7.42}$ [c]	3.5	0.2	3.3	AA1R, ACM1, AG2R, NTR1
Lys296$^{7.43}$ [b]	10.3	2.9	7.4	5H2A, AA1R, AA2A, D2DR, P2YR, PAFR,

Note: Receptor names are abbreviated according to their SWISS-PROT Annotated Protein Sequence Database entry names: http://www.expasy.ch/cgi-bin/lists?7tmrlist.text. Ser186–Ile189 forms β4 in E2 (see text).

Residue indexing: To facilitate comparison of aligned residues in related GPCRs, the most conserved residue in TMX is given the index number X.50, and residues within a given TM are then indexed relative to the "50" position [6]. The most highly conserved residues in this family are indicated in Fig. 3 in an alignment of rhodopsin with a selected group of class A GPCRs. (Note that the "50" position does not require the residue to be in the middle of the TM; for example, the highly conserved Arg$^{3.50}$ is at the cytoplasmic end of TM3 in the conserved E/D–R–Y sequence.).

[a]The calculation is based on chain A of the refined bovine rhodopsin structure (PDB code 1HZX). NR, the SASA is calculated without retinal; WR, the SASA is calculated with retinal; Δ, the difference between NR and WR. The values shown are the surface area of each residue that is exposed to solvent.

[b]Protected residues in D_2 receptor SCAM experiments [14,23–26].

[c]Accessible but protection not tested due to small effect size [26].

Source: Adapted from Table 3 of Ballesteros, J. *et al*., *Mol. Pharmacol.* 60, 1–19, 2001, which contains the references for the studies in the representative receptors, except for 3.40 [27,28] and 4.56 [29,30].

note that, in the dark, retinal is an inverse agonist, a particular type of antagonist that stabilizes the inactive state of the receptor and contributes to the very low constitutive activity of rhodopsin in the dark (see Chapter 22). Upon photoisomerization by light, retinal adopts a different configuration in the binding pocket and becomes an agonist. The high-resolution rhodopsin structure was obtained in the dark in an inactive state, and because there is no high-resolution active structure, the conformational changes involved in receptor activation have been studied using indirect methods.

The residues identified by the SASA analysis in rhodopsin are highlighted in Figs. 2 and 3A, in which retinal is shown within the binding-site crevice formed by the retinal-protected residues. The positions of these residues are remarkably consistent with those of previously identified ligand–receptor contact sites for aminergic GPCRs (Table 1), as well as with our substituted-cysteine accessibility method (SCAM) studies of the dopamine D_2 receptor (reviewed in Ballesteros *et al.* [4]). Figure 3B illustrates this agreement with the backbone of the rhodopsin structure and the side chains of the β2-AR that have been shown to interact

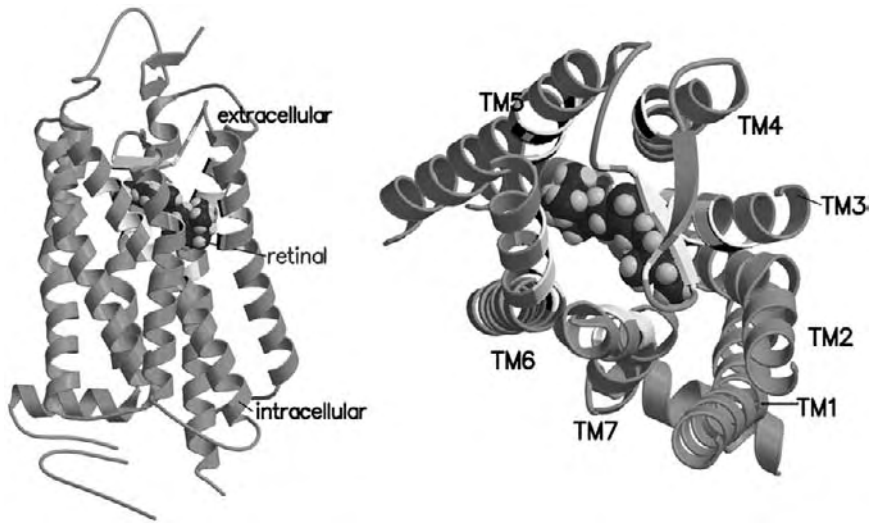


Figure 2 Schematic three-dimensional representations of the bovine rhodopsin structure. The 7 α-helical TMs are shown as green ribbons. The β-strands in the extracellular domain are indicated by large strand arrows. The positions of the residues that are in direct contact with the ligand retinal are colored yellow. The left panel shows a side view, and the right panel shows an extracellular view of the 7 α-helical TMs and E2, but with the amino terminus, E1, and E3 removed.

directly with catecholamine agonists. These residues include Asp113$^{3.32}$, which interacts with the protonated amine of biogenic amines; Ser203$^{5.42}$, Ser204$^{5.43}$, and Ser207$^{5.46}$, which interact with the *meta*-OH and *para*-OH of catecholamines; Asn293$^{6.55}$, which interacts with the β-OH of epinephrine; and Phe208$^{5.47}$, Trp286$^{6.48}$, Phe289$^{6.51}$, and Phe290$^{6.52}$, which form a cluster of aromatic residues in TM5 and TM6 that interact with the aromatic ring of ligands. Even without modification of the rhodopsin backbone, it is clear that epinephrine fits remarkably well within the binding site formed by these critical residues.

In Fig. 3C, we illustrate a β2-AR antagonist affinity label, docked within the TMs of the rhodopsin structure, with all the residues from Table 1 mutated to the aligned β2-AR residues. Again, the ligand is bound to essentially an overlapping set of the residues that contact retinal in rhodopsin. It is important to note that, although some of the residues in the binding-site crevice of these receptors are conserved, most are not. Thus, these residues have apparently evolved to impart specificity within a certain receptor. Consequently, what is "conserved" among these receptors are the positions of the residues involved in ligand binding and thus the particular surface that serves the role of ligand binding.

It seems likely that many, although certainly not all, GPCRs will share similar locations of their binding pockets. Indeed, although peptide ligands bind to extracellular loops, at least in certain cases, there is also evidence that parts of peptides dip down into the transmembrane domain and contact some of the same positions found to be critical for binding to rhodopsin and the aminergic GPCRs [7–9]. It is likely, however, that structurally dissimilar ligands bind to some extent in different orientations, and these modes of binding can be extremely difficult to predict [10].

A Role of the Second Extracellular Loop in Ligand Binding

A surprising feature of bovine rhodopsin is the highly structured extracellular N terminus and extracellular loops [3]. In particular, the second extracellular loop (E2), which connects TM4 and TM5, dives down into the transmembrane domain and forms a "plug" that contacts retinal (Table 1 and Fig. 2). This loop also contains a highly conserved Cys that is disulfide-bonded to another highly conserved Cys at the top of TM3 [11]. E2 contains two stretches of β-strand, one of which, β4, lies directly over retinal [3]. E2 thus forms a lid over retinal and protects it from the extracellular milieu. Given the high degree of conservation of the amino acids in the β4 strand in vertebrate opsins, and the variability within this region in other class A receptors, the prevailing view was that the β4 strand might serve specifically to define the retinal-binding pocket in vertebrate opsins and not other GPCRs [12,13]. We suggest that this response is at least partly wrong, for a number of reasons discussed below.

We do not yet know of any structural similarity of E2 between rhodopsin and other class A receptors beyond the shared disulfide bond, but the sequence at the extracellular end of TM4 and the beginning of E2 is highly conserved among functionally related receptors and among species variants of these receptors, despite the fact that the sequence of E2 is highly variable across class A receptors [14]. In addition, this region has been identified as the site of covalent attachment of photoaffinity derivatives of agonist and antagonist ligands of the α2-AR [15], and mutations in this region have ligand-specific effects (reviewed in Javitch *et al.* [14]). Moreover, known ligand binding sites in other TMs are predicted to be in spatial proximity to this region. It is likely, therefore, that this region plays a functional role, and it

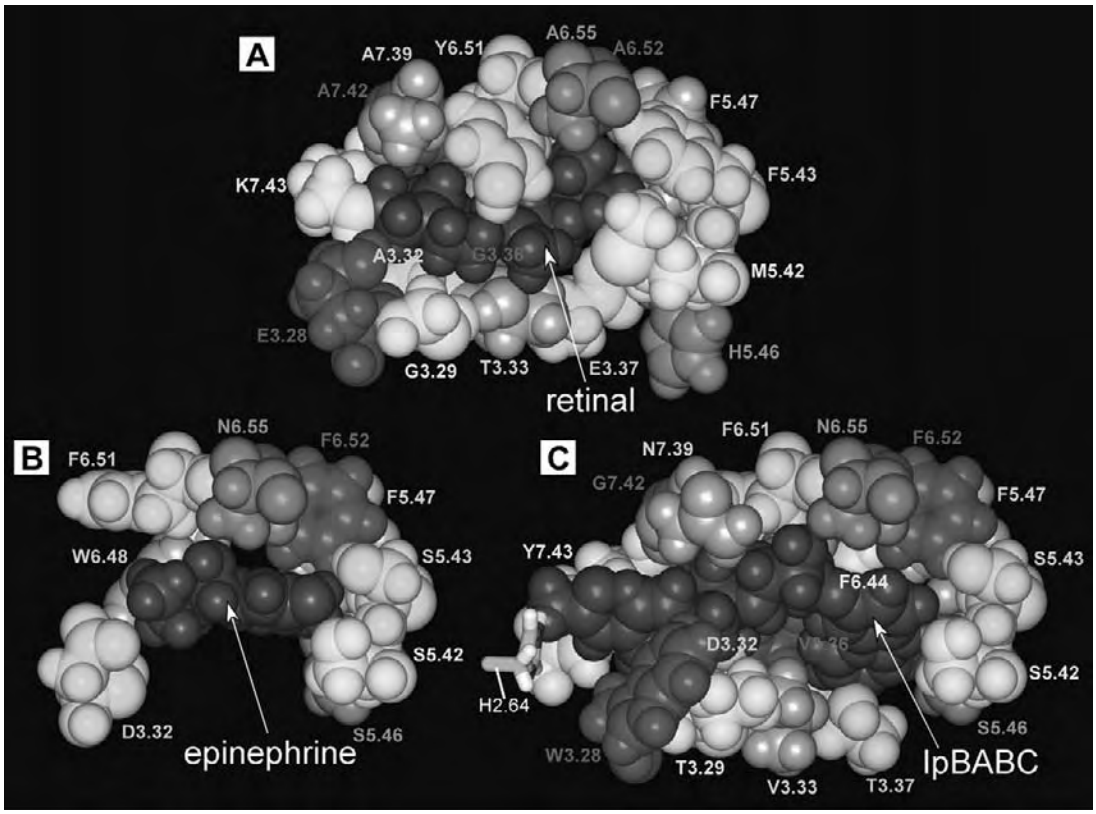


Figure 3 Ligand binding crevice. In (A), the residues in the TMs that were identified from the SASA analysis (see text, Table 1, and Fig. 2) are shown in van der Waals representation, with retinal bound within the surface created by these residues. In (B), the side chains of residues from the β2-adrenergic receptor are shown on the backbone of the rhodopsin structure. These residues have been experimentally determined to interact with catecholamine ligands, and include Asp113$^{3.32}$, Ser203$^{5.42}$, Ser204$^{5.43}$, Ser207$^{5.46}$, Phe208$^{5.47}$, Trp286$^{6.48}$, Phe289$^{6.51}$, Phe290$^{6.52}$, and Asn293$^{6.55}$, a subset of the positions shown in (A). In (C), IpBABC (p- (bromoacetamido) benzyl-1-[^{125}I]iodocarazolol), an affinity label derivative of pindodol, is docked within the TMs of the rhodopsin structure with the TM residues from Table 1 mutated to the aligned β2-adrenergic receptor residues. IpBABC is shown covalently attached to His93$^{2.64}$ in TM2. Residues with the same index number are shown in the same color in all three panels. The residues displayed next to each other are shown in different colors. (From Ballesteros, J. *et al*., *Mol. Pharmacol.* 60, 1–19, 2001. With permission.) A color representation of this figure is available on the CD version of the *Handbook of Cell Signaling*.

is possible that an orientation of E2 similar to that in rhodopsin may explain these findings.

Several reports implicate E2 in ligand specificity in a number of small molecule-ligand GPCRs. Perez and colleagues found that substitution of three consecutive residues in E2 changed the ligand specificity for particular antagonists from that of α_{1B}AR to that of α_{1A}AR, and vice versa [16]. Similarly, substitution of E2 and TM5 altered the subtype specificity of the 5-HT$_{1D}$ receptor to that of the 5-HT$_{1B}$ receptor and vice versa [17], and substitution of a single residue in E2 was also sufficient to interconvert the pharmacological specificity of canine 5-HT$_{1D}$ and human 5-HT$_{1D}$ receptor [18]. In adenosine receptor, in which the binding site is also formed in the transmembrane domain [18], several glutamate residues in E2 are critical for ligand recognition [20,21]. Although it is currently difficult to envision the entrance route of ligands into the binding-site crevice and the potential associated conformational rearrangements of E2, these data nonetheless suggest a direct role of residues in E2 in ligand binding in other class A receptors [22].

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CHAPTER 27

Glycoprotein Hormone Receptors: A Unique Paradigm for Ligand Binding and GPCR Activation

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Introduction

The glycoprotein hormones (GPHs) and their receptors (GPHrs) constitute an interesting paradigm of agonist–receptor coevolution. The hormones of pituitary or placental origin, lutropin (LH), chorionic gonadotropin (CG, with lutropin activity), follitropin (FSH), and thyrotropin (TSH), are dimeric glycoproteins of about 30 kDa made of a common alpha subunit and a specific beta subunit endowed with the functional and binding specificity. In all vertebrates, beta subunits are encoded by paralogous genes. The tridimensional structures of CG and FSH have been solved at 2.6- and 3-Å resolution, respectively [1,2]. To these three different hormone activities correspond the three GPHrs, namely the LH/CGr [3], FSHr [4], and TSHr [5,6]. The GPHrs have a bipartite structure reflecting a dual evolutionary origin; they are made of a serpentine portion with seven transmembrane alpha helices, typical of rhodopsin-like G-protein-coupled receptors (GPCRs), and a large (350 to 400 residues) amino-terminal extracellular domain containing nine motifs characteristic of the family of leucine-rich repeat (LRR) proteins. All three receptors are preferentially coupled to Gs, although at high agonist concentrations they also couple to Gq [3,4,7]. Mining of genomes and low-stringency PCRs have revealed additional GPCRs with a similar structural organization; these will not be dealt with further here [8].

Molecular Pathophysiology

Amino acid substitutions at each of 20 separate positions cause constitutive activation of TSH or LH/CG receptors [9]. The TSH receptor has been particularly fertile in activating mutations because of the fact that somatic mutations leading to activation of the TSHr cause a readily detectable thyroid phenotype (i.e., autonomous toxic adenomas) [10]. Much less frequent are germline mutations with similar activating effects. When affecting the TSHr or LH/CGr, they cause autosomal dominant hyperthyroidism or pseudo precocious puberty of the male, respectively [3,9]. Except for a single anecdotal case, no disease has been associated with activating mutations of the FSHr [4].

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Structure Function Relationships of the Glycoprotein Hormone Receptors

The particularly wide spectrum of activating mutations identified in the TSHr correlates with the observation that wild-type TSHr displays readily detectable basal activity [10,11], whereas gonadotropin receptors are virtually silent in the absence of their ligands [12]. Another peculiarity of the TSHr is the spontaneous cleavage of a proportion of the molecules present at the cell surface into two subunits that remain linked by disulfide bridges [6].

The GPHrs show clear structural dichotomy between the ligand-recognizing amino-terminal ectodomain and the serpentine rhodopsin-like portion that transmits the signal to the G protein. How does binding of the hormone to the ectodomain result in the activation of the serpentine domain? We will summarize the three key steps in this process: recognition and binding of the hormones, activation of the serpentine portion of the receptors, and intramolecular transduction of the activation signal between the ectodomain and the serpentine portion.

Structure and Function of the Ectodomain of Glycoprotein Hormone Receptors

The ectodomain of all three receptors is made of nine leucine-rich repeats (LRRs), each ≈ 25 amino acids, flanked by two-cysteine containing domains (Fig. 1A,B). LRR-containing proteins constitute a large family of both intra- and extracellular molecules specialized in protein–protein

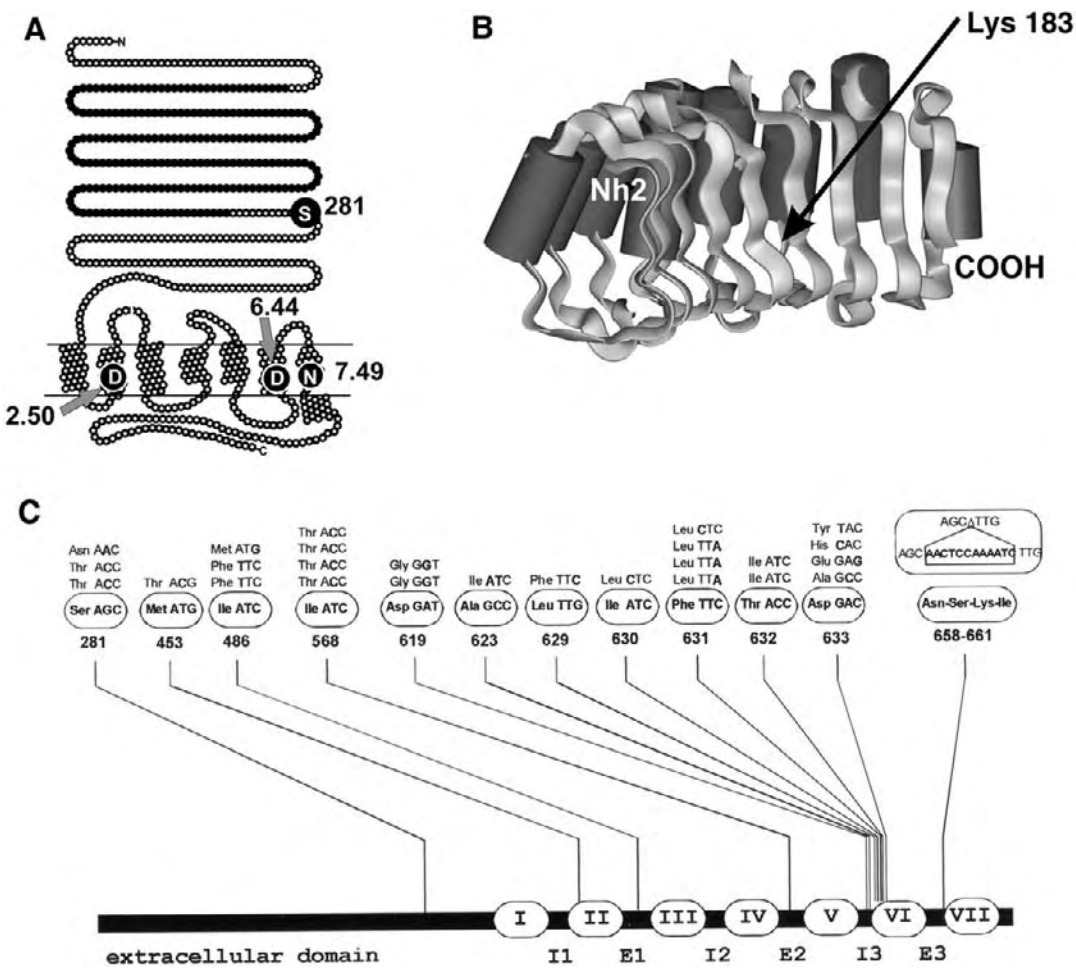


Figure 1 Schematic representation of the TSH receptor (A), ribbon representation of the model of the leucine rich repeats region (B), and illustration of the position of a series of activating mutations (C). (A) The seven transmembrane helices are drawn as helical nets, respecting the helice ends as observed in the crystal structure of rhodopsin [51]. Closed circles in the N-terminal extension represent the portion of the domain modeled by Kajava *et al.* [14], comprising residues 54 to 254. Some of the key residues discussed in the text are indicated as black circles with an indication of the amino acid and the position (numbering system of Ballesteros *et al.* [50] (B) The α-helices are drawn as solid tubes. The "horseshoe" curvature is clearly visible, and the position of the residue mutated in pregnancy hyperthyroidism (Lys 183) is indicated. (C) The TSH receptor is represented linearly, with the transmembrane helices indicated in Roman numerals. The positions of a series of activating mutations are indicated with the nature of the amino acid substitutions. The numbering is TSHr specific (e.g., D633 corresponds to D6.44). (Adapted from Smits, G. *et al.*, *Mol. Endocrinol.*, 16, 722–735, 2002; Parma, J. *et al.*, *J. Clin. Endocrinol. Metab.*, 82, 2695–2701, 1997. With permission.)

interactions [13]. Structural models for the ectodomains of the TSHr and LH/CGr were elaborated on the basis of the three-dimensional structure of an LRR protein, ribonuclease inhibitor [14,15]. The models predict that the LRR portion of the ectodomains of the receptors would adopt a horseshoe (or segment of doughnut) shape, with alpha helices and beta sheets making the convex and concave surfaces of the structure, respectively (Fig. 1B). For the ribonuclease inhibitor, direct crystallographic evidence indicated that the concave surface was responsible for the majority of the binding interactions with the ligand (ribonuclease) [16]. The pertinence of this model has been tested for the LH/CGr, essentially by means of loss-of-function mutations [15,17,18]. In the case of the TSHr, a gain-of-function mutation was identified in a family presenting with pregnancy-dependent hyperthyroidism due to a K183R amino acid substitution, located in the middle of the LRR portion of the ectodomain of the receptor (Fig. 1B), predicted to face inside the putative hormone binding domain [19]. Functional studies in transfected C cells show that the K183R mutant becomes abnormally sensitive to the pregnancy hormone hCG [19]. The gain of function, though modest, is enough to cause disease because of the extremely high concentration of hCG achieved during the first trimester of pregnancy. Extensive site-directed mutagenesis based on the putative structural model suggested that the gain of function was due to the unmasking of the negative charge of glutamic acid in position 157 from a salt bridge with lysine 183, not achieved with the arginine replacement [20]. Any amino acid substitution in position 183 causes a gain of function similar to that of K183R. Definitive validation of the model of the TSHr based on the structure of the ribonuclease inhibitor has been obtained very recently. Conversion of eight carefully selected residues of the putative binding surface of the TSHr to their LH/CGr homologs yields a TSHr mutant displaying a sensitivity to hCG comparable to that of wild-type LH/CGr (G. Smits *et al.*, in preparation).

A posttranslational modification with important functional significance has recently been identified in all three GPHrs. Close to the border between the ectodomain and the first transmembrane segment of the serpentine, the three receptors harbor a motif that undergoes tyrosine sulfation just before insertion of the molecule into the plasma membrane [21]. The sulfated tyrosines are an important component of the binding surface, as mutant receptors unable to become sulfated lose sensitivity to their hormones by one order of magnitude [21]. This identifies the sulfated tyrosines as an important participant in the known ionic interactions between GPH and their receptors [22].

Activation of the Serpentine Portion

The GPHr and, in particular, the TSHr can be activated by a wide spectrum of amino acid substitutions or deletions affecting mainly but not exclusively the serpentine domain (Fig. 1C) [9]. Some of these are homologous to activating mutations identified initially in adrenergic receptors [23,24]. Others involve residues specific to the GPHr subfamily. Despite their high sequence similarity, the three receptors display great differences in the propensity to be activated by mutations, with the TSHr being more prone to activation than the LH/CGr and the FSHr being particularly refractory [25]. The structural bases for these differences are still unknown. Among the spontaneous gain of function mutations, those affecting residue D6.44, in the sixth transmembrane segment (numbering system of Ballesteros *et al.* [26]) deserve special attention. This residue is part of one of the sequence signatures specific to the GPHr in transmembrane VI [27]. D6.44 (D633 or D578 in the TSHr- or LH/CGr-specific numbering systems, respectively) is one of the residues most frequently mutated in precocious puberty of the male and toxic thyroid adenomas.

Experiments performed with the TSHr were driven by the observation that in LGR1 (a glycoprotein hormone receptor homolog of *Drosophila*) [28] Asp and Asn residues were naturally exchanged between 6.44 and 7.49, suggesting that these residues of transmembrane segments VI and VII interact with each other. Functional studies of single and double mutants transfected in COS cells led to the following model: In the inactive state of the receptor, D6.44 and N7.49 interact; release of the side chain of N7.49 from this interaction, caused by mutation of D6.44 (e.g., D6.44A), would make it available for interactions involved in stabilization of an active state of the receptor [29]. This conclusion is also drawn from the observation that the N7.49A mutant loses the ability to be stimulated by TSH. Addition of the N7.49A mutation to constitutively active TSHr mutants dramatically reduces their activity, to the level of the wt receptor or below [30]. These results are in agreement with others that point to N7.49, one of the most conserved residues in rhodopsin-like GPCRs, as a key residue involved in stabilizing both the inactive and the active conformations (see discussion in Meng and Bourne [31] and Lu *et al.* [32]). The partner(s) of N7.49 in the active conformation is (are) still subject to intense investigation; in several other GPCRs, experimental evidence points to D2.50 [33–36]. It is likely that a complex network of interactions implicating N7.49 and D2.50, but also other residues (e.g., N1.50), stabilizes the active conformation [31,32].

Intramolecular Signal Transduction Between the Ectodomain and the Serpentine Domain

The observation that ectodomains of the GPHr can bind their agonists with high affinity in the absence of the serpentine domain [1,37,38] is compatible with two models for the activation of the receptors. According to the first, high-affinity binding of the agonist would position the hormone for a low-affinity interaction with the extracellular loops (and/or crevice) of the serpentine, leading to activation. A candidate for this activating interaction is the alpha subunit common to the three hormones. Experimental support for this model has been provided by site-directed mutagenesis experiments introducing reciprocal mutations in the LH/CGr and hCG and by affinity labeling [39,40].

The above model, however, does not account for the capacity of the three receptors to be fully activated by point mutations in their ectodomain. A serine in position 281 of the TSHr was found mutated to threonine, asparagine, or isoleucine in autonomous thyroid adenomas [41–43]. Subsequently, it was found that mutations introduced at homologous positions in the LH/CGr (S277) and FSHr (S273) were similarly active [44]. This led to the notion that the ectodomain normally exerts a silencing effect on the serpentine domain and that activation of the GPHr results from the release of this inhibitory interaction. Direct evidence for this silencing role of the ectodomain was obtained in two types of experiments. In the first, constructs containing only the serpentine domain of the TSHr were shown to increase basal cAMP levels when expressed in transfected cells [45,46]. In the second, chimeric molecules were made containing segments of LGR2 [47] (a *Drosophila* homolog of the GPHr with a high basal activity) and the LH/CGr (which is virtually devoid of basal activity). The results indicated the establishment of silencing interactions between a segment of the ectodomain (containing serine 277 of LH/CGr, see above) and the second extracellular loop of the transmembrane domain, provided they both originate from the LH/CGr [8]. From these experiments, one could propose that activation of GPHrs by their agonists results from the release of a silencing effect exerted by the unliganded ectodomain on an intrinsically active serpentine.

Whereas this would be in agreement *qualitatively* with the above experiments, it does not account for the observation that, when normalized to the level of receptor expression at the cell surface, the basal activity of serpentine-alone TSHr constructs is much lower than the maximal activity achieved after stimulation by saturating concentrations of the hormone, or in the most active serine 281 mutants [45]. In an attempt to integrate available information, we have proposed a model for the activation of the TSHr in which the ectodomain would act as a molecular switch (Fig. 2) [45]. In the "off" position, in the absence of hormone, the ecto domain acts as a tethered inverse agonist of the serpentine domain, minimizing basal activity. Binding of the hormone to the receptor stabilizes the "on" position, in which the ectodomain now behaves as a tethered full agonist. Mutations affecting serine 281 of the ectodomain similarly puts the switch in the "on" position. The relative potency of individual amino acid substitutions at S281 indicates a direct relation between the destructuring effects of the mutations and constitutive activity [44,48], suggesting that the gain of function results from a local loss of structure in the ectodomain.

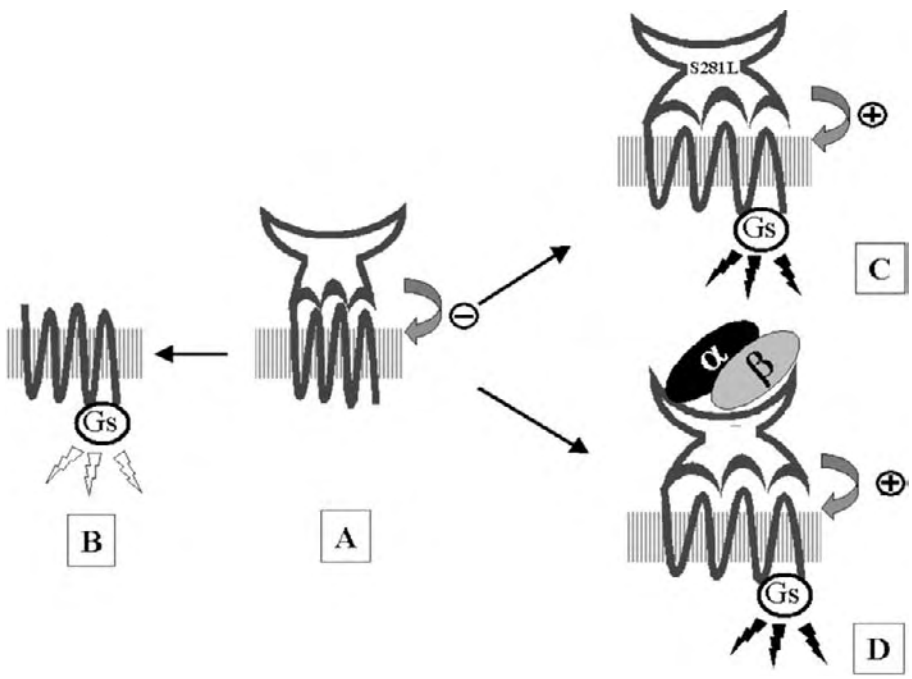


Figure 2 Putative model of the intramolecular interactions involved in the activation of the TSH receptor. (A) The basal state of the receptor is characterized by an inhibitory interaction between the ectodomain and the serpentine domain; the ectodomain would function as a tethered inverse agonist. (B) Removal of the ectodomain releases the serpentine domain from the inhibitory interaction, resulting in partial activation. (C) Mutation of Ser281 into Leu switches the ectodomain from an inverse agonist into a full agonist of the serpentine domain. (D) Binding of TSH to the ectodomain is proposed to have a similar effect, converting it into a full agonist of the serpentine portion. It must be stressed that the scheme is purely illustrative. It emphasizes that, according to the model, activation does not require a direct interaction between the hormone and the serpentine domain. Such an interaction, however, is by no means excluded. (Adapted from Vlaeminck, V. *et al.*, Activation of the cAMP pathway by the TSH receptor involves switching of the ectodomain from a tethered inverse agonist to an agonist. *Mol. Endocrinol.*, 16, 736–746, 2002.)

A last set of experiments suggests that the molecular switch controlling activation of the serpentine domain must be a composite structure combining a portion of the ectodomain and the extracellular loops of the serpentine. A spectrum of well-defined activating mutations of the TSHr were engineered, either on a holoreceptor background or in serpentine-alone constructs. Whereas the mutations in the transmembrane segments or intracellular loops were equally effective on both backgrounds, mutations of the extracellular loops with a strong effect on the holoreceptor were totally ineffective on the serpentine-alone constructs [45]. This model does not rule out that activation of GPHr involves a direct interaction between the hormones and the serpentine portion of the receptors, but it indicates that such an interaction is not required to account for most observations. In the case of the TSHr, it also provides a rationale for the activation of the receptor by autoantibodies present in the plasma of patients with Graves'disease [6]. According to this model, stimulating autoantibodies would only need to have a "destructuring" effect on a segment of the ectosomain controlling the molecular switch.

Conclusions and Perspectives

With their bipartite structure already present in primitive marine invertebrates [49], the GPHr have evolved a specific way to become activated after binding of their hormones to the ectodomain. On the other hand, their membership in the rhodopsin-like family of GPCRs implies that basic molecular mechanisms implicated in the activation of their serpentine domain must be shared with this protein family. We believe that these peculiarities provide a unique opportunity to dissect the molecular steps of activation of type I GPCRs. The particularly wide spectrum of activating mutations in GPHr are expected to mimic (and allow us to explore) the sequential conformational changes that begin after binding of agonists and terminate with activation of the G protein.

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CHAPTER 28

Protease-Activated Receptors

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Introduction

G-protein-coupled receptors have evolved a variety of mechanisms to acquire information about the environment of a cell. They sense light, odorants, ions, lipids, nucleotides, amino acids and their derivatives, small peptides, polypeptides, and large glycoprotein hormones. This chapter describes a subfamily of heptahelical receptors known as protease-activated receptors (PARs), which have evolved to sense proteases [1–8]. PAR1, the prototype for the PAR family, was identified in the context of an effort to understand how the coagulation protease thrombin activates platelets and other cells. Thrombin is a multifunctional serine protease generated at sites of tissue injury. In addition to cleaving fibrinogen, thrombin triggers a variety of cellular responses from platelet aggregation to endothelial display of adhesion molecules to fibroblast proliferation. These actions of thrombin raised the question of how a protease can function like a hormone to regulate cell behavior. PARs provide an answer.

Mechanism of Activation

Thrombin activates PAR1 by binding to and cleaving its amino terminal exodomain (Fig. 1). This cleavage event is both necessary and sufficient for receptor activation. Mutation of the cleavage site ablates receptor signaling, and substitution of cleavage recognition sites for another protease for the thrombin site confers signaling in response to that protease. Thus, the essential role of the protease in activating PARs is cleavage of the receptor at a single site within its amino-terminal exodomain. How does cleavage of this apparently flexible, unstructured domain send information across the cell membrane? The synthetic peptide SFLLRN, which mimics the first six amino acids of the new amino terminus unmasked by receptor cleavage, functions as an agonist for PAR1 and activates the receptor independent of thrombin and proteolysis. Moreover, removal of the amino terminal exodomain of the receptor yields a receptor that responds to SFLLRN but not to thrombin. These and other data support a model in which receptor cleavage serves to unmask a new amino terminus that then functions as a tethered peptide ligand and binds to the heptahelical segment of the receptor to effect transmembrane signaling and G-protein activation. Thus, PAR1 is in essence a peptide receptor that carries its own ligand, and this ligand remains hidden until revealed by selective cleavage of the amino terminal exodomain of PAR1. This mechanism raises several interesting questions, addressed below.

How is it that the PAR1 tethered ligand remains inactive in the uncleaved receptor and is activated by cleavage? Addition of even one amino acid to the N terminus of the SFLLRN agonist peptide ablates agonist activity, as does removal of its N-terminal protonated amino group. In the uncleaved receptor, the cognate nitrogen atom is part of the peptide bond between Arg41 and Ser42, the P1 and P1' amino acids of the thrombin cleavage site. Ser42 is also the N-terminal amino acid of the tethered ligand. Thus, the proteolytic switch that activates the cryptic peptide ligand appears to involve removal of amino terminal sequence that sterically hinders ligand function as well as generation of a new and functionally important protonated amino group at the N terminus of the ligand (Fig. 2). Parallels with zymogen activation of serine proteases are apparent. In conversion of trypsinogen to trypsin, precise proteolytic cleavage generates a new amino terminus that bears a new protonated amino group; this then docks intramolecularly to trap the protease in its active conformation.

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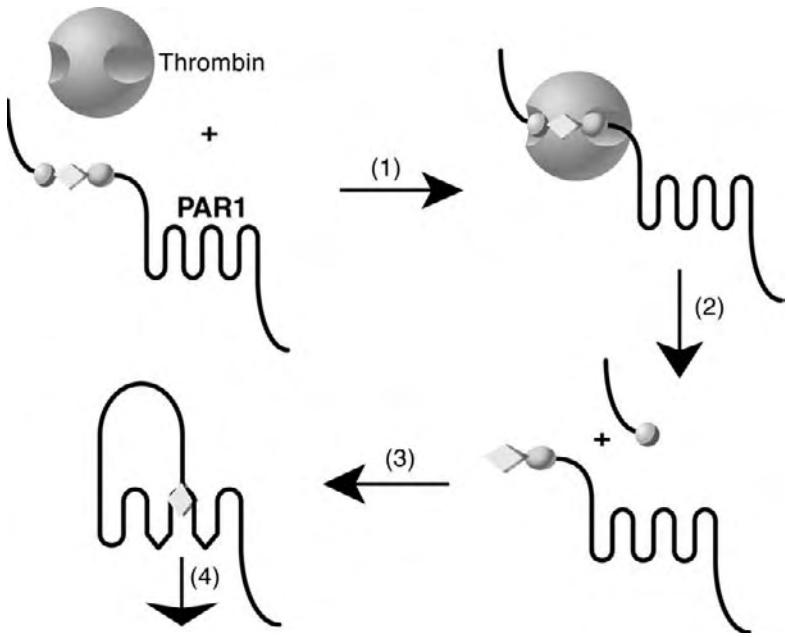


Figure 1 Model for proteolytic mechanism of PAR activation. (1) Thrombin, the green sphere, binds to the amino-terminal exodomain of PAR1. Receptor amino acids $L^{38}DPR^{41}$ (small blue sphere) interact with the active center of thrombin, and receptor amino acids $D^{50}KYEPF^{55}$, the so-called hirudin-like domain (pink oval), interact with exosite 1 of thrombin. (2) After binding, thrombin cleaves the receptor between R^{41} and S^{42} to create a new amino terminus beginning with the sequence S^{42}FLLRN (yellow diamond). (3) Once unmasked, SFLLRN serves as a tethered peptide ligand that binds to the heptahelical domain to effect transmembrane signaling and G-protein activation (4).

Does the tethered ligand bind intramolecularly? Where does it bind and how does such binding yield G-protein activation? These questions are both basic and practical. One strategy for blocking PAR1 function is to block binding of the tethered ligand, and the SFLLRN tethered-ligand peptide has served as a pharmacophore for antagonist development.

Intramolecular binding of the ligand to the receptor to which it is tethered would clearly be favored unless prevented by specific structural constraints. Assuming an ≈50-amino-acid tether localized the ligand to a hemisphere of radius ≈100 Å, the effective concentration of the tethered ligand would be on the order of 1 m*M*; micromolar concentrations of SFLLRN peptide in solution suffice to activate PAR1. Structure–function studies indeed suggest that intramolecular ligation is the predominant mode for PAR activation, and the relative ineffectiveness of PAR1 antagonists at blocking cellular responses to thrombin versus SFLLRN is consistent with favored, intramolecular binding of the tethered ligand. Intermolecular ligation of PARs can be demonstrated in certain settings. It is worth noting that intermolecular ligation of receptors in stable dimeric or oligomeric complexes would not be readily distinguished from intramolecular ligation of monomers, but there is as yet no compelling evidence that PARs form such complexes.

Studies with chimeric receptors, receptor mutations that complement loss of function substitutions in agonist peptide, and blocking antibodies all point to the exofacial domain of PAR1 as being critical for recognition of agonist peptides. Such studies also suggested that PAR exofacial domains interact to form a structure necessary for receptor function. The crystal structure of rhodopsin reveals that the N-terminal exodomain and extracellular loops of rhodopsin interact to form a cap over the heptahelical core of the receptor. Thus, the exofacial domain of PAR1 might be the binding site for the tethered ligand or, alternatively, might function as a kind of template or keyhole that determines access of the tethered ligand to a site deeper in the heptahelical core. A satisfying answer will await a crystal structure.

The mechanism of PAR1 activation is strikingly irreversible. Cleavage of PAR1 by thrombin is irrevocable, and the tethered ligand generated cannot diffuse away from the receptor. How then is PAR1 shut off? Activated PAR1 is rapidly phosphorylated and uncoupled from signaling, then internalized and degraded in lysosomes—a disposable receptor. In fibroblasts and endothelial cells, an intracellular pool of naïve receptors can refresh the cell surface without need for new receptor synthesis. These observations suggest a plausible answer to another question raised by the fact that thrombin functions like a hormone. Because thrombin is an enzyme, one molecule of thrombin should be able to activate more than one receptor; in the limiting case, one thrombin molecule might eventually activate all molecules of PAR1 on a cell. How, then, does PAR1 mediate graded responses that are proportional to thrombin concentration? Because each activated PAR1 signals only transiently (and because the second messengers formed are themselves

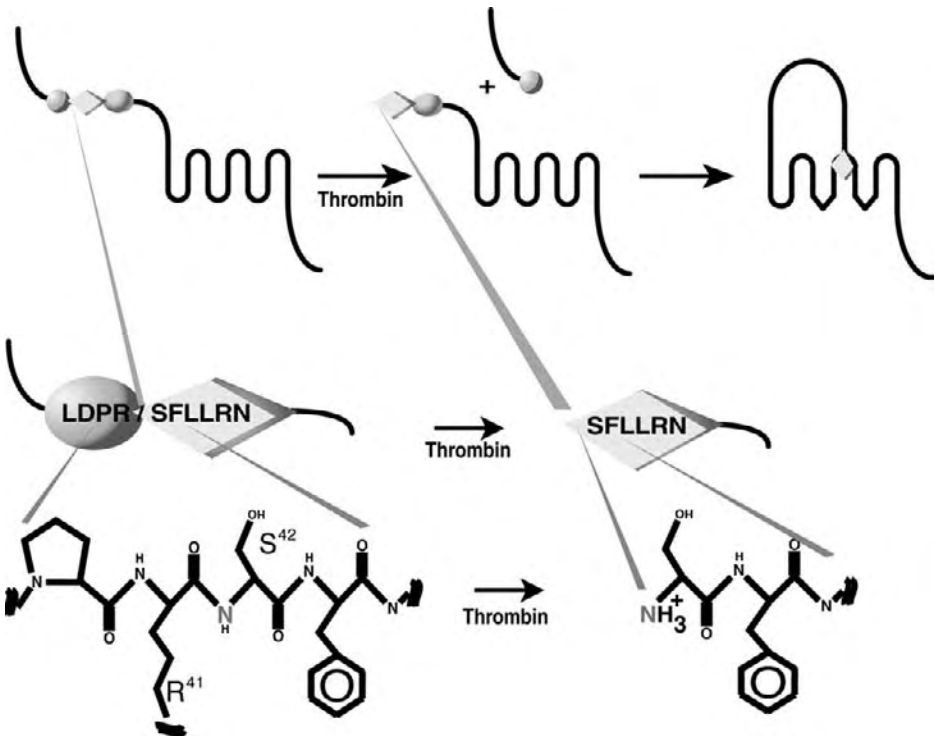


Figure 2 Details of the PAR1 proteolytic switch. A synthetic peptide of sequence SFLLRN (the tethered ligand sequence) can activate PAR1 independent of thrombin and receptor cleavage. Adding a single amino acid to the N terminal of SFLLRN or removing its N-terminal protonated amino group inhibits agonist activity. This suggests that cleavage of PAR1 between R^{41} and S^{42} accomplishes two things to switch on the cryptic ligand; it removes an activation peptide that sterically inhibits SFLLRN and creates a new protonated amino group (red N) that is important for agonist function.

short lived), the magnitude of the response is related to the rate of receptor cleavage and activation and hence to thrombin concentration. The existence of different PARs that are cleaved more or less efficiently by thrombin may also contribute to differential responses over a range of thrombin concentrations.

Protease-Activated Receptor Family

Four PARs are now known (Table 1). PAR1, PAR3, and PAR4 can be activated by thrombin. PAR2 can be activated by trypsin, mast cell tryptase, tissue factor/VIIa complex, factor Xa, and membrane-tethered serine protease-1, but not by thrombin. PAR1 and human PAR3 have a recognizable thrombin binding sequence and respond to thrombin at subnanomolar concentrations. PAR4 requires higher but probably still physiological levels of thrombin for activation, perhaps because it lacks the hirudin-like thrombin binding sequence that is present in PAR1 and PAR3. It is very likely that PAR1, PAR3, and PAR4 are activated by thrombin *in vivo*. Indeed, these receptors seem to account in large part for the ability of thrombin to activate platelets, and recent knockout studies suggest an important role in hemostasis and thrombosis. It is possible that other proteases are also physiological activators of PAR1, PAR3, and PAR4. Similarly, it is certainly possible that one or more of the PAR2-activating proteases listed above are its physiological activators, but this remains to be established.

PAR1 can activate members of the $G_{12/13}$, G_q, and G_i protein families, consistent with the pleiotrophic effects of PAR1 in platelets and other cells which include cytoskeletal reorganization, secretion of granule contents, mobilization of transmembrane adhesion proteins to the cell surface, and metabolic and transcriptional responses. PAR4 couples to G_q and probably $G_{12/13}$, but not to G_i. PAR2 and human PAR3 couple to G_q; their ability to activate other G proteins remains to be explored (Table 1).

Roles of PARs *In Vivo*

Because of the importance of platelet activation in myocardial infarction and stroke, defining the role of PARs in platelet activation by thrombin and the relative importance of this pathway in hemostasis and thrombosis has been a priority. A useful working model is in place. In human platelets, PAR1 appears to be the main thrombin receptor and mediates platelet activation at low concentrations of thrombin. In the absence of PAR1 function, PAR4 can mediate platelet activation, but relatively higher concentrations of thrombin are required. In addition to being activated by thrombin, PAR4 can be activated by the neutrophil granzyme cathepsin G, and PAR4 signaling is shut off more slowly than that of PAR1.

Table I Properties of Human PARs

Receptor	Chromosome	Activated by	Coupled to			Rapid agonist-triggered phosphorylation, internalization, and degradation	Expressed by	Possible roles *in vivo*
			$G_{12,13}$	G_q	G_i			
hPAR1	5q13	Thrombin, trypsin, Xa	Yes	Yes	Yes	Yes	Platelets, megakaryocytes, endothelial cells, mast cells, vascular smooth muscle, glia, fibroblasts, T cells, cardiac mycocytes, skeletal myoblasts, etc.	Hemostastis—platelet secretion, aggregation, ? procoagulant activity Inflammation— leukocyte and platelet recruitment, increased permeability Inflammation— degranulation Inflammation—neurogenic inflammation and pain perception ? Contraction and repair ? Repair ? Repair Unknown
hPAR2	5q13	Trypsin, mast cell tryptase, TF/VIIa, Xa, TF/VIIa/Xa, MTSP1	?	Yes	Probably	Yes	Most epithelial cells including intestine, vascular endothelial cells, neurons, mast cells	Possible cytoprotective role Inflammation—leukocyte and platelet recruitment, ? increased permeability Inflammatory—neurogenic inflammation and pain perception Inflammation—degranulation
hPAR3	5q13	Thrombin, Xa	?	Yes	?	?	Multiple organs northern plot	Unknown (not yet shown to be a major contributor to thrombin signalling)
hPAR4	19p12	Thrombin, cathespin G	Probably	Yes	No	No (shutoff of signalling is slow)	Platelets, megakaryocytes endothelial cells under some conditions	Hemostasis—platelet secretion, aggregation, procoagulant activity unknown

Note: The genes encoding PAR1, PAR2, and PAR3 are located in tandem on chromosome 5; the PAR4 gene is on chromosome 19. A partial list of proteases capable of activating the different PARs is shown. Thrombin is capitalized to indicate that there is good evidence that it is a physiological activator. TF/VIIa, tissue factor/factor VIIa complex; TF/VIIa/Xa, the cognate ternary complex. The G-protein families activated by each PAR and their shutoff properties are listed along with a partial description of expression patterns and probable *in vivo* roles. The latter description is by no means complete and focuses responses relevant to tissue injury. Roles in normal embryonic development and homeostasis are emerging.

It is not known whether these differences between PAR1 and PAR4 are important *in vivo*; PAR4 might simply be redundant and/or provide robustness in an important system.

In contrast to human platelets, mouse platelets utilize PAR3 and PAR4 to mediate thrombin thrombin signaling. Interestingly, the mouse homolog of PAR3 appears incapable of mediating transmembrane signaling by itself. Instead, it functions as a cofactor to promote cleavage and activation of PAR4 at low thrombin concentrations. There is as yet no evidence that PAR3/PAR4 heterodimers are required for the cofactoring activity of PAR3, and available data are consistent with PAR3 simply localizing thrombin to the cell surface. This paradigm is not novel from the perspective of the coagulation cascade, which is replete with examples of cofactors that localize proteases to the plasma membrane and/or bring protease and substrate together. It does, however, represent an interesting mode of interaction among heptahelical receptors in which one receptor localizes ligand to the cell surface for the ultimate ligation of another.

The model of thrombin signaling in mouse platelets predicts that platelets from PAR4-deficient mice should be unresponsive to thrombin. This was indeed the case, and mice lacking PAR4, while grossly normal, had markedly prolonged bleeding times and were protected from thrombosis—strong genetic evidence that, despite the existence of multiple redundant mechanisms for platelet activation, platelet activation by thrombin appears to be necessary for normal hemostasis and important in at least one model of thrombosis [9].

Recent studies suggest interesting roles for PARs in other cell types. For example, activation of endothelial PARs may help trigger recruitment of platelets and leukocytes in response to vascular injury. Activation of PARs on sensory neurons may contribute to neurogenic inflammation and edema and modulate sensitivity to painful stimuli [10,11]. Like the role for PARs in hemostasis, these roles are consistent with the general view that PARs mediate cellular responses to tissue injury. Roles in other settings, such as blood vessel development during embryogenesis, are emerging [12].

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CHAPTER 29

Constitutive and Regulated Signaling in Virus-Encoded 7TM Receptors

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Virus-Encoded Proteins Are Developed through Targeted Evolution *In Vivo*

Large DNA viruses, in particular herpes- and poxviruses, have evolved a number of proteins that function as mimics of or as decoys for endogenous proteins of the host organism. Often the virus uses such proteins to evade key components of the immune system. The virus-encoded proteins are elegant examples of targeted evolution, where the virus has captured a gene from its host and through "combinatorial chemistry" varied its structure and thereby its function randomly through mutagenesis. Unlike biotech entrepreneurs, the virus has the advantage of being able to select the mutant protein with the optimal pharmacological property through *in vivo* screening in the intact organism. The virus with the most useful protein—for example, the most potent or broad-spectrum antagonist—will prevail. One example is the vMIP-II chemokine of human herpesvirus 8, which acts as an efficient blocker of a surprisingly large number of structurally different chemokine receptors. The chemokine system in general is a favored target for virus-encoded proteins. Many chemokine receptors have been hijacked by viruses and optimized for ligand recognition and signaling properties (Fig. 1A).

The Redundant Chemokine System Is an Optimal Target for Viral Exploitation

Chemokines are chemotactic cytokines, which primarily control the migration but also the activation and differentiation of all subsets of leukocytes and play important roles in angiogenesis, organogenesis, and carcinogenesis [1]. Chemokines act through a large family of G-protein-coupled receptors, which are divided into subfamilies of CXC, CC, and CX3C receptors. This nomenclature refers to a fingerprint sequence in the ligands where the first two Cys residues are either neighbors (CC) or separated by one (CXC) or three (CX3C) residues. Although a few chemokine receptors are regulated by only a single chemokine protein, the system is generally characterized by a high degree of redundancy, in which a given chemokine receptor is activated by more than one ligand and a given chemokine acts through more than one receptor within a chemokine subfamily. Thus, the chemokine system is not only the key to the control of the immune system, but it is also an optimal target for viral exploitation, due to the redundancy among multiple endogenous proteins.

The endogenous chemokine receptors all signal rather similarly via the pertussis toxin sensitive G_i pathway. Calcium mobilization mediated mainly by the $\beta\gamma$ subunit of the heterotrimeric G protein is a generally used, robust readout. In fact, non-chemokine receptors, that signal through G_i can also mediate cell migration when expressed in chemotactic cells. Surprisingly, the chemokine receptors are distributed uniformly in the membrane of the migratory cell, and the directional migration apparently depends on an asymmetric distribution of effector molecules downstream to the G proteins. Some chemokine receptors in addition activate $G\alpha_q$ and $G\alpha_{i6}$. The downstream signaling events involve various kinases, including MAP kinases such as p38, which appears to be important for chemotaxis, as well as PI3-Kγ

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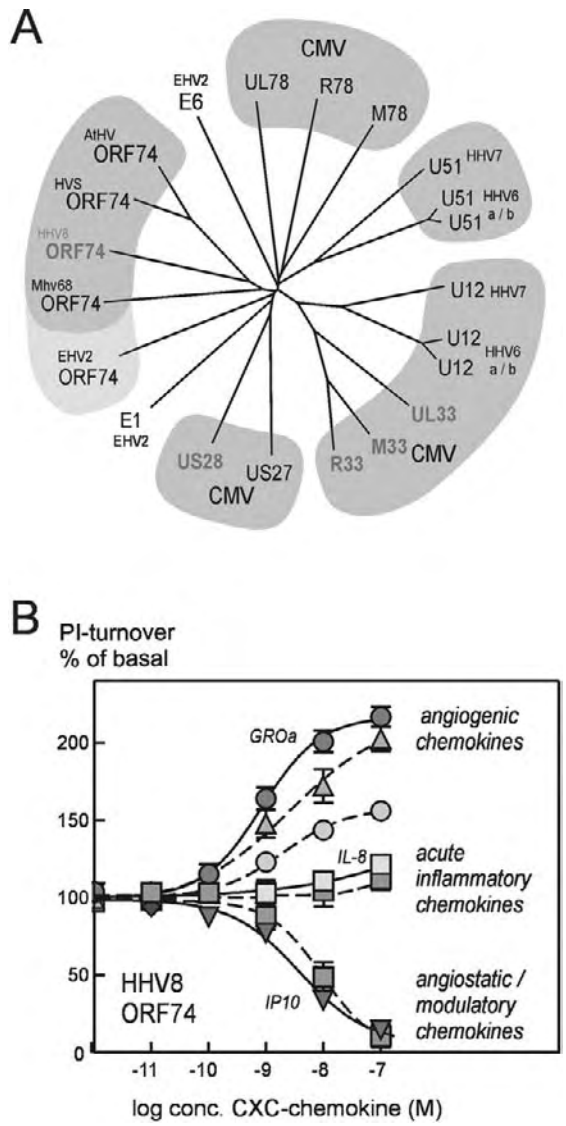


Figure 1 Structural relationship between herpesvirus-encoded chemokine receptors (A) and highly constitutive but regulated signaling of the prototype virus-encoded receptor ORF74 (B). In the dendrogram of herpesvirus-encoded chemokine receptors structural groupings are marked with shaded areas and receptors with known constitutive activity are highlighted. Panel (B) shows how the high constitutive signaling of the most well-characterized virally encoded receptor, ORF-74 from HHV8 (Kaposi's sarcoma-associated herpesvirus), is tuned by angiogenic chemokines acting as agonists and by angiostatic/angiomodulatory chemokines acting as inverse agonists.

and tyrosine kinases, which link the receptors to activation of small GTP-ases [2].

Endogenous chemokine receptors are strictly ligand regulated and normally do not display constitutive signaling activity. This is in accordance with the fact that the receptors are involved in directional migration (i.e., chemotaxis), controlled by a chemokine gradient built in the interstitial tissue through attachment of the secreted chemokines to glycosaminoglycans. Constitutive signaling of a chemokine receptor in a migratory cell would conceivably lead to chemokinesis (i.e., undirected cell movement), which perhaps in conjunction with binding to cell adhesion molecules could be useful in the extravasation process.

Multiple Virus-Encoded 7TM Receptors

More than 20 G-protein-coupled receptors have been identified in various herpes- and poxviruses (Table 1 and Fig. lA). Most of these receptors display key structural elements, that identify them as belonging to the family of chemokine receptors. In general, however, it is impossible to identify a specific endogeneous chemokine receptor as the original scaffold hijacked by the virus. The extensive subsequent mutational effort performed by the virus which has generated the desired pharmacological profile has at the same time significantly altered the primary structure of the receptor. Conceivably a multitude of more or less silent mutations have accompanied the functionally important substitutions that produced the useful property of the viral receptor. A few chemokine-like virus-encoded receptors nonetheless have been convincingly de-orphanized. That is, their endogenous chemokine ligand has been identified. The best examples are US28, a broad-spectrum CC and CX3C chemokine receptor from human cytomegalovirus (CMV), and ORF74, a broad-spectrum CXC chemokine receptor from human herpesvirus 8.

Constitutive Signaling through Altered Pathways

The virus-encoded receptors have often chosen G proteins and downstream effector molecules different from those of the endogenous chemokine receptors. Moreover, whereas most endogenous chemokine receptors are rather silent in the absence of agonist, the virus-encoded receptors often display clear constitutive signaling activity, which may or may not be subject to further fine-tuning or regulation by endogenous ligands.

ORF74, also named KSHV-GPCR (i.e., Kaposi's sarcoma-associated herpesvirus GPCR), is the prototype of a constitutively active, virus-encoded receptor. Multiple signal transduction pathways have been demonstrated for this receptor, involving a variety of G proteins, with G_q signaling dominating, in contrast to the G_i signaling of endogenous receptors. Small GTPases, kinases including MAP kinases, and many transcription factors are also involved in ORF74 signaling (Fig. 2). The receptor activates NFκB, for instance, via $G_{i/o}$, G_{a13} RhoA pathway, G_q, βγ subunits, and P13-Kγ. VEGF secretion, possibly regulated by a transcription factor (HIF-1a) controlled by MAP kinases, may mediate ORF74's ability to induce angiogenic lesions *in vivo*. The constitutive activity of other transcription factors (CREB and NFAT) downstream of ORF74 could be important for lytic replication of the virus, as both pathways have been shown to contribute to HHV8 reactivation. ORF74 also promotes cell survival through activation of PKB/Akt and NFκB.

US28, from human cytomegalovirus, is another highly constitutively active, virus-encoded receptor. In contrast to ORF74,

Table I Properties of Known Herpes and Poxvirus-Encoded Chemokine Receptors. Constitutive and Regulated Activities are Listed Together with the Proposed Functions in Virus Life-Cycle

Virus class and name	Receptor name	Constitutive activity	Regulated activity	Function in virus life-cycle
β-Herpesvirus				
Human CMV	US27	–	–	–
	US28	PLC, Erk2, NFkB, CREB and NFAT activation	Sparse regulation of constitutive activity by chemokines	Chemokine sequestration, cell programming, migration of smc
	UL33	PLC and CREB activation	no described ligand	
	UL78	–	–	
HHV6A and B	U12	–	Ca^{2+} release by CCLs	Viral replication and survival
	U51	–	Binding of various CCLs[a]	Viral replication and survival
HHV7	U12	–	–	–
	U51	–	–	–
Murine CMV	M33	CREB and NFkB activation[b]	no described chemokine ligand	Viral replication, survival and targeting to salivary glands
	M78	–	–	Viral replication and survival
Rat CMV	R33	PLC activation. CREB, NFkB activation	no described chemokine ligand	Viral replication, survival and targeting to salivary glands
	R78	–	–	Viral replication and survival
γ-Herpesvirus				
HHV8	ORF74	see Fig. 2	Regulation of constitutive activity by CXCLs[c] (Fig. 1B and 2)	Angiogenesis, cell-survival, reactivation of viral replication
EHV-2	E1	–	Ca^{2+} release by CCL11	
	E6	–	–	
	ORF74	–	–	
HVS	ORF74	–	Ca^{2+} release by ELR+CXCLs	
AtHV	ORF74	–	–	
Mhv68	ORF74	–	–	
Poxvirus				
Swinepox	K2R	–	–	
Capripox	Q2/3L	–	–	

[a]Various CC-chemokines bind to U51 (HHV6B) transfected cells, but no signaling has ever been shown.
[b]Unpublished data about activity by M. Waldhoer.
[c]CREB and NFAT activation are unpublished data from K. McLean, P. Holst, MMR and TWS.
[d]Abbreviations: CMV, cytomegalovirus, HHV, human herpesvirus; Mhv68, Murine γ-herpesvirus 68; EHV2, Equine Herpesvirus-2; HVS, Herpesvirus Saimiiri; AtHV, Ateles Herpesvirus; ORF74, open reading frame 74; PLC, phospholipase C; AC, adenylate cyclase; PI3K-phosphatidylinositol 3 kinase; RAFTK, Related Adhesion Focal Tyrosine Kinase/or Proline-rich Tyrosine Kinase 2; DAG, Diacylglycerol; IP3, Inositol-3-phosphat; PKA/B/C, protein kinase A/B/C; MAPK-mitogen activated protein kinase; Erk, extracellular regulated kinase; JNK/SAPK, Jun N-ternimal kinase/stress activated protein kinase; CREB, cAMP responsive element-binding protein; NFAT, Nuclear Factor of Activated T-cells; NFkB, Nuclear Factor kB; AP-1 (Fos-Jun); HIF-1a, Hypoxia inducible factor 1a; VEGF, vascular endothelial growth factor; Smc, smooth muscle cell.

this receptor is constitutively internalized and accumulates mainly in the late endocytotic pathway in multivesicular bodies. Other CMV-encoded receptors such as US27 and UL33 also accumulate in multivesicular bodies, a location where the virus is believed to pick up its envelope. Accordingly, these receptors are found on the virions and may be transferred to the target cell during the initial fusion phase of infection [3].

Viral Receptors Recognize Multiple Ligands with Variable Function

The constitutive signaling of ORF74 from HHV8 is fine-tuned by a number of endogenous CXC-chemokines. Thus, ORF74 responds to certain angiogenic CXC ligands (called ELR+CXCLs) as agonists and angiostatic ELR−CXCLs as inverse agonists (ELR refers to the conserved amino acids located just prior to the first Cys in the protein). CXC chemokines that are involved especially in acute inflammatory reactions (for example, IL-8) do not affect the high level of constitutive signaling (Fig. 1B). Interestingly, ORF74 in fact *binds* basically all human CXC chemokines, including IL-8, with high affinity. Competition binding experiments with multiple radiolabeled ligands have revealed different active and inactive conformations that apparently do not readily interchange. The constitutive activity per se as well as the regulated activity are important functions for oncogenesis [4,5].

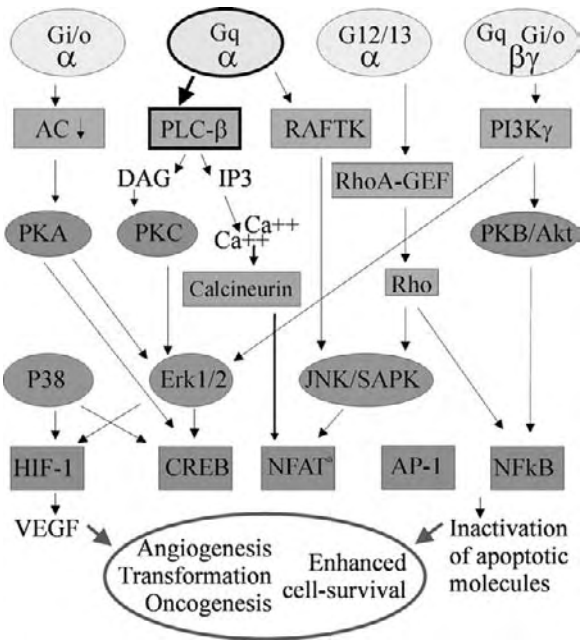


Figure 2 Multiple downstream signaling pathways are activated by ORF74 from HHV8. Several levels of effector molecules have been implicated as being part of the signaling cascade elicited by ORF74 from the G-protein activation (yellow) over a variety of signaling molecules to gene transcription regulation through control of at least five different transcription factors (orange) in the cell nucleus. The diagram is grossly simplified, and several suggested cross-regulations between the depicted enzymes have been excluded. For abbreviations, see footnote for Table 1.

Although US28 binds all human CC chemokines with nanomolar affinity, *none* of the ligands appears to affect signaling by the receptor. It has been suggested that the receptor could function as a scavenger, picking up the CC chemokines from around the infected cell and rapidly inactivating them through internalization. The favored ligand for US28, however, is membrane-bound CX3C chemokine fractalkine, which acts as a partial inverse agonist. Surprisingly, the fractalkine binding cannot be blocked by the otherwise high-affinity binding of CC chemokines. The membrane-anchored fractalkine may serve as a cell-entry gateway for human CMV via interaction with US28 expressed on the surface of infected cells and the virion [6].

Attempts To Identify the Function of Virus-Encoded Receptors *In Vivo*

None of the virus-encoded 7TM receptors is essential for viral replication in cell cultures. *In vivo* studies with rodent CMV, however, consistently demonstrated decreased virulence of viruses in which R33, M33, R78, or M78 receptors had been selectively knocked out. In the cases of R33 and M33, the receptor-deleted virus replicated normally in the bone marrow, but not in salivary glands. This could indicate that the M33/R33 receptor is essential either for targeting of the virus to the salivary gland (a "taxi" function where the receptor provides the infected cell with a new homing address) or for viral replication in this tissue, which would be crucial for the spread of the virus between animals.

Transgenic expression of ORF74 from HHV8 under the CD2 promoter in mice resulted in a phenotype with striking similarities to Kaposi's sarcoma, in regard to both location and histopathology of the highly vascularized lesions [7]. By selectively eliminating either the high constitutive activity of the receptor or the ability of the receptor to be controlled by ligands it was demonstrated that both these properties were required in order to obtain the angiogenic lesions [5]. This is especially interesting, because the virus apparently has optimized the ORF74 receptor to be regulated positively by endogenous angiogenic chemokines—as agonists—and negatively by angiostatic or angiomodulatory chemokines—as inverse agonists (Fig. 1B).

In summary, virally encoded chemokines and receptors have evolved through a massive *in vivo* selection process performed by opportunistic organisms trying to exploit our endogenous cellular communication systems. Consequently, each of these molecules is an interesting pointer or showcase for key aspects of signal transduction by 7TM receptors.

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CHAPTER 30

Frizzleds as G-Protein-Coupled Receptors for Wnt Ligands

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Introduction

The *frizzled* (*fz*) gene family encodes predicted seven-transmembrane proteins that serve as receptors for the Wnt family of secreted glycoprotein ligands [1–3]. Together, Wnts and Fzs stimulate signaling pathways integral to development and implicated in disease. A persistent problem in Wnt and Fz signaling, until recently, has been the identity of intracellular signaling molecules activated directly by Fz [2]. In one pathway, intracellular signaling by Fz requires the cytoplasmic phosphoprotein Dishevelled (Dsh), shown genetically to be the most immediate cytoplasmic protein involved in Fz signaling. Fz and Dsh have never been shown to interact biochemically, however, leaving a gap in this important signaling pathway. Although the sequence of Fz does not fit the classical G-protein-coupled receptor (GPCR) mold [4–6], it has for some time been attractive to imagine that the Fz family of proteins may indeed be GPCRs. An abundance of recent evidence has now demonstrated that Fzs require G proteins in two Wnt/Fz signaling pathways (discussed below). Fzs are therefore now known as GPCRs for Wnt ligands, which begins to explain how Fzs activate downstream signaling molecules such as Dsh. Here, after a brief overview of Wnt signaling pathways, we will review the lines of evidence supporting the characterization of Fzs as GPCRs.

Wnt Signaling

Fzs bind and synergize with Wnts to activate two signaling pathways in vertebrates referred to as the Wnt/β-catenin and Wnt/calcium pathways [2,7]. The canonical or Wnt/β-catenin pathway (Fig. 1A) promotes the interaction between β-catenin and the Lef/Tcf family of transcription factors [1] to regulate cell proliferation and cell fate determination. Upon binding Wnt, Fz signals to Dishevelled (Dsh), which inhibits the "destruction complex" [2]. The destruction complex is composed of a large assembly of proteins, including Axin, APC, PP2A, and GSK-3 that continually promotes the ubiquitination and proteosomal degradation of β-catenin in the absence of active Wnt signaling. Once Dsh has inactivated this complex, β-catenin accumulates and interacts with the Lef/Tcf family of transcription factors to activate transcription of Wnt-responsive genes. In *Xenopus* and mammalian cells, strong evidence shows that Fz signaling to Lef/Tcf occurs via G-protein subunits (discussed below). A lack of biochemical data showing how G proteins might then regulate the function of Dsh and ultimately Lef/Tcf transcription factors represents a significant gap in our knowledge of Wnt/β-catenin signaling. (Detailed maps of this pathway can be found at http://www.ana.ed.ac.uk/rnusse/pathways/cell2.html and http://stke.sciencemag.org/cgi/cm/CMP_5533.)

Although the net effect of activation of the Wnt/Ca^{++} pathway (Fig. 1B) is poorly understood, at a minimum it regulates cell behavior and some cell fates [7]. Activation of this pathway has also been reported to oppose the effects of Wnt/β-catenin pathway activation. Fz stimulates G proteins, which activate phopholipase C to turn on Ca^{++} signaling. Wnts and Fzs increase the release of Ca^{++} from intracellular stores and activation of the Ca^{++}-sensitive

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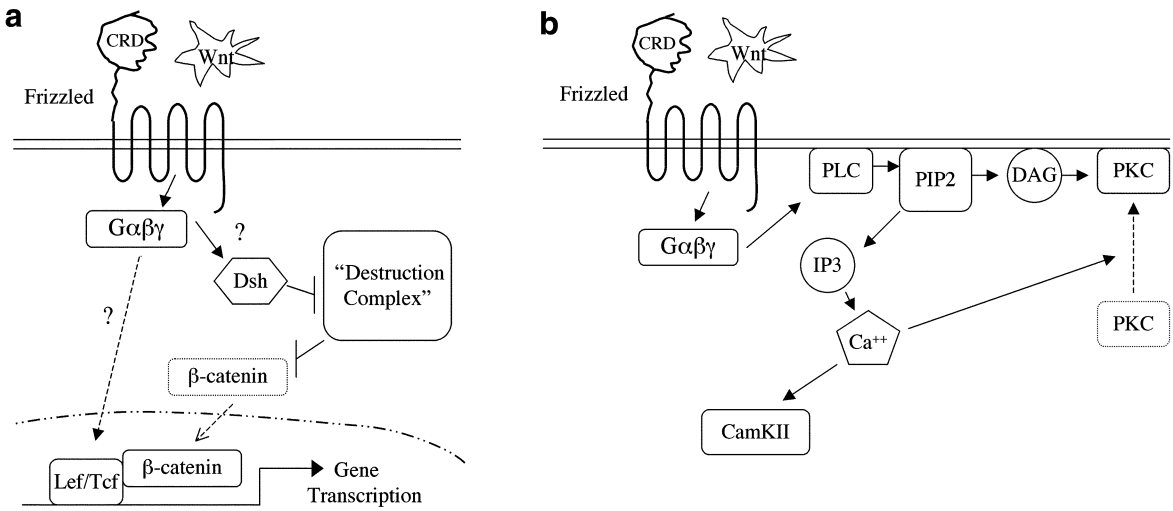


Figure 1 The two vertebrate Wnt signaling pathways are discussed in detail in the text. *Abbreviations:* cysteine-rich domain (CRD); Dishevelled (Dsh), phospholipase C (PLC), phosphatidylinositol 4,5-bisphosphate (PIP2), diacylglycerol (DAG), inositol 1,4,5-triphosphate (IP3), protein kinase C (PKC), and calcium-/calmodulin-dependent protein kinase II (CaMKII).

protein kinase C (PKC) and calcium-/calmodulin-dependent kinase II (CaMKII).

Evidence for Frizzleds as G-Protein-Coupled Receptors

Structural comparison to GPCRs and interesting experimental findings argue that Frizzleds signal through heterotrimeric G proteins. The seven hydrophobic domains of the predicted Fz protein, the predicted NH2-terminal signal sequence, and the potential signal peptidase cleavage site suggest topological homology to all known GPCRs [8,9]. Phylogenetically, *fz* is most closely related to *smoothened* (*smo*) [5], which was recently reported to signal through G proteins [10]. Some reports have noted that *fz* has no amino acid sequence similarity to the *rhodopsin* superfamily of GPCRs [4,5,11,12]. However, reevaluation of Fz predicted protein sequences reveals that Fzs share more characteristics with established GPCR families than was previously thought (Table 1) [4,11,13]. Because Fzs are phylogenetically linked to a known GPCR, Smo, and Fzs contain several GPCR sequence motifs, Fzs might also share with GPCRs a mechanism of conformational change that can activate G proteins.

Fzs not only resemble GPCRs but experimental evidence also argues that Fzs rely upon G proteins for signaling. Recent work examined Fzs as GPCRs using rat Fz-2 signaling in the Wnt/Ca^{++} pathway and rat Fz-1 signaling in the Wnt/β-catenin pathway. The first report showing a requirement for G proteins by Fz came from the analysis of intracellular calcium in zebrafish [14]. An increase in the frequency of intracellular calcium transients was measured in zebrafish embryos over-expressing Wnt-5A or rat Fz-2. Whether G proteins were required for this phenomenon was tested by treating embryos expressing rat Fz-2 with several G-protein inhibitors. The elevation of Ca^{++} stimulated by rat Fz-2 was blocked by the G-protein inhibitors GDPβS, which prevents G-protein activation; pertussis toxin, which adenosine diphosphate (ADP) ribosylates and which specifically inhibits guanosine diphosphate (GDP)–guanosine triphosphate (GTP) exchange on $G_{\alpha i}$, $G_{\alpha o}$, and $G_{\alpha t}$; and α-transducin, which sequesters βγ subunits. Subsequent studies in *Xenopus* embryos found that rat Fz-2 requires G proteins to activate two Ca^{++}-sensitive enzymes, PKC [15] and CaMKII [16]. Activation of both these enzymes by rat Fz-2 was also inhibited by pertussis toxin and α-transducin, confirming that Wnt/Ca^{++} signaling by rat Fz-2 is mediated, directly or indirectly, by G proteins.

In order to determine whether G proteins mediate Fz signaling directly, chimeric receptors were constructed to control the activation state of Fz. The intracellular loops of rat Fz-1 and -2 were substituted for the cognate loops of the

Table I Conserved Sequence Characteristics in the G-Protein-Coupled Receptors of the Rhodopsin (Rho) and Smoothened (Smo) Families and Frizzleds (Fz)

Conserved sequence characteristic	Rho	Smo	Fz
Putative signal peptide	Y	Y	Y
Potential N-linked glycosylation sites	Y	Y	Y
Cysteine-rich domain	N	Y	Y
Seven predicted transmembrane domains	Y	Y	Y
Cysteines in extracellular loops 1 and 2	Y	Y	Y
DRY or ERW motif	Y	N	N
Prolines in transmembrane domains 4–6	Y	4,5 only	Y
Leucine-rich transmembrane domain 5	Y	Y	Y
Lys–X–X–Lys in intracellular loop 3	Y	N	Y
Cysteine in intracellular COOH terminus	Y	Y	N

Table II G-Protein Subunits Required for Wnt-Fz Signaling

Activator	System	Response	Required[a]	Not required
Xwnt-8	*Xenopus*	Axis duplication	$G_{\alpha q}$	$G_{\alpha i}$, $G_{\alpha o}$, $G_{\alpha t}$
rat Fz-1	*Xenopus*	Gene transcription	$G_{\alpha i}$, $G_{\alpha o}$, $G_{\alpha t}$	—
β2-AR/Fz-1	F9 cells	Topflash activation	$G_{\alpha o}$, $G_{\alpha q}$	$G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha 11}$, β
rat Fz-2	*Zebrafish*	Intracellular [Ca^{++}]	$G_{\alpha i}$, $G_{\alpha o}$, $G_{\alpha t}$, β	—
rat Fz-2	*Xenopus*	PKC activation	$G_{\alpha i}$, $G_{\alpha o}$, Gαt, βγ	—
β2-AR/Fz-2	*Xenopus*	CaMKII activation	$G_{\alpha i}$, $G_{\alpha o}$, $G_{\alpha t}$	—
β2-AR/Fz-2	F9 cells	Primitive endoderm	$G_{\alpha o}$, $G_{\alpha t}$, β	$G_{\alpha q}$, $G_{\alpha i}$, $G_{\alpha 11}$
β2-AR/Fz-2	F9 cells	Ligand affinity shift	$G_{\alpha o}$, $G_{\alpha t}$	$G_{\alpha s}$

[a]Inhibition of these G-protein subunits interfered with Fz signaling.

β2-adrenergic receptor (β2-AR), so that Fz signaling domains could be kept in an inactive state using a β2-AR antagonist and quickly activated by a β2-AR agonist [17,18]. Stimulation of the β2-AR/rat Fz-2 chimera activated CaMKII within just10 minutes, and that effect was inhibited by treatment with pertussis toxin [16]. The dependence on G proteins for such a rapid response to Fz signaling indicated that G proteins must be integral to Fz signaling. Actual binding of G proteins to Fz has not been reported but can be inferred from the observation of a shift in agonist affinity of the β2-AR/rat Fz chimeras in the presence of a nonhydrolyzable GTP analog. The presence of GTP causes a reduction in the affinity of most GPCRs for their agonists; the decrease correlates with dissociation of the GPCR from the G protein. The β2-AR/rat Fz chimeras exhibit this classic affinity shift, suggesting that intracellular residues of rat Fz-1 [17] and rat Fz-2 [19] directly bind G proteins also. Together, these experiments suggest that Fzs interact directly with G proteins to activate cytoplasmic signaling molecules.

Additional work aimed to show that Fzs require G proteins to mediate cellular and physiological processes. First, it was observed that a GPCR known to stimulate Ca^{++} signaling, 5-HT1c, and Xwnt-5A produce the same overexpression phenotype in *Xenopus* embryos [20]. It was then shown that Wnts require G proteins to produce the classic duplicated-axis over-expression phenotype. The regulator of G-protein signaling (RGS4), which enhances the intrinsic GTPase activity of $G_{\alpha i}$ and $G_{\alpha q}$ subunits blocked the ability of Wnt, but not Dsh, to induce duplicated axes in *Xenopus* embryos [21]. This observation placed G proteins between Fz and Dsh for the first time. In cultured mammalian F9 cells, pertussis toxin and oligonucleotides antisense to specific G proteins inhibited both induction of primitive endoderm by rat Fz-1 [22] and the β2-AR/rat Fz-2 chimera [18] and activation of a Lef/Tcf specific reporter gene by rat Fz-1. This result was confirmed in *Xenopus* embryos where pertussis toxin blocked activation of Wnt-responsive genes by rat Fz-1 [17]. Recently it has been shown that activation of Frizzled-2 in mouse tititpotent F9 cells involves activation of cyclic GMP phosphodiesterase and a sharp decline in the intracellular concentration of cyclic GMP [23]. Inhibitors of cyclic GMP phosphodiesterases block aspects of Frizzled-2 signaling in the F9 cells as well as in zebrafish oocytes. Wnt-5A and G-protein signaling are required also for collagen-induced DDR1 receptor activation and normal cell adhesion [24]. Taken together, these studies indicate that Fzs not only require G proteins to activate intracellular signaling enzymes, but also couple to G proteins to regulate physiologically relevant events.

Perspective

Understanding basic development and human disease requires better understanding of Fz signal transduction. Recent work demonstrates that G proteins are directly required for Fz signaling, supporting the inclusion of the Fz family within the greater GPCR superfamily. Rat Fz-1 and rat Fz-2 were used as model Fzs and demonstrated different but overlapping G-protein requirements (Table 2). Because these studies relied upon inhibiting effects of Wnts or involved over-expression of Fzs and not endogenous cellular processes, it remains to be seen specifically which Fzs couple to which G proteins during endogenous signaling events. The identification of Fz coreceptors combined with the large number of Wnts, Fzs, and G proteins, which are often expressed in tissue-specific patterns, increases the complexity of defining these important signaling pathways.

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CHAPTER 31

Agonist-Induced Desensitization and Endocytosis of G-Protein-Coupled Receptors

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Introduction

Multiple mechanisms contribute to the physiological regulation of G-protein-coupled receptors (GPCRs). Early studies delineated the existence of distinct functional processes of receptor regulation in natively expressing cells and tissues [1,2]. More recent studies have led to an explosion of new information regarding cellular and molecular mechanisms of receptor regulation [3–5]. We begin this chapter by reviewing some functional processes of GPCR regulation defined in early studies, followed by a review of our current understanding of some specific mechanisms that mediate (or contribute to) these processes of regulation. In doing so, we focus on relatively well-characterized mechanisms of desensitization and endocytosis that are relevant to the regulation of a large number of GPCRs. Finally, we briefly mention insights from recent studies suggesting some previously unanticipated features of GPCR desensitization and endocytosis.

General Processes of GPCR Regulation

Desensitization and Resensitization: Rapid Regulation of the Functional Activity of Receptors

Many GPCRs are regulated very rapidly after agonist-induced activation, a process that has been characterized in considerable detail in studies of the β2 adrenergic receptor (β2-AR) [3–5]. Upon binding of agonist the β2-AR promotes guanine nucleotide exchange on its cognate heterotrimeric G protein (G_s), which thereby activates downstream effectors such as adenylyl cyclase. Receptor-mediated signaling via this pathway occurs within seconds after the initial addition of agonist to cells or tissues. Within several minutes the ability of the same concentration of the same agonist to stimulate adenylyl cyclase diminishes greatly. This process of *rapid desensitization* can make the tissue refractory to even high concentrations of agonist. In some cases, the physiological responsiveness of the tissue can return quite rapidly (within several minutes) after agonist washout, allowing the cell or tissue to respond again when rechallenged with the same agonist. This recovery of signaling potential from the desensitized state is called *resensitization*. Rapid desensitization of β2-AR-mediated signaling can occur without significant effects on other signaling pathways and without any detectable change in the total number of receptors present in cells or tissues. These processes of desensitization and resensitization were therefore proposed to reflect primarily changes in the *functional activity* of receptors.

Sequestration: Rapid Regulation of the Subcellular Localization of Receptors

Agonists can also cause a pronounced decrease in the number of receptors present in the plasma membrane, usually within several minutes after the onset of rapid desensitization.

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This process, called *sequestration*, was defined originally by pharmacological studies investigating the number of receptor sites accessible to membrane-impermeant radioligands in intact cells. In general, sequestration occurs without any change in the total number of receptors present in cells or tissues, as detected using membrane-permeant radioligands or disrupted membrane preparations [6]. Therefore, it was proposed that sequestration represents primarily a change in the subcellular *localization* of GPCRs.

Downregulation and Upregulation: Slower Modulation of the Total Number of Receptors

The term *downregulation* refers to a distinct process associated with reduced responsiveness of cells or tissues that occurs much more slowly than the process of rapid desensitization. Instead of occurring in seconds or minutes, downregulation is often observed hours or even days after exposure of cells or tissues to ligands. The process of downregulation is characterized pharmacologically by a decrease in total number of receptor sites (B_{max}), detected in radioligand binding assays using membrane-permeant compounds or disrupted membrane preparations, and is not associated with a change in ligand binding affinity (K_d) [1,7]. Recovery of receptor number (and signaling responsiveness) after downregulation is a slow process that requires biosynthesis of new receptor protein. Some ligands (typically antagonists) can induce the opposite process, *upregulation*, which refers to a gradual increase in the B_{max} detected by radioligand binding [8]. Thus, downregulation and upregulation primarily reflect changes in the total *number* of GPCRs.

Mechanisms of GPCR Desensitization and Endocytosis

Functional Uncoupling of GPCRs from Heterotrimeric G Proteins Mediated by Receptor Phosphorylation

Extensive studies of certain GPCRs, such as rhodopsin (a light-activated GPCR) and β2-AR (a ligand-activated GPCR), established a highly conserved mechanism that regulates the functional activity of many GPCRs [3–5,9]. This mechanism involves the phosphorylation of receptors by a specific family of G-protein-coupled receptor kinases (GRKs) followed by the interaction of phosphorylated receptors with cytoplasmic accessory proteins called *arrestins*. Arrestin-bound receptors are unable to couple to heterotrimeric G proteins and disrupt the pathway of GPCR-mediated signal transduction at the earliest stage (Fig. 1A and B).

Biochemical studies of signal transduction in isolated rod outer segment preparations identified a protein, *rhodopsin kinase* (or GRK1), that inhibited the ability of light-activated rhodopsin to activate transducin. Light-activated rhodopsin is a good substrate for phosphorylation by rhodopsin kinase, whereas rhodopsin that has not been activated by light is a poor substrate [10]. Phosphorylated rhodopsin was only partially inhibited in activating transducin. A second protein, *visual arrestin*, was identified from cytoplasmic fractions of rod cells according to its ability to completely inhibit, or arrest, activation of transducin by phosphorylated rhodopsin [11].

Studies using functional reconstitution of β2-AR-mediated activation of adenylyl cyclase provided strong evidence for a role of phosphorylation in mediating rapid desensitization of a ligand-activated GPCR [12]. Biochemical purification of the cytoplasmic activity responsible identified a protein called β adrenergic receptor kinase (BARK, or GRK2), which preferentially phosphorylates agonist-occupied receptors [13] and is similar in structure to rhodopsin kinase [14]. Biochemical reconstitution studies indicated that increasingly purified fractions of BARK exhibited reduced ability to attenuate β2-AR-mediated signal transduction in reconstituted membrane preparations. Further analysis of this effect led to the identification of a distinct protein component that was lost in increasingly purified fractions and which increased functional desensitization when added back to highly purified fractions of BARK [15,16]. This protein cofactor turned out to be a protein similar to visual arrestin and was therefore named *nonvisual arrestin*, or β arrestin (βArr). cDNA cloning has identified a family of arrestins involved in regulating the function of phosphorylated GPCRs [5].

It turns out that agonists regulate not only phosphorylation of GPCRs by GRKs but also the affinity with which phosphorylated receptors bind to arrestins [17]. Such dual control by agonist of a single regulatory mechanism is an example of coincidence detection, an important principle guiding many other signaling processes. One role of coincidence detection in GRK/arrestin-mediated regulation may be to assure definitively that only those receptors actually activated by agonist are desensitized. In this way, other receptors that are not activated (including coexpressed GPCRs that recognize other ligands but are potentially upregulated by the same desensitization mechanism) are not affected. Indeed, GRK-mediated phosphorylation and subsequent binding of arrestins is generally considered to be a paradigm for *homologous desensitization*, a form of desensitization that is specific only to the activated GPCR at hand and is not influenced by (or extended to) activation of other receptors in the same cell [13]. Coincidence detection may serve other important functions in GPCR regulation. For example, one might imagine that transient or low-frequency activation of GPCRs could promote GRK-mediated phosphorylation of receptors without much binding of arrestins, thus causing only partial desensitization of receptors (because phosphorylated receptors can still interact weakly with heterotrimeric G proteins). More prolonged or higher frequency activation of receptors by strongly promoting both phosphorylation of receptors and arrestin binding (which essentially blocks receptor–G protein coupling), could lead to a more profound desensitization of signal

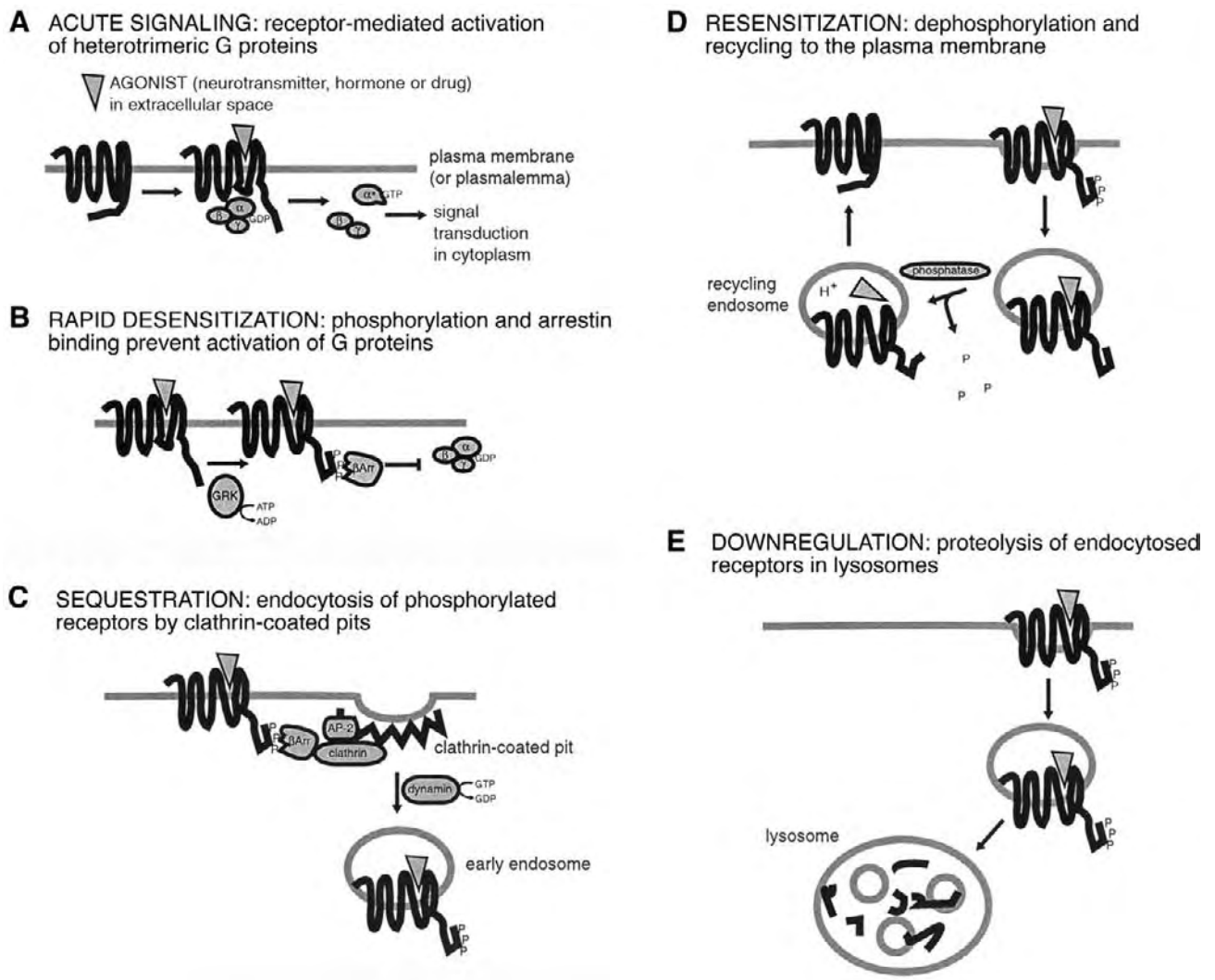


Figure 1 Major mechanisms of GPCR desensitization and endocytosis. (A) Classical pathway of GPCR signaling via receptor-mediated activation of heterotrimeric G proteins. (B) Rapid desensitization (functional uncoupling) of GPCRs mediated by GRKs and arrestins. (C) Role of GRKs and non-visual (beta-) arrestins (βArr) in promoting endocytosis of GPCRs via clathrin-coated pits. (D) Role of endocytosis in mediating resensitization of GPCRs. (E) Role of endocytosis in mediating downregulation of GPCRs by proteolysis in lysosomes.

transduction. In this way, functional desensitization could be modulated both by the strength and kinetics of receptor activation.

Desensitization of GPCRs by Other Kinases: Example of a Mechanism Mediating Heterologous Desensitization

Other kinases, such as the so-called second-messenger-regulated kinases, are also implicated in mediating desensitization of GPCRs. For example, the β2-AR can be phosphorylated by cyclic-AMP-dependent protein kinase (PKA). PKA-mediated phosphorylation of a single residue located in the third intracellular loop of the β2-AR impairs the ability of the receptor to couple to G_s and thereby attenuates receptor-mediated activation of adenylyl cyclase [18–20]. Phosphorylation of this residue is thought to impair receptor–G protein coupling directly, without requiring any known protein cofactor such as an arrestin. An important feature of PKA is that this kinase can phosphorylate β2-ARs whether or not they have been activated by ligand, in contrast to the preferential phosphorylation of agonist-activated receptors by GRKs. Because PKA is activated by cyclic AMP (a signaling intermediate produced as a result of β2-AR activation), PKA-mediated phosphorylation of the β2-AR is an example of feedback inhibition by a second messenger. In addition, because activation of any other receptor that stimulates adenylyl cyclase can also activate PKA, phosphorylation of the β2-AR by PKA is generally considered to be a paradigm for *heterologous desensitization*—that is, desensitization of one type of GPCR that is induced by activation of another (heterologous) receptor. Heterologous desensitization of GPCRs by kinases such as PKA, in contrast to homologous desensitization mediated by GRKs, is thought to play

important roles in integrating and controlling "cross-talk" between diverse signaling pathways in the same cell.

Agonist-Induced Endocytosis of GPCRs

Pharmacological studies of the process of sequestration led to the hypothesis that certain GPCRs are removed from the plasma membrane within minutes after agonist-induced activation [6,21]. Biochemical and immunochemical methods have demonstrated that this is indeed the case, both in cultured cells and certain native tissues [22–24]. Rapid endocytosis of the β2-AR is mediated by an agonist-dependent lateral redistribution into clathrin-coated pits [25]. Coated pits then pinch off from the plasma membrane to form endocytic vesicles, a process dependent on the cytoplasmic GTPase *dynamin* [26–29]. Subsequent studies have demonstrated that regulated endocytosis of several other GPCRs is also mediated by a dynamin-dependent mechanism, suggesting a conserved role of clathrin-coated pits in mediating endocytosis of many GPCRs.

Clathrin-coated pits play a general role in mediating rapid endocytosis of a large number of cell-surface components besides signaling receptors, many of which are endocytosed constitutively (i.e., in a ligand-independent manner). This has raised the question of how GPCR endocytosis is regulated by ligands. It turns out that GRKs and arrestins, in addition to their previously established role in mediating functional uncoupling of receptors from heterotrimeric G proteins, also play an important role in regulating endocytosis of certain GPCRs. In particular, nonvisual (or β-) arrestins can promote the concentration of phosphorylated receptors in coated pits by binding simultaneously, via distinct protein interaction domains, to both receptors and the clathrin-containing lattice structure, thereby functioning as adapters linking specific GPCRs to endocytic membranes [30,31] (Fig. 1C). Despite the highly conserved nature of this endocytic mechanism, there are also examples of GPCRs that either are not rapidly endocytosed or are endocytosed by a different mechanism [32]. This diversity of GPCR membrane trafficking, although not yet understood at the mechanistic level, has important implications for the physiological regulation of distinct GPCRs [33].

Functional Consequences of GPCR Endocytosis

Role in Rapid Desensitization of GPCRs

In many cases, endocytosis is not thought to play a primary role in mediating rapid desensitization of many GPCRs, although the precise role of endocytosis in this process may depend on receptor expression level. Endocytosis of μ-opioid receptors does not contribute significantly to functional desensitization in cells expressing relatively high levels of receptor protein but does appear to cause desensitization in cells expressing lower levels of receptor [34]. Studies of the β2-AR emphasize that GRK- and arrestin-dependent uncoupling of receptor from G protein (Fig. 1B) occurs in the plasma membrane before endocytosis begins, and desensitization of the β2-AR is not affected by blockade of receptor endocytosis [35].

Role in Resensitization of GPCRs

In contrast to its limited role in mediating rapid desensitization, endocytosis of certain GPCRs is thought to play a major role in mediating the distinct process of receptor resensitization [4,36,37]. It is believed that the reason for this is that endocytosis brings receptors in close proximity to an endosome-associated phosphatase, which dephosphorylates receptors that were previously phosphorylated (hence, *desensitized*) at the cell surface. Dephosphorylated receptors are then recycled back to the plasma membrane in a "resensitized" state, which is fully functional to mediate subsequent rounds of signal transduction upon re-exposure to agonist [35,38]. This proposed mechanism of GPCR resensitization is shown in Fig. 1D.

Role in Mediating Proteolytic Downregulation of GPCRs

Endocytosis is also thought to play an important role in mediating downregulation of many GPCRs by promoting proteolysis of receptors. The best characterized pathway mediating proteolytic downregulation of GPCRs involves endocytosis of receptors followed by membrane trafficking to lysosomes (Fig. 1E). Additional proteolytic machinery, such as proteasomes or cell-associated endoproteases, are also implicated in mediating downregulation of certain GPCRs [39]. GPCRs may be targeted to lysosomes after initial endocytosis by clathrin-coated pits or may follow a distinct membrane pathway involving alternate mechanism(s) of endocytosis [7,39]. In some cases it is clear that receptors endocytosed by clathrin-coated pits can be targeted to a rapid recycling pathway mediating resensitization of receptors as well as to a degradative pathway mediating receptor trafficking to lysosomes. Furthermore, distinct GPCRs differ in their sorting between divergent membrane pathways when coexpressed in the same cells [40,41]. Recent studies have identified cytoplasmic sequences present in certain GPCRs that promote sorting of internalized receptors to lysosomes [42], as well as sequences that promote [43] or prevent [44] rapid recycling of receptors from endocytic vesicles to the plasma membrane. It is likely that there exist multiple biochemical mechanisms which distinguish the postendocytic sorting of specific GPCRs and that these distinct mechanisms play critical roles in determining the precise functional consequences of agonist-induced endocytosis.

Role in Controlling the Specificity of Signal Transduction

Endocytosis of GPCRs may also control the specificity with which receptors signal to or via certain downstream effectors, such as mitogen-activated protein (MAP) kinase

modules, although the physiological significance of this regulation has not yet been established. A number of mechanisms have been proposed, generally involving the formation on endosome membranes of a protein complex including internalized GPCRs and signal-transducing kinases (such as c-Src) recruited from the cytoplasm [45,46] or receptor tyrosine kinases (such as epidermal growth factor receptors) co-endocytosed from the plasma membrane [47].

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CHAPTER 32

Functional Role(s) of Dimeric Complexes Formed from G-Protein-Coupled Receptors

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G-protein-coupled receptors (GPCRs) form the largest known gene superfamily and are involved in the regulation of numerous physiological processes, including hormonal signaling, neurotransmission, and reception of sensory stimuli; not surprisingly, these receptors are targets for the large majority (≈90%) of clinically used drugs. All GPCRs share the same basic topology (an extracellular N terminus, seven transmembrane domains, and an intracellular C terminus) and are classified into six large families, A through F, based on sequence homology. Classically, GPCRs were thought to function as monomers; however, higher molecular weight complexes were often observed on protein electrophoretic gels, suggesting the existence of dimeric or higher order macromolecular complexes. The presence of receptor homo- and heterodimers in living cells was recently confirmed for many different GPCRs using novel experimental approaches such as fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), functional complementation assays, and studies of receptor trafficking. However, questions remain as to whether dimerization is constitutive or ligand induced for various GPCRs and what its functional significance may be. Table 1 classifies the types and functions of GPCR oligomers that have been documented.

The best examples of constitutive GPCR dimerization are found in the GPCR family C, which includes metabotropic glutamate receptors (mGluRs), $GABA_B$ receptors, extracellular Ca^{2+}-sensing receptor (CaR), and some pheromone and taste receptors. mGluR1, mGluR5, and CaR are known to form constitutive homodimers, and it was recently shown that CaR dimers represent a true signaling unit [1]. For both mGluRs and CaR, dimerization is mediated by both noncovalent and covalent interactions of the extracellular N-terminal domains [2–7]. In contrast, $GABA_B$ receptors are obligate heterodimers [8–11], and individual receptor subunits do not form functional receptors even if expressed on the plasma membrane [12–14]. The known $GABA_B$ receptor subunit interactions include the C-terminal coiled–coil interaction, important for the regulation of assembly-dependent receptor trafficking [12,14], and N-terminal domain interactions, important for ligand binding and intramolecular signaling [15,16]. Similar obligate heterodimerization has recently been reported for T1R2/T1R3 sweet taste receptors, which also belong to family C [17]. The importance of dimerization for family C GPCRs is underscored by crystallographic analysis of the mGluR1 N-terminal ligand-binding domain; this domain, homologous to bacterial periplasmic amino acid binding proteins and conserved among family C GPCRs, was shown to exist in the dimeric form even in the absence of ligand [18]. Taken together, these findings suggest that family C GPCRs are expressed on the plasma membrane as preformed constitutive dimers and that dimerization is a prerequisite for signaling in this receptor family.

What about non-family C GPCRs? Basal homodimerization was demonstrated for yeast α-factor receptors using FRET [19], β2-adrenergic [20] and thyrotropin-releasing hormone receptors [21] using BRET, and δ opioid receptors

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Table I GPCR Oligomers

Type of GPCR oligomerization and its potential functions	Examples with references
Constitutive homomerization: A role in receptor/G-protein coupling?	*Energy transfer experiments:* Yeast α-factor receptor [19] β2-Adrenergic receptor [20] δ Opioid receptor [22] Thyrotropin-releasing hormone receptor [21] *Dominant-negative trafficking effect experiments:* D2 dopamine receptor [23] V2 vasopressin receptor [24] Platelet-activating factor receptor [26]
Ligand-induced homomerization: A role in receptor maturation and trafficking?	*Energy transfer experiments:* Gonadotropin-releasing hormone receptor [28] SSTR5 somatostatin receptor [29] *Coimmunoprecipitation experiments:* B2 bradykinin receptor [27]
Heteromerization: Receptor synergy Novel agonist selectivity Novel signaling properties Novel trafficking properties	*Constitutive (coimmunoprecipitation experiments):* κ/δ opioid receptors [35] μ/δ opioid receptors [37,39] δ opioid/β2-adrenergic receptors [40] κ opioid/β2-adrenergic receptors [40] Angiotensin AT1/bradykinin B2 receptors [36] Dopamine D1/adenosine A1 receptors [38] Purinergic P2Y/adenosine A1 receptors [41] *Ligand-induced (energy transfer experiments):* SSTR5/SSTR1 somatostatin receptors [29] Somatostatin SSTR5/dopamine D2 receptors [42] δ Opioid/β2-adrenergic receptors [22]
A special case—family C GPCRs: Constitutive homo- or heterodimerization of individually nonfunctional subunits results in signaling-competent receptors	mGluR1 metabotropic glutamate receptor [4,5] mGluR5 metabotropic glutamate receptor [2,6] Ca^{2+}-sensing receptor [1,3,7] $GABA_B$ GABA-ergic receptor [8–16,33] T1R2/T1R3 sweet taste receptor [17]

using both BRET and time-resolved FRET [22]. In addition, for dopamine D2 receptors [23], vasopressin V2 receptors [24], chemokine CCR5 receptors [25], and platelet-activating factor receptors [26], it was shown that plasma membrane expression of wild-type receptors can be suppressed by coexpression of mutant receptors, suggesting ligand-independent, constitutive dimerization in the endoplasmic reticulum. In contrast, ligand-induced rather than constitutive homodimerization was observed for bradykinin B2 receptors using coimmunoprecipitation [27] and for gonadotropin-releasing hormone (GnRH) receptors [28] and somatostatin SSTR5 receptors [29] using FRET. The reason for these apparent differences among different GPCRs is not clear. However, it is worth noting that, at least for B2 and GnRH receptors, this agonist-induced homooligomerization was not related to receptor activation and may thus not be relevant for receptor signaling. For example, N-terminal truncation of B2 receptors eliminates bradykinin-induced receptor dimerization as well as receptor phosphorylation and downregulation, without affecting receptor function [27]. Similarly, agonist-exposed GnRH receptors exhibit slow and long-lasting microaggregation [28] not clearly related to receptor signaling. It is also worth noting that the efficiency of energy transfer depends on both the distance and the orientation of the two fluorophores; absence of FRET or BRET does not necessarily indicate absence of dimerization. The available data are thus consistent with the possibility that GPCRs constitutively dimerize, probably while still in the endoplasmic reticulum. Interestingly, in all known cases, except for the two family C examples, these preformed dimers are homomeric in nature.

What might be the functional significance of constitutive dimerization? The surface area of a GPCR monomer is barely large enough to contact the α and βγ subunits of the trimeric G protein simultaneously, and it was suggested that a receptor dimer might be necessary for G protein activation [30–32]. For $GABA_B$ receptors, it was demonstrated that while the intracellular segments of GB2 subunit were required for the G protein activation, the GB1 C terminus could be deleted and GB1 intracellular loops could be replaced with those of G_I- or G_q-coupled family C receptors without impairing function [33]. These data suggest that a single GPCR may provide all the specific G protein contacts. However, it remains to be determined whether the other subunit in a dimer provides nonspecific contacts for the βγ and/or the conserved part of the α subunit, or whether it

does not participate in G-protein activation. In addition to this potential role in G-protein coupling, constitutive GPCR dimerization may be important in receptor maturation and/or trafficking (reviewed in Bouvier [30] and Milligan [34]).

What about GPCR heterodimerization? Aside from family C receptors, the evidence for constitutive heterodimerization is based mainly on coimmunoprecipitation experiments [35–41]. Notably, no constitutive and only ligand-induced dimerization was reported for somatostatin receptor SSTR5/SSTR1 [29], SSTR5/dopamine D2R [42], and δ opioid/β2-AR complexes [22] using energy transfer approaches. Interestingly, ligand-induced δ/β2 receptor heterodimerization was observed using BRET but not the time-resolved FRET (which detects only the plasma membrane receptors). These data suggest that, at least in this case, ligand-induced oligomerization might underlie not the signaling but the trafficking events, or that ligand binding induces conformational changes that alter the efficiency of energy transfer [22]. An interesting possibility is that preformed GPCR (homo)dimers might form ligand-regulated heterooligomeric complexes on the plasma membrane, resulting in novel signaling and/or trafficking properties. This possibility is supported by several recent studies that explored the functional consequences of heterooligomerization between GPCRs that form functional monomeric or homomultimeric receptors. Receptor synergy, where application of one agonist resulted in the leftward shift of the dose–response curve for the other agonist, was reported for heterooligomers of dopamine D2R and somatostatin SSTR5 receptors [42], opioid μ and δ receptors [39], opioid κ and δ receptors [35], and angiotensin AT1 and bradykinin B2 receptors [36]. The formation of receptors with novel agonist selectivity and/or signaling properties was reported for opioid κ/δ [35] and μ/δ heterooligomers [37], as well as for heterooligomers of adenosine A1 receptors with ATP P2Y1 receptors [41] or dopamine D1 receptors [38]. These findings are particularly interesting in light of some long-standing discrepancies between pharmacologically and structurally defined receptor subtypes. For example, pharmacological properties of κ/δ heterooligomers are identical to the previously reported κ2 subtype [35]. Finally, changes in trafficking were observed with AT1/B2 [36], SSTR5/ SSTR1 [29], D1/A1 [38], and β2/δ and β2/κ receptor heterodimers [40].

In summary, it is clear that many GPCRs constitutively dimerize early in the synthetic pathway. In addition, it appears that ligand application can result in the formation of higher order heterooligomeric complexes with novel signaling and/or trafficking properties. It is hoped that future studies will elucidate whether these findings apply to all GPCRs or whether qualitative differences exist between different GPCRs with respect to their oligomerization.

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CHAPTER 33

The Role of Chemokine Receptors in HIV Infection of Host Cells

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Introduction

For retroviruses to infect cells, they must transfer their genetic material across two membranes: the envelope that surrounds the virus, and the membrane of the cell. To accomplish this, retroviruses encode an integral membrane envelope (Env) protein that not only attaches virus to the cell surface but also mediates membrane fusion. The human immunodeficiency virus (HIV) Env protein attaches viruses to the surface of CD4-positive T cells and cells of the monocyte/macrophage lineage by binding directly to CD4. However, CD4 binding by itself does not trigger the fusion activity of Env. Surprisingly, the seven-transmembrane (7TM) chemokine receptors CCR5 and CXCR4 hold the key to unlocking the fusion potential of Env; sequential binding of CD4 and one of these chemokine coreceptors by the viral Env protein leads to a series of conformational changes that ultimately result in membrane fusion, virus entry, and replication. This entry pathway provides new targets for antiviral approaches and helps to explain viral tropism and pathogenesis. In addition, use of the CCR5 and CXCR4 7TM receptors by HIV raises the possibility that virus-induced signaling could impact virus infection. In this chapter, we provide an overview of 7TM coreceptor use for HIV entry, summarize what is known about the structural basis for envelope–coreceptor interactions, and indicate how signaling via 7TM receptors may influence HIV infection.

HIV Entry

Human immunodeficiency virus type 1 and 2 (HIV-1/HIV-2) are members of the lentivirus genus of the Retroviridae family and are the causative agents of acquired immune deficiency syndrome (AIDS) [1,2]. While all HIV strains infect primary $CD4^+$ T-cells, they can differ in their relative tropism for macrophages and human T-cell lines, both of which also express CD4. Some HIV strains can infect T-cell lines but not macrophages, some can infect macrophages but not T-cell lines, and others can infect both cell types. With some exceptions, HIV tropism is largely explained by the differential expression of CCR5 and CXCR4 on these $CD4^+$ cell types, coupled with the fact that some HIV strains use CCR5 (R5 strains), some use CXCR4 (X4 strains), and others can utilize both molecules after binding to CD4 to enter cells (R5X4 strains) [3]. That tropism can be controlled merely by the presence of the correct viral coreceptor can be demonstrated with T-cell lines that typically express CXCR4 but not CCR5. Expression of CCR5 in T-cell lines makes them permissive to infection by R5 virus strains. Likewise, introduction of CXCR4 into a $CD4^+$ cell that otherwise lacks this coreceptor confers susceptibility to infection by X4 virus strains.

How does HIV use CD4 and a coreceptor to enter a cell? The viral Env protein on the surface of virions can be triggered to undergo a dramatic conformational change that allows it to fuse the virus membrane with that of a cell. Triggering is caused by receptor engagement, which explains why the presence or absence of CCR5 or CXCR4 along with CD4 governs the susceptibility of a cell to HIV infection.

The HIV Env protein is first synthesized as a 160-kDa precursor glycoprotein (gp160) in the endoplasmic reticulum. Like many other viral membrane proteins, including those of other retroviruses, Env assembles into homotrimers after which each subunit is cleaved by a host protease into

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two parts, which in the case of HIV are termed gp120 and gp41. The gp120 subunit is external to the viral membrane and is noncovalently associated with the gp41 subunit, which traverses the virus membrane. This cleavage event is essential to render Env fusion active [4]; in the absence of cleavage, Env is expressed on the cell surface, is incorporated into virus particles, and can bind to its receptors; however, it cannot cause membrane fusion. The reason is that the cleavage liberates what is now the N-terminus of the gp41 subunit. The hydrophobic N-terminal domain of gp41 acts as a fusion peptide that functions much like a harpoon, ultimately spearing the membrane of the cell during the fusion process. Many other membrane-bound viruses express proteins that, like Env, are cleaved by a host cell protease in a manner that liberates a hydrophobic fusion peptide, suggesting a common membrane fusion mechanism.

How does the fusion peptide actually cause membrane fusion? It is thought that this hydrophobic domain is hidden in the native Env protein. During virus entry, the gp120 subunit of Env interacts with CD4. This induces conformational changes within Env that then enable an interaction with either CCR5 or CXCR4. Coreceptor binding is thought to be the final trigger that induces further conformational changes in Env that result in exposure of the hydrophobic fusion peptide in gp41. The fusion peptide is then thought to insert into the membrane of the host cell, making gp41 an integral component of two membranes: the viral membrane in which it is lodged and the cellular membrane it has speared. To actually cause membrane fusion, the lipids in both membranes must be brought together. This, too, is accomplished by gp41, which folds back on itself, much like closing a jackknife. This final conformational change is associated with a significant change in free energy that likely provides the force required to elicit a fusion pore [5]. A model for HIV entry is depicted in Fig. 1.

Coreceptor Use *In Vivo*

Regulating expression of CD4, CCR5, and CXCR4 on cell lines *in vitro* controls whether or not a cell can be infected by HIV. Receptor expression, as well as virus tropism, also regulates virus infection *in vivo*. For HIV-1, R5 viruses are predominantly transmitted, are the major virus population in asymptomatic individuals [6,7], and usually remain present throughout the course of infection [8–10]. R5X4 viruses may precede the evolution to X4 tropism, which occurs in less than 50% of AIDS patients [6,11]. A broadening of coreceptor specificity and the development of X4 tropic strains is linked with disease progression in HIV-1 infection [6]. The importance of CCR5 in transmission of HIV-1 was revealed with the discovery of a 32-bp deletion in the CCR5 gene (Δ32 CCR5) that ablates cell-surface expression [12–14]. Approximately 1% of the Caucasian population are homozygous for this deletion, and these CCR5-negative individuals are highly resistant to HIV infection [7,12–14]. Only a few cases of HIV infection in Δ32/Δ32 CCR5 individuals have been reported resulting from heterosexual, homosexual, and blood transfusion transmission routes, with X4 tropic viruses being transmitted where characterized [15–20]. Heterozygosity for Δ32 CCR5 may confer very modest protection against HIV-1 transmission [14,21,22] but is clearly associated with delayed progression to AIDS [12,23–25].

Env Domains Involved in Coreceptor Interactions

The gp120 subunit of Env binds to both CD4 and CCR5/CXCR4. It contains five variable regions (V1–V5) interspersed between five conserved domains (C1–C5), and is very heavily glycosylated. The third variable region, or V3-loop, plays a major role in governing whether Env interacts with CXCR4, CCR5, or both coreceptors. The first and second variable loops, termed the V1/V2 region, play a more subsidiary role. Amino acid changes in the V3 loop can result in a coreceptor switch, while changes in V1/V2 do so less commonly [26–29]. Other regions of gp120 are also involved in coreceptor binding as revealed by the crystal structure of a core of HIV-1_{HXBc2} gp120 glycoprotein coupled with site-directed mutagenesis (see Fig. 2) [30,31].

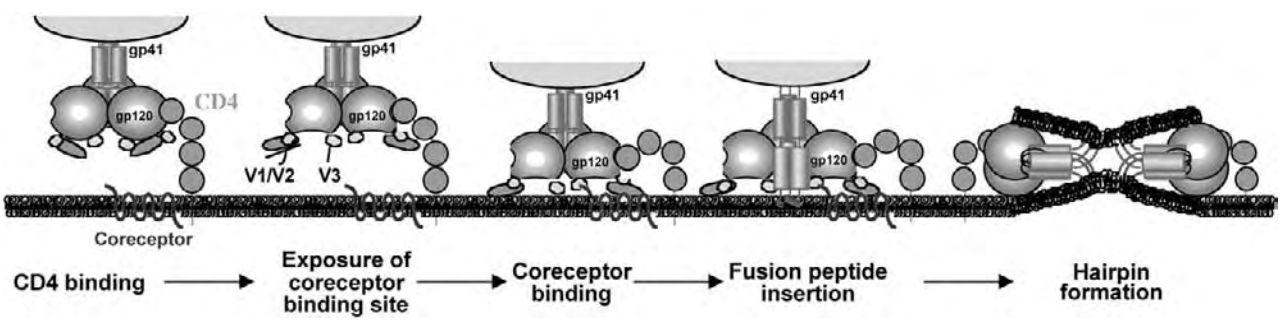


Figure 1 Model of HIV entry. The interaction of gp120 with CD4 at the cell surface induces conformational changes in Env that result in the exposure of a coreceptor binding site. Binding to a 7TM-coreceptor molecule induces further conformational changes that may involve fusion peptide insertion into the target cell membrane. The ectodomain of gp41 is then likely to fold back upon itself to form a coiled-coil bundle, causing apposition then fusion of viral and cellular membranes. (Adapted from Doms, R. W., *Virology*, 276, 229–237, 2000.)

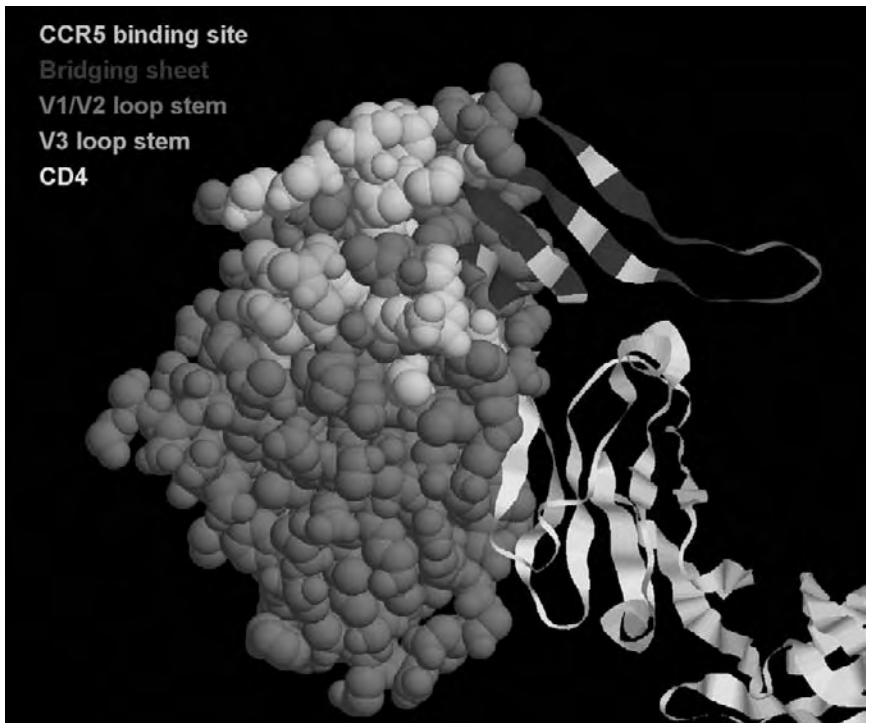


Figure 2 CCR5 binding site on gp120. Space-filling model of gp120 with the conserved bridging sheet domain and two-domain sCD4 in ribbon format. Residues involved in CCR5 binding are indicated, as are the V1/V2 and V3 loop stems. Model was rendered with RasMol 2.7.1 from the Protein Databank file 1GC1.PDB, gp120 crystal structure [30].

To aid in crystallization, the gp120 was deglycosylated, the V3 and V1/V2 loops were genetically deleted, and the protein was cocrystallized with a two-domain soluble CD4 molecule and a Fab fragment of a monoclonal antibody that binds to an epitope in gp120 that is induced by CD4 binding (Fig. 2). The epitope recognized by this antibody is highly conserved across different virus strains and is located between the base of the V3 loop and the base of the V1/V2 region, near or within the bridging sheet domain (Fig. 2). Interestingly, mutations in this conserved domain diminish the ability of gp120 to bind to CCR5 [31]. The model that logically follows is that CD4 binding results in the exposure or generation of this highly conserved portion of gp120, perhaps as the result of repositioning of the V2 and V3 loops [30,32]. This conserved domain and V3 together form a CCR5 receptor-binding domain. This is in some ways reminiscent of how chemokines bind to their receptors, which is thought to likewise involve two regions of the chemokine.

Coreceptor Domains Involved in HIV Infection

The structure of rhodopsin and the presence of conserved Cys residues in each of the extracellular domains of CCR5 and CXCR4 suggest that the four extracellular regions interact closely with each other [33]. All extracellular domains of CCR5 (N terminus [Nt]; extracellular regions 1 [E1], 2 [E2], and 3 [E3]) have been implicated in mediating infection of various R5 tropic HIV strains, with Nt and E2 being required by the majority [34–38]. Additionally, HIV-1 strains that use both CCR5 and CXCR4 have been found to interact differently with CCR5 compared to R5 viruses [37]. CCR5-restricted viruses may evolve to become R5X4 tropic by acquiring the ability to interact with the extracellular loops of CXCR4 while retaining the ability to interact with the Nt of CCR5 [39]. The involvement of multiple coreceptor domains in Env binding is perhaps therefore not surprising. As for CCR5, multiple regions of CXCR4 are important for coreceptor activity, with the E2 loop being particularly important [39–43]. The surface of CXCR4, and particularly E2, has a greater negative charge than that of CCR5, perhaps explaining why X4 tropic V3 loops are more positively charged than those of R5 viruses.

Receptor Presentation and Processing

The expression of receptors on a particular cell type does not necessarily indicate susceptibility to infection. As for other enveloped viruses, fusion mediated by the HIV Env protein is likely to require several Env trimers, each in turn triggered by independent receptor binding events (reviewed in Doms [44]). Thus, receptor density is clearly a factor that impacts the efficiency of virus infection. The fact that Δ32-CCR5 heterozygotes exhibit delayed progression to AIDS despite only a modest reduction in CCR5 expression levels indicates that coreceptor expression can be limiting for virus infection *in vivo*. Thus, cells that express higher levels

of coreceptor may be more susceptible to virus infection. The requirement for multiple coreceptor binding events may in part explain why some viruses that bind to CXCR4 with low affinity cannot infect macrophages, which express low levels of CXCR4. If CXCR4 expression is increased, infection of macrophages by these viruses can occur [45]. Thus, the ability of some primary X4 virus strains to infect macrophages may be due to their increased affinity for this coreceptor relative to TCLA virus strains [46–48]. Finally, in addition to receptor density, receptor conformation and processing may influence virus infection. Both CCR5 and CXCR4 have been shown to exist in antigenically distinct conformations, not all of which may function equally well as virus coreceptors [49,50]. Posttranslational modifications of CCR5 and CXCR4 can also impact their coreceptor activity. There are two N-linked glycosylation sites in Nt and E2 of CXCR4, and mutation of these sites can enhance HIV fusion and infection several-fold [40–42]. The N-terminal domain of CCR5 contains tyrosine residues that are sulfated, and sulfation contributes substantially to infection by HIV-1 [51].

Role of Signaling in HIV Infection

Env protein binding to CCR5 and CXCR4 can trigger signaling via these receptors, though the importance of this for virus infection is not known. Several studies have shown conclusively, through the expression of signaling-deficient receptors, that receptor internalization or intracellular signaling through inhibitory guanine nucleotide-binding regulatory (G_i)-proteins is not required for viral entry of cell lines [34,39,42,52,53]. However, a number of studies indicate that signaling may facilitate HIV infection of primary cells and may be especially important for post-entry viral events (reviewed in Kinter *et al.* [54]). These inferences are not definitive due to their reliance on signaling inhibitors that can exert their effects by other means.

Signaling mediated by chemokines may also impact virus replication, though these effects can be double-edged. Chemokines that bind to CCR5 or CXCR4 can block virus infection by preventing Env-coreceptor interactions. However, these same chemokines may also be able to enhance HIV infection and replication in T cells, in part by activation of signal transduction pathways [55–58]. The mechanisms of this enhancement have been reported to involve either tyrosine kinase or G_i protein-dependent signaling cascades, to increase viral attachment via glycosaminoglycans, to enhance CXCR4 transcription and thus viral attachment, or to enhance CD4 : CXCR4 colocalization. The mechanisms by which HIV or chemokines induce receptor signaling, the signaling pathways that are activated, and their effects on different steps in the virus life cycle clearly must be examined in more detail, as this may help explain the post-entry restriction of some viruses that fail to replicate in macrophages [59–62] as well as lead to the development to new therapeutics.

Summary

The discovery that HIV uses the chemokine receptors CCR5 and CXCR4 in conjunction with CD4 to infect cells helped explain viral tropism and pathogenesis. The expression pattern of CCR5 and CXCR4 coupled with their differential use by diverse virus strains largely explains HIV tropism, at least at the level of entry. Their discovery led directly to the identification of receptor-related polymorphisms that determine the genetic basis for the resistance to infection exhibited by some individuals and help explain the variable progression rates to AIDS of others. Subsequent structural studies have revealed the presence of highly conserved domains in the viral Env protein that are responsible for receptor binding and membrane fusion and that are real or potential targets for antiviral agents and neutralizing antibodies. The Env protein can also induce signaling via either CCR5 or CXCR4. While receptor signaling is not required for virus replication in transformed cell lines, it may modulate virus infection of primary cell types. The utilization of CCR5 and CXCR4 for the membrane fusion reaction is clear, but the role of signaling in the infection of primary cells is not and is clearly an area that requires further investigation.

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CHAPTER 34

Chemotaxis Receptor in Bacteria: Transmembrane Signaling, Sensitivity, Adaptation, and Receptor Clustering

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While the chemotactic signaling pathway of bacteria has been established [1,2] well enough to enable accurate computer simulation of the migration of virtual bacteria [3], detailed molecular mechanisms underlying ligand-mediated transmembrane signaling, high receptor sensitivity, receptor adaptation, and one broad dynamic range of sensitivity to attractants remain to be elucidated.

Bacteria rapidly respond to changes in concentrations of critical chemicals in their environment by chemotaxis—that is, a swimming pattern biased toward or away from particular stimuli [4]. The chemotaxis pathway includes chemosensory receptors and a phosphotransfer system known as the two-component signal transduction pathway [5–8]. It is well established that bacteria sense their environmental changes over time [9]. Like many other sensory perception processes, bacterial chemotaxis has high sensitivity and broad dynamic range [10–12]. The sensitivity allows the binding of attractants to less than 1% of the receptors to induce increased swimming motion of *Escherichia coli* [13]; this high sensitivity does not appear to be due to a signal amplification step downstream of the receptor [14]. As for the dynamic range, bacteria can detect the gradient of attractants such as aspartate under background concentrations (from nanomolar to millimolar) spanning five to six orders of magnitude [13,15,16].

Most bacterial chemoreceptors belong to a family of transmembrane methyl-accepting chemotaxis proteins (MCPs). MCPs can be divided into at least four subfamilies on the basis of the disposition of hydrophobic regions that are predicted to be transmembrane helices by hydrophathy analysis [17]. Family A includes two transmembrane helices separated by a substantial periplasmic domain (Fig. 1). Whereas the other families possess less periplasmic and less transmembrane components (for a recent review, see Falke and Kim [18]). Family A also includes the chemoreceptors for aspartate, serine, ribose and galactose, peptide, citrate, and osmolarity [19,20]. The architectures of selected family chemoreceptors are well understood and are likely to represent many other members of family A. The folding unit of the chemoreceptor is a stable homodimer [21]. Each receptor monomer consists of a ligand binding region (periplasmic sensory domain) made up by an anti-parallel, four-helix bundle (α1, α2, α3, and α4), an antiparallel two-helix (TM1, TM2) transmembrane region, and a long two-helix hairpin region (cytoplasmic signaling domain) (Fig. 1). The receptor dimer is thus an elongated helical bundle thought to be oriented normal to the membrane plane [22–27]. The length of one *E. coli* serine receptor model spans $\approx$380 Å from one end to the other [27]. The incoming signal propagates through the cytoplasmic domain, which is coupled by a scaffolding protein CheW to the signaling kinase CheA [8,28]. CheA in turn regulates two response regulators: CheY (in the phosphosignaling branch of the pathway) and CheB (in the adaptation branch). Attractant binding attenuates CheA activity and, ultimately, via the phosphosignaling branch, reduces the overall frequency of tumbling (by clockwise rotation of flagella) (Fig. 2) [8].

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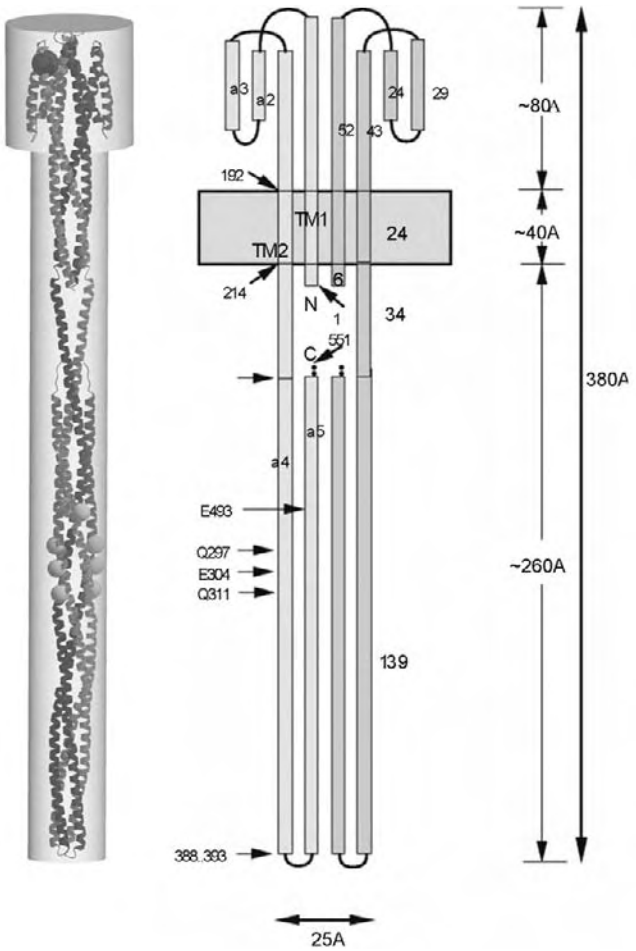


Figure 1 A model of intact *E. coli* Tsr chemotaxis receptor dimer [27]: one monomer is blue, the other pink. The presumed membrane bilayer is represented by a gray horizontal band. The model is about 380 Å long and consists of about 80-Å-long ligand binding domains, an about 40-Å long transmembrane domain, and an about 260-Å long cytoplasmic domain. The length of each domain is shown. (Left) Ribbon diagram of the intact Tsr dimer model viewed perpendicular to the non-crystallographic two-fold symmetry axis. The dimensions are scaled to match those of the schematic figure at right. One monomer is purple, the other cyan. Methylation sites are marked by yellow balls in one monomer and orange balls in the other, and the ligand serine is red (partially hidden at upper left corner). Some landmark residues are shown.

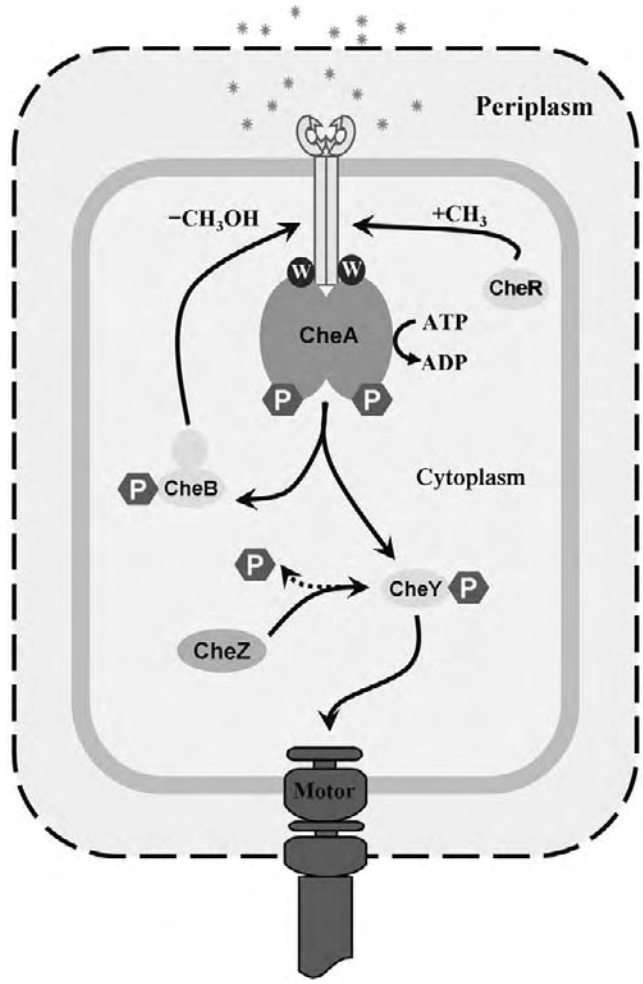


Figure 2 A schematic presentation of the chemotaxis pathway. The chemotaxis receptors are coupled to the histidine kinase CheA by CheW. Binding of attractant at the periplasmic domain of the receptor downregulates the phosphorylation rate of CheA. CheA is phosphorylated by hydrolyzing adenosine triphosphate (ATP). The phosphorylated CheA activates two response regulators CheB and CheY. Phosphorylation of CheB activates its effector domain, which demethylates four glutamate sites of the receptor cytoplasmic domain, while a methyltransferase CheR constitutively methylates these sites. Phosphorylation of CheY modulates the flagella motor. Phosphorylated CheY docks the motor switch apparatus and controls the direction of motor rotation (clockwise rotation of flagella causing tumbling behavior of bacteria). Both CheY and CheB catalyze autodephosphorylation. CheZ, a phosphatase, enhances the dephosphorylation rate of CheY.

Signaling at Periplasmic Ligand Binding Domain

In contrast to many other receptors, such as growth hormone receptors, bacterial chemotaxis receptors do not signal by horizontal aggregation of the receptor monomers; instead, ligand binding induces small conformational changes, which are assumed to be transmitted through the transmembrane helices to the cytoplasmic domain and to affect the phosphorylation rate of the bound histidine kinase (recently reviewed by Falke and Hazelbauer [29]). Crystal structures of the ligand binding domain of a *Salmonella tryphimurium* aspartate receptor (Tar) mutant [22] in apo and liganded (Asp bound) forms and of the wild-type Tar of apo and liganded forms [30] revealed one Asp bound per dimeric receptor, which was also shown to be true in solution [31]. The difference distance matrix method of comparing apo and Asp bond forms suggested a small but significant intersubunit conformational change ($\alpha 1$ with respect to $\alpha 1'$) [30] and even smaller intrasubunit conformational changes ($\alpha 1$ with respect to $\alpha 4$) [32]. The main difference between structures of the apo and complex forms of the periplasmic domain is a small, approximately 4°, rigid-body rotation of one monomer subunit with respect to the other in the dimeric domain [22,30,33]. The crystal structures of *E. coli* Tar periplasmic domain in true apo and pseudoligand-bound forms [34] provided further evidence in favor of dimeric signaling models [32,35] involving intersubunit motion. Because the apo and Asp bound domains are in two different crystalline environment, it is not clear whether these small conformational changes are functionally relevant or caused by differences in crystal packing.

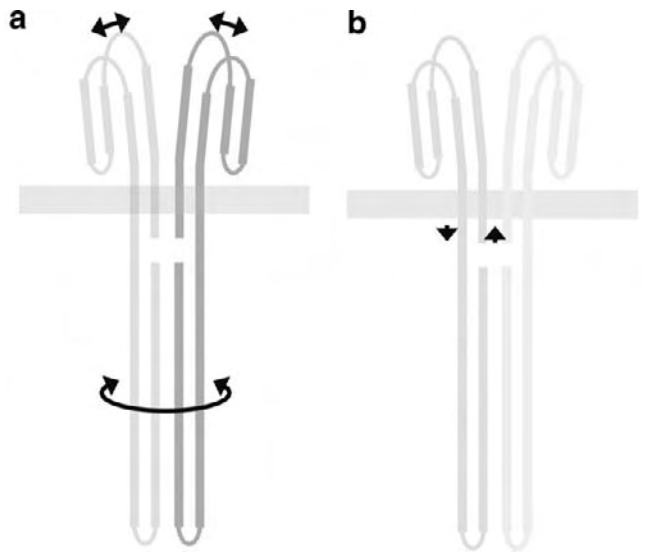


Figure 3 Two models of transmembrane signaling mechanism. (a) Dimeric signaling model; (b) monomeric signaling model.

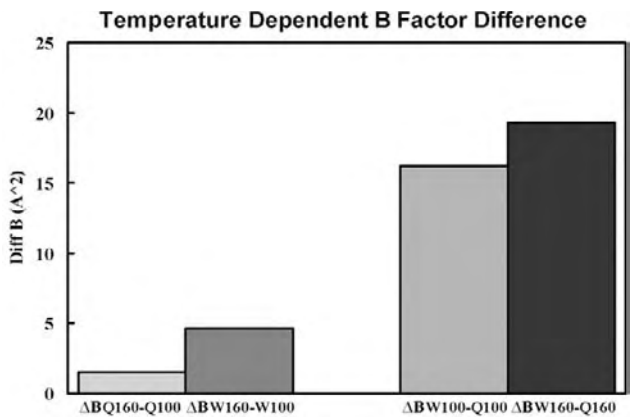


Figure 4 Cyrstallographic B factors of a molecule reflect the sum of two properties: dynamic flexibility and static disorder of the molecule. The ΔBs are calculated using the average B factors of the entire molecule. Q160, all Q mutant at160 K; Q100, all Q mutant at 100 K; W160, wild type at 160 K; W100, wild-type at 100 K. The B factors of either the wild type or Q mutant change little between temperatures 60 K apart; however, the wild type displayed a much higher temperature factor when compared with the Q mutant at both temperatures.

In contrast to crystallographic data, biochemical and other biophysical studies provided information that led to monomeric signaling models of transmembrane mechanism. Results from site-directed disulfide chemistry [24,25,32,36–39], site-directed spin labeling electron paramagnetic resonance (EPR) [40], and nuclear magnetic resonance (NMR) [41,42] are best accounted for in terms of a piston type motion that involves a modest (1–2 Å) sliding of the transmembrane helices across the membrane within a monomer, which subtly rearranges packing of the cytoplasmic four-helix bundle, thereby transmitting the signal [29].

In both the dimeric signaling and monomeric signaling models (Fig. 3), it is generally accepted that the ligand binding induces a small conformational change resulting in subtle movement in the periplasmic domain as well as transmembrane helices, which, in turn, is transmitted to the cytoplasmic domain. However, conformational differences as small as those observed are subject to other interpretations.

Signaling at the Cytoplasmic Domain

In a mutant of the *E. coli* serine receptor (Tsr), Q mutant, all four methylation site residues of the Tsr cytoplasmic domain are glutamines, thus mimicking the fully methylated state of the receptor. The phosphorylation activity of this mutant receptor is very high [43]. The Q mutant effectively locks the receptor in a signal "on" state. In contrast to the ligand binding domains, crystal structures of the cytoplasmic domains of the Q mutant [27] and the wild-type receptor [44] show no conformational differences. The crystallographic data described in Fig. 4 [44] revealed the presence of significant differences in dynamic flexibilities of the domains, however, suggesting that modulation of or changes in the dynamic properties of the receptor may be the language in which the signal is transmitted from the chemotaxis receptor to CheA, its immediate downstream effector. The structural basis for the reduced dynamic flexibility in the Q mutant is probably the extensive inter-helical hydrogen bonding formed by the glutamine residues at the four methylation sites.

Adaptation

The adaptation branch of the chemotaxis pathway enables the cell to adapt to a constant background stimulus so that it can chemotax up a small concentration gradient superimposed on a large background level of attractant. Covalent modifications of the methylation sites of the cytoplasmic domain are responsible for the sensory adaptation. Chemo-receptors possess multiple glutamate residues on the cytoplasmic domain surface that can be reversibly methylated by methyltransferase, CheR, and demethylated by methylesterase, CheB. CheR activity is constitutive, whereas CheB is activated by phosphorylated CheA (Fig. 2). Attractant binding or demethylation of the cytoplasmic domain down-modulates the CheA phosphorylation rate [45,46]. The relative rates of methylation and demethylation define the steady-state level of the receptor methylation [8]. Methylation of the receptor cytoplasmic domain reduces the ligand binding affinity to the periplasmic domain in the receptor–CheW–CheA ternary complex of *E. coli* Tsr [47]. Thus, an elevated level of receptor methylation, which occurs when bacteria encounter persistently high concentrations of attractant, desensitizes the periplasmic domain by lowering ligand binding affinity. In the opposite scenario, reduced receptor methylation increases the sensitivity in a low attractant concentration environment.

The mechanism by which methylation affects the ligand binding affinity and the rate of Che Y phosphorylation is not known. One possibility is that methylation changes the dynamic mobility of the receptor; inter-helical favorable hydrophobic interactions involving the methyl groups may reduce the dynamic flexibility of the receptor, thereby influencing the ligand binding and/or Che A activity.

Clustering of the Chemoreceptor and Sensitivity

A higher order structure of chemoreceptors was demonstrated first in *E. coli* cells by immuno-electron microscopy experiments [48]. These studies showed that chemoreceptor–CheW–CheA complexes are clustered in large arrays localized at the flagellum-bearing pole of the bacterial cells. It was later observed that the leucine-zipper fused cytoplasmic domain of Tar forms a cluster of ≈14 receptor signaling domains in the presence of CheA and CheW *in vitro* [49]. *In vivo* clusters of intact receptors and active complexes in the cell membrane may nucleate formation of additional further active complexes, thus amplifying the signal at the receptor level. The positive cooperativity observed for ligand-induced inhibition of CheA activity *in vitro* added to the evidence that receptor cluster have functional importance [47] Bray *et al.* [50] and Zhang and Kim [51] proposed mathematical models for the high sensitivity by proposing that one ligand-bound receptor can convert the conformation of many neighbors in the cluster. They further showed that the dynamic range can be modeled mathematically if one assumes that the receptors exist as clusters as well as single dimeric receptors and that the degree of receptor clustering depends on the concentration of the ligand. Clustering may promote interaction between receptors, thereby propagating the signal to neighboring receptors. A structural basis for receptor clusters is suggested by the crystal structure of the *E. coli* Tsr cytoplasmic domain [27].

A recent computer modeling of the clustering of chemotaxis receptor [44] based on the crystal structure and packing of the cytoplasmic domain of a serine chemotaxis receptor [27] reveals that each receptor dimer may contacts two other receptor dimers at the cytoplasmic domain and two yet different receptor dimers at the ligand binding domain, thus, making an infinitely extendable two-dimensional sheet of receptors (Fig. 5). In this "trussing" arrangement each receptor

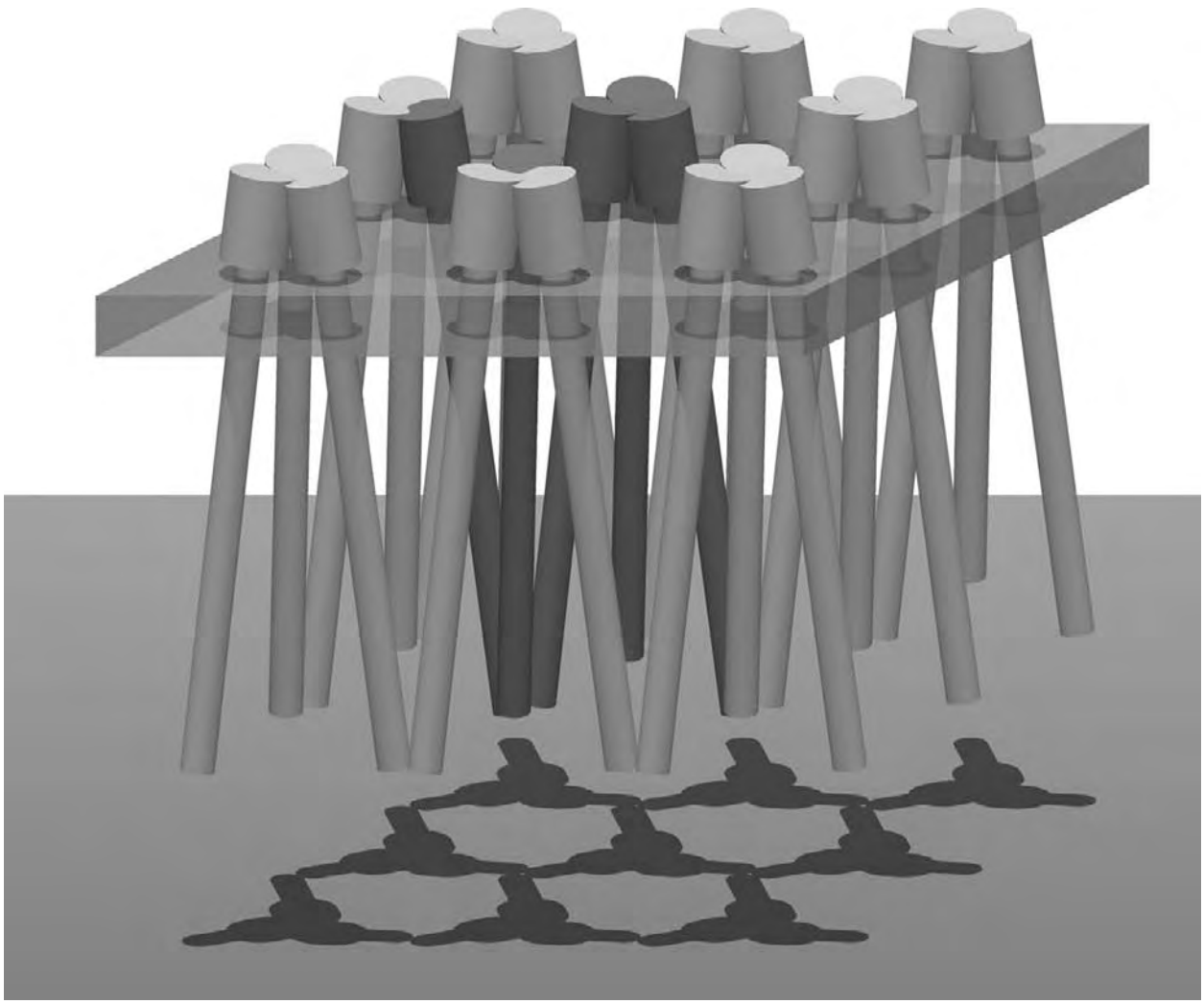


Figure 5 A schematic drawing of the receptor dimer clustering model. Each cylinder represents a dimer of chemotaxis receptors as in Fig. 1 (left). On repellent binding to or departure of bound attractant from one receptor, the dynamic property of the receptor is reduced (shown in blue) and the reduction is "felt" progressively by the first order and second order neighbors (indicated by milder blue colors) propagating through the trussed slab of the receptors. The cluster cast by a vertical light source reveals the contact network in 2-dimension graphically.

dimer is in contact with total of four other receptor dimers. This inter-connection of the receptors is proposed to be the structural basis for the high sensitivity of the bacterial chemotaxis receptors. Furthermore, absence of any significant differences between the average structures, but the presence of significant differences in dynamic flexibilities of signal active and inactive states of the structure suggest that the modulation of dynamic property of the receptor may be the language of the receptor signaling [44].

Future Studies

Our understanding of bacterial chemotaxis at the level of individual molecules, although based on a tremendous body of knowledge, must be combined with studies of chemotaxis at the level of molecular complexes. Furthermore, receptor clustering and high-order interaction among receptor complexes may lead to fundamental explanations of the extraordinary sensitivity and dynamic range of gradient sensing in bacterial chemotaxis.

Acknowledgment

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CHAPTER 35

Overview: Function and Three-Dimensional Structures of Ion Channels

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Introduction

Actions speak louder than words. The molecular roots of our actions and the thoughts and feelings that drive us to act are ion channels, proteins that form macromolecular pores in cell membranes. These transmembrane proteins generate and propagate the electrical signals that allow us to sense our surroundings, process information, make decisions, and move.

Ion channel proteins act as gates that span the lipid bilayer that surrounds all cells where they open and close to allow the flow of ions down their electrochemical gradients (Fig. 1). The ion flux through a channel pore can be extremely high, $\approx 10^6$ ions per second [1]. Because of their central role in the function of the excitable tissues such as heart, brain, muscles, and nervous system, investigators have long sought to understand ion channel properties from a molecular perspective. Decades of biophysical measurements and functional studies have been devoted to understanding ion channel function [1]. Yet, the very nature of these molecules—transmembrane proteins that are difficult to obtain in the large quantities and high purity necessary for structural investigation—has impeded attempts to obtain the most essential information for understanding their functions, a three-dimensional description of their molecular architectures at high resolution. In the past 5 years, the once impregnable barrier separating biophysicists and neuroscientists from this essential information has been breached. The first high-resolution structures of ion channels and ion-channel-associated proteins are providing the substrates for sophisticated tests of the mechanisms of channel gating and permeation. This chapter touches briefly on these pioneering studies and the questions they raise.

Ion channels perform two basic functions. They open and close to control the passage of ions across the cell membrane (see Chapter 36) and they sense and respond to signals that drive them between open and closed states (see Chapters 35 and 37 to 40). The response times of channels to these inputs can be very fast, on the order of tens of microseconds to a few milliseconds [1]. Different classes of ion channels have been designed by nature to respond to the three types of signals one can imagine sensing in a membrane environment: extracellular signals such as neurotransmitters (e.g., acetylcholine and glutamate receptors; see Chapters 37 and 38), transmembrane voltage changes (typified by voltage-sensitive cation channels; see Chapter 35), and intracellular signals such as calcium and cyclic nucleotides (see Chapters 39 and 40). While channels are generally classified based on the primary signal that opens them, many channels serve as integrators and respond to some combination of signals.

The pore-forming domains of most ion channels are multimeric assemblies possessing cyclic symmetry in a general architecture known as *barrel-stave* (Fig. 2). A fixed number of subunits assemble around the axis of the ion conduction pore.

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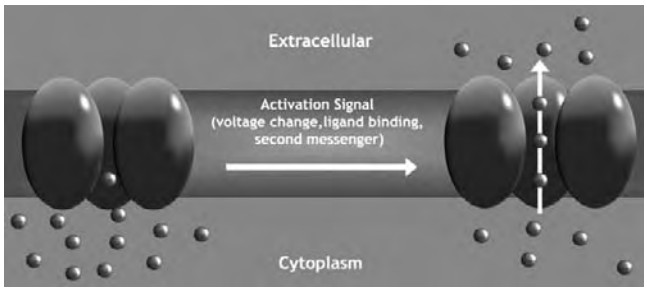


Figure 1 Schematic of ion channel function as viewed from the plane of the membrane. Three subunits of an ion channel are shown in magenta; ions are shown as green spheres. Upon activation by a stimulus, such as a transmembrane voltage change or ligand binding, the channel undergoes a conformational change that opens a pore formed by the protein. Ions flow through the open pore in a direction that is determined by the electrochemical gradient.

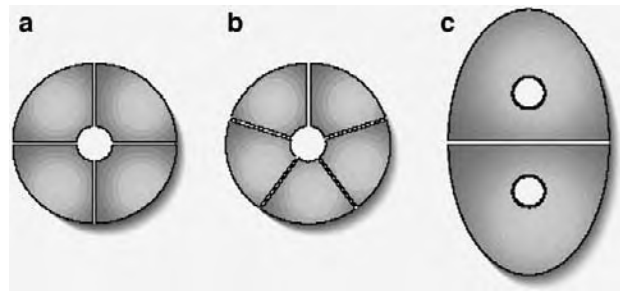


Figure 2 General architecture of ion channels. Parts (a) and (b) show the barrel-stave characteristics of the voltage-gated cation channel family and the nicotinic acetylcholine receptor family. In each of these, the channel subunits are arranged around the pore through which the ions flow. Part (c) shows the general architecture of voltage-gated chloride channels. These channels are dimers in which each subunit makes its own pore. (Adapted from Jentsch, T., *Nature*, 415, 276–277, 2002.)

The number of subunits is roughly related to the size and selectivity characteristics of the channel. For example, the most selective channels, such as voltage-gated sodium channels and voltage-gated potassium channels, are tetramers in which four identical or highly homologous subunits are arranged around the pore. Pentameric channels such as the nicotinic acetylcholine receptor (nAChR) have larger pores and generally discriminate between positive and negative ions but not among ions within these general classes. Hexameric channels such as gap junctions allow ions and small solutes to pass [2]. The barrel-stave channel arrangement has been a boon to structure–function studies, as the channel symmetry imposes strong constraints on the likely location of amino acids close to the pore. Nature, however, does not always follow this plan when constructing ion channels. Voltage-gated chloride channels have two pores that are formed from a dimer of subunits in which each subunit makes its own ion passageway (Fig. 2c) [3].

Studies of Full-Length Ion Channels

X-ray crystallographic and nuclear magnetic resonance experiments are the most powerful tools for obtaining information about the atomic structure of macromolecules. Unfortunately, it is still extremely difficult to use these methods to study membrane proteins such as ion channels. Ion channels have domains that reside in the hydrophobic environment of the cell membrane as well as domains that reside in the aqueous intra- and extracellular spaces. To keep the transmembrane domains soluble upon removal from the cell membrane, reagents such as detergents or lipids must be used in the purification and handling of full-length channels. The search for the precise detergent or lipid that will work for a given channel complicates purification attempts as well as the search for conditions that produce diffraction-quality protein crystals, the necessary prerequisite for any X-ray crystallographic study. The large size of most ion channel proteins places them outside what is currently possible with the most sophisticated nuclear magnetic resonance (NMR) methods. Furthermore, good overexpression systems for producing eukaryotic membrane proteins in the quantities required for high-resolution studies are not currently available. Solving this technical problem is one of the major requirements for routine high-resolution investigation of membrane protein structure.

Electron microscopy studies have proven particularly useful in obtaining low- to medium-resolution descriptions of eukaryotic ion channels and the conformational changes that accompany ion channel opening. Studies of ion channels found in high abundance in the electric organs of electric rays and electric eels, such as the nicotinic acetylcholine receptor and the voltage-gated sodium channel [4,5], reveal the general cyclic symmetric architecture of both of these channels (Fig. 3). While difficult, these studies require much less protein than other structural methods, and information can be obtained from two-dimensional crystals, tubular membrane crystals, and even single particles.

General Pore Features Revealed by Bacterial Channels

The problems with obtaining material for ion channel structural studies can be overcome by turning to bacterial ion channels. These molecules can be more readily expressed and purified in large quantities than their eukaryotic counterparts. The atomic details of the inner workings of an ion channel were first seen in the X-ray crystallographic structures of the bacterial potassium channel, KcsA (Fig. 4a) [6–9]. The KcsA structure revealed many of the general features of ion channel pores that had been anticipated from careful biophysical studies coupled with structural reasoning (see Chapter 36). For instance, many channels seem to be made on a funnel-shaped plan with a large entryway that tapers to a narrow constriction that can serve as a selectivity filter that allows only particular types of ions to pass.

Potassium channels are remarkable for their ability to discriminate between potassium and sodium ions with very high precision, preferring potassium by a factor of $\approx$ 10,000:1 [1].

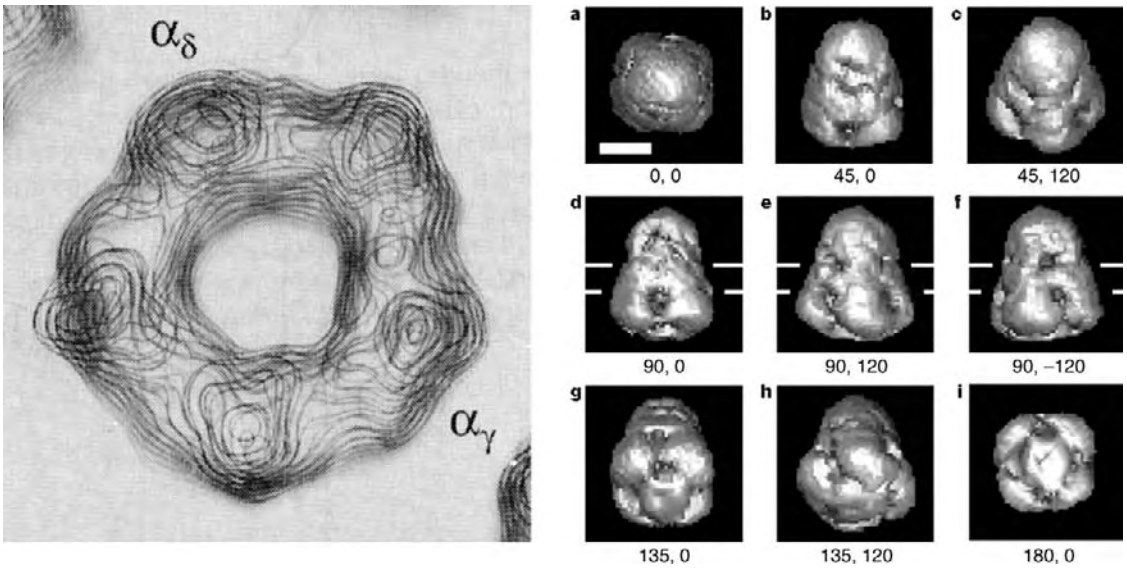


Figure 3 Electron microscopy reveals the general features of the nictotinic acetylcholine receptor seen from the extracellular space at 9-Å resolution, left, and the voltage-gated sodium channel at 25 Å, right. The panels for the sodium channel show successive rotations of a surface representation of the channel and start from the extracellular side (0, 0) through the intracellular side (180, 0). The pairs of numbers indicate the degrees of rotation around the *x* and *y* axes. (Adapted from Unwin, N., *Nature*, 373, 37–43, 1995; Sato, C. *et al.*, *Nature*, **409**, 1047–1051, 2001.)

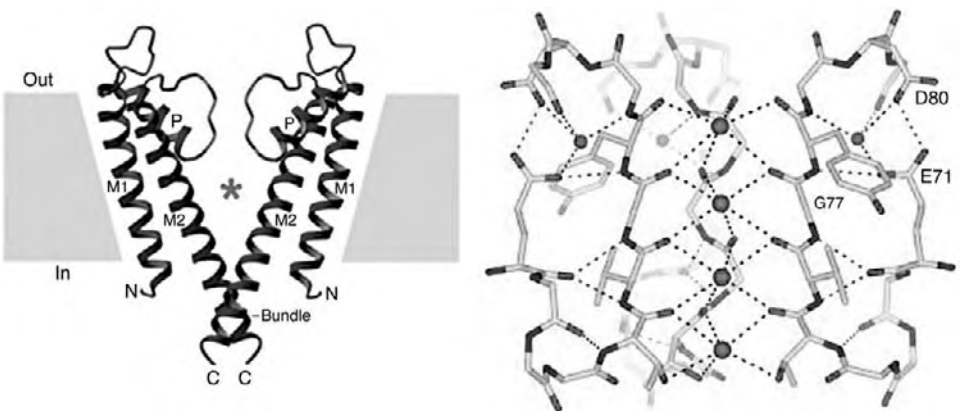


Figure 4 Structural elements of a potassium channel pore. (Left) Two subunits from the KcsA potassium channel are shown. The transmembrane segments are labeled M1 and M2. M2 subunits cross at the region marked "Bundle" and restrict access to the channel pore. The pore helix is indicated by "P" and the selectivity filter is shown in yellow. The red star marks the inner cavity of the channel. (Right) Close-up view of the intimate contacts between the KcsA selectivity filter oxygens (red) and potassium ions (green spheres). (Adapted from Jiang, Y. *et al.*, *Neuron*, 29, 593–601, 2001; Zhou, Y. *et al.*, *Nature*, 414, 43–48, 2001.)

Both ions are monovalent cations. A sodium ion has a radius of 0.95 Å, while potassium has a radius of 1.33 Å. How does the larger potassium ion pass through the potassium channel selectivity filter while the smaller sodium ion does not? Chemistry. All ions have shells of closely associated water molecules in solution [1]. For an ion to enter the filter, it must shed its waters of hydration. The selectivity filter of potassium channels is arranged in a way that displays rings of carbonyl oxygen atoms from the protein backbone at the exact diameter of a potassium ion. Thus, the waters of hydration surrounding a potassium ion are exactly replaced by oxygen atoms from the protein, creating a perfect chemical and energetic match as the ion enters the selectivity filter (Fig. 4b) [7,9]. Although the smaller sodium ion can pass through the filter, it is much more energetically costly, as fewer of its lost water ligands can be replaced by the channel. Other selectivity filters may work in a similar way in which the protein makes intimate contact with the permeant ion.

Pore Helices: Electrostatic Aids to Permeation

A second feature that is common in the high-resolution ion channel structures (bacterial potassium and bacterial

chloride channels) is the use of the N- or C-terminal ends of short α-helices (known as pore helices) to stabilize the ion as it passes through or near the points of narrow constriction in the channel pore. For example, the pore helices of KcsA have their C-terminal ends aimed at the ion conduction pathway (Fig. 5, left). It is thought that the negative end of the helix dipole (the C-terminal ends of helices bear a small net negative charge, while the N-terminal ends bear a small net positive charge [10]) stabilizes the potassium ion on its journey through the channel [11]. Likewise, the recent structure of a bacterial chloride channel shows that the N-terminal ends (positive end) of two helices that form the narrowest part of the ion conduction pathway form a binding site for the negatively charged chloride ion (Fig. 5, right) [3].

Open Channels

The key thing that ion channels do is open and close. Structural studies are beginning to reveal the general rearrangements that occur when channels are prompted to move between closed and open states. Electron microscopy studies of the nAChR show that ligand binding to the extracellular domain causes a twisting of the subunits that is propagated some 60 Å away to the narrowest part of the channel pore embedded deep in the membrane. This conformational change widens the narrow constriction, or gate, that prevents ion flow in the closed state [4] (Fig. 6).

Potassium channels also use a distinct portion of the protein as a gate to prevent ion flow in the closed state. X-ray crystallographic comparisons of the homologous pore regions of open [12] and closed [6] bacterial potassium channels suggest that the lower part of the inner helix moves during gating to widen the narrow constriction formed by the bundle crossing of the inner helices [13] (Fig. 6). These conformational changes occur below the selectivity filter, which remains largely unchanged. This mechanism of opening is likely to be conserved among many diverse types of potassium channels.

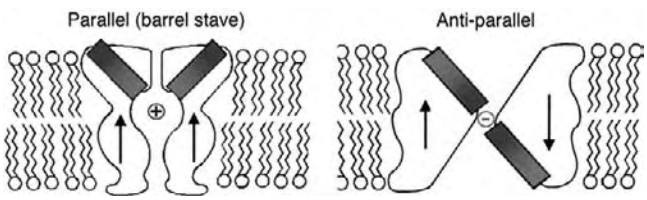


Figure 5 Examples of how pore helices stabilize permeant ions for cation channels (left) and anion channels (right). (Adapted from Dutzler, R. *et al.*, *Nature*, 415, 287–294, 2002.)

Eukaryotic Ion Channels at High Resolution: Divide and Conquer

Bacterial channels have provided insight into the guts of ion channel permeation machineries, revealing the intimate details of permeation pathways that are likely to be conserved and recapitulated in their larger eukaryotic cousins. In contrast to prokaryotic membrane proteins (which are difficult to obtain in their own right), eukaryotic membrane proteins are currently extremely difficult to obtain in the quantities required for high-resolution study. Eukaryotic channels often contain a host of extramembranous regulatory domains and subunits that are essential for their activity, signal sensing, and gating. These domains have proven to be a tractable entry point for the study of eukaryotic ion channel structure and function.

A number of groups have successfully "liberated" extramembranous domains from the membrane-spanning part of a variety of ion channels so that they can be expressed, purified, crystallized, and treated like soluble proteins.

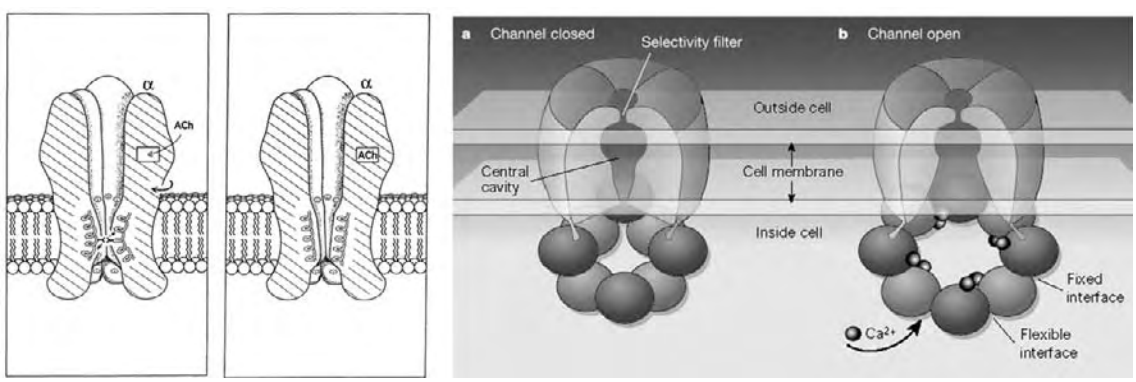


Figure 6 Opening mechanisms of ion channels. (Left) Simplified diagram of the opening mechanism of the nAChR. Acetylcholine (Ach) binds to the extracellular domain of the receptor initiates a rotation that causes the closest approach of the ring of pore-lining helices to widen creating a pathway for the ions. (Right) Schematic model for the opening of a bacterial calcium-gated potassium channel. Calcium binding to the cytoplasmic domains causes a conformational change that is propagated to the inner pore-lining helices. This opens the pathway for the ions to enter the channel. (Adapted from Unwin, N., *J. Struct. Biol.*, 121, 181–190, 1998; Schumacher, M. A. and Adelman, J. P., *Nature*, **417**, 501–502, 2002.)

This divide-and-conquer approach has proven particularly powerful for illuminating channel-gating mechanisms when the high-resolution information about these domains is incorporated into structure–function studies of the intact channel. For example, studies of an assembly domain from eukaryotic voltage-gated potassium channels led to the discovery of a new role for this domain in channel gating [14,15]. The structure of a soluble homolog of the extracellular domain of the nAchR found in snail glial cells has provided molecular landmarks for interpreting decades of study by chemical modification, mutagenesis, and electron microscopy of the intact receptor [16]. Similarly, structures of the ligand binding domains of glutamate receptors [17] and calmodulin-activated potassium channels [18] have led to detailed models of channel gating and ligand recognition. This divide-and-conquer approach is likely to remain a fruitful endeavor in the near future while better methods for purifying ion channels from native sources and new means for expressing full-length ion channels are developed.

Ion Channel Accessory Subunits: Soluble and Transmembrane

Many eukaryotic ion channels have soluble subunits that associate with and regulate the properties of the channel *in vivo*. For example, some voltage-gated potassium channels associate with soluble β subunits that affect their ability to rapidly inactivate. Curiously, the structure of the Kvβ subunit reveals a structure that is common to oxidoreductase enzymes [19], complete with a firmly bound nicotinamide adenine dinucleotide phosphate (NADP) molecule. This structural observation suggests that Kvβ may act as some sort of enzyme that depends on the activity of the channel; however, to date, no functional data support this hypothesis.

Many other channel subunits exist. Some are soluble proteins, such as the calcium channel β subunit, and many are transmembrane proteins that bear intra- and extramembranous domains that affect the function of the pore-forming subunit [1]. Little is known about the structure of any of these molecules.

The Future: Ion Channels as Electrosomes

Beyond the classical pore-forming subunits and auxillary subunits that comprise ion channels, it is becoming ever more clear that, in real biological settings, ion channels are part of large protein networks. These networks include cytoskeletal components, signaling proteins such as protein kinases and phosphatases, and channel-associated proteins that recruit these signaling molecules to the channel to modify its function. To understand the biological structure of ion channels, it will be necessary to move from thinking about channels as proteins that simply form ion conduction pores to thinking about them as electrical signaling centers (*electrosomes*), large, multiprotein macromolecular complexes that not only generate electrical signals or changes in the membrane potential but also generate and respond to other chemical signals within a cell.

Perhaps the best example of a channel as an electrosome is the voltage-gated calcium channel [1]. When these channels open, they provide a means to depolarize the cell by allowing calcium entry. Calcium influx through the channel pore interacts with a channel-resident, calcium-sensing protein (calmodulin) that accelerates channel inactivation [20] and also causes the activation of signals that lead to alterations in transcription in the cell nucleus [21]. Together, these actions affect both the immediate electrical properties of the neuron as well as its long-term adaptation to activity. Understanding the activity of channels, the systems that regulate their action (such as G-protein-coupled receptors), and the complex interplay between chemical and electrical signaling pathways in cells will be essential for developing an molecular understanding of complex processes such as the regulation of heartbeat and the molecular basis of learning and memory.

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CHAPTER 36

How Do Voltage-Gated Channels Sense the Membrane Potential?

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Introduction

Voltage-gated ion channels transduce changes in the membrane electric field into changes in membrane permeability. These channels consist of four similar or identical subunits, each containing six α-helical transmembrane segments (S1–S6) and a pore-forming loop (P-loop) (Fig. 1A). The subunits may assemble from one continuous polypeptide chain, as is the case for voltage-gated Na^+ and Ca^{++} channels, or from four separate chains, as is the case for K^+ channels. Each subunit of a voltage-gated channel contains two functionally distinct domains.

The first of these is a pore domain (Fig. 1C) composed of the S5, S6, and P-loop of each subunit. This domain forms the permeation pathway, which includes the narrow ion selectivity filter, an internal activation gate, and an external slow inactivation gate. The pore domain is homologous to 2-TM channels such as the inward rectifier K^+ channel and the bacterial KcsA channel (see Chapter 34). The second domain, composed of four transmembrane helices (S1–S4), surrounds the pore domain and regulates opening and closing of the pore gates.

Depolarization drives conformational changes in the voltage-sensing domains that cause the activation gate to open and the slow inactivation gate to close. Channels conduct ions when both gates are open simultaneously. Usually the activation gate responds more quickly, yielding a transient current at depolarized potentials; however, in some channels (e.g., hERG), the inactivation gate responds more quickly, resulting in a transient current upon repolarization [41]. When present, a separate fast inactivation gate (made of the N terminus of K^+ channels or the linker between subunits III and IV in Na^+ channels) closes by blocking the internal mouth of the open channel.

The Voltage-Sensing Gating Particle

Before the advent of molecular biology, through classical electrophysiology experiments, Hodgkin and Huxley [14] established the physical basis for action potential generation and propagation. Working in squid giant axon, they demonstrated that the action potential consists of two voltage-dependent currents carried by an initial inward Na^+ flux followed by an outward K^+ flux. Hodgkin and Huxley hypothesized that voltage controlled the Na^+ and K^+ conductances by biasing the equilibrium of charged *gating particles* between two stable positions (resting and activated) within the transmembrane electric field. Activation of four independent gating particles in K^+ channels and three in Na^+ channels was proposed to turn on the conductances. This led to the prediction that the transmembrane motion of the charged gating particles would generate a small *gating current*. Because all of the gating particles would have to be activated before channels conduct, the movement of the *gating charge* (the charge carried by the gating particle) was predicted to precede the ionic current and be less steep in voltage dependence than the conductance (Fig. 2).

Principles of Voltage-Sensing in Proteins

How could nature have evolved a gating particle/voltage sensor? In principle, any charged particle (q)—for example,

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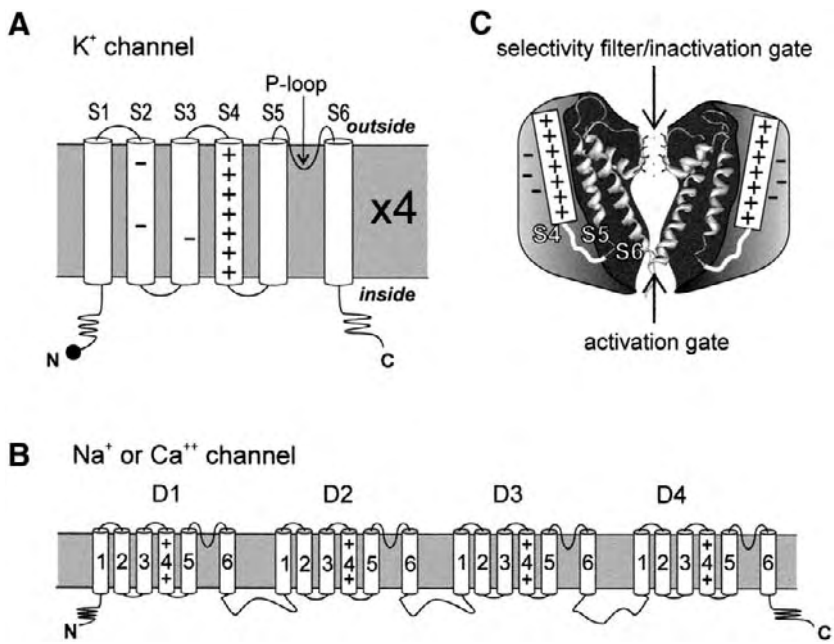


Figure 1 Structure of voltage-gated channels. (A) Membrane topology of one subunit of a voltage-gated K^+ channel. S1–S4 form the voltage sensing domain. S5, S6, and the P-loop form the pore domain. A series of conserved positive charges in S4 interact with negative counter-charges in S2 and S3. The fast inactivation gate is shown as an N-terminal ball. (B) Membrane topology of voltage-gated Na^+ and Ca^+ channels. (C) Side view of two subunits from (A). The pore domain is shown as a gray ribbon. The internal activation gate is located at the S6 bundle crossing. The selectivity filter and slow inactivation gate are located near the external end of the pore. Voltage-sensing domains surround the pore domain and regulate opening and closing of the gates.

the formal charge of a positive (R, K) or negative (E, D) amino acid or the partial charge of a dipole moment of an α-helix—will experience an electromotive force (**F**) when placed in an electric field (**E**), with $\mathbf{F} = q\mathbf{E}$. A typical neuronal resting potential of −70 mV exerts a force of ≈ 3 pN on a single charge in the membrane. For comparison, the typical forces generated by the motor proteins myosin and kinesin are 1.5 and 3 pN, respectively. Depolarization changes the electrical force, allowing the charge to relax to a new position in the electric field. The motion of the charge could be small, on the order of angstroms, or significantly larger. For example, a charged side chain could reorient itself in the electric field due to rotations around its chemical bonds. Alternatively, a rigid body motion of the protein backbone could move several charged side chains on a protein segment together through the electric field.

Simple Models of Voltage-Sensing Protein Structures

It is energetically unfavorable to place a charge in the low dielectric, nonpolar environment of the membrane core. The pore has solved this problem for permeant ions by providing a polar pathway made of backbone oxygens, partial charges, and water (see Chapters 34 and 36). The same energetic constraints apply to the voltage sensor. Charges on the voltage sensor must either be stabilized by counter-charges or be hydrated by water. An early model of the voltage sensor paired oppositely charged surfaces in two membrane-spanning protein segments [2], providing a favorable pathway for charges to move across the membrane by ratcheting through a series of intermediate positions (Fig. 3A). Alternatively, charges may move from exposure to water on one face of the membrane to exposure on the opposite face through a short, polar pathway analogous to the selectivity filter (Fig. 3B and C). In either case, the pathway of the charge movement, or *gating canal*, must be constricted enough to prevent the leak of ions and contain stabilizing counter-charges.

S4 Is the Primary Voltage Sensor

S4 Sequence and Charge Pairing

A high-resolution structure of the voltage-sensing domain of an ion channel has yet to be obtained; however, some things are already known about the structure of this domain. S1 to S6 form transmembrane helices [8,13,14,19,25,33]. The S4 helix is conserved across voltage-gated cation channels and contains a positively charged arginine (R) or lysine (K) at every third position (Fig. 4). Because S4 spans the membrane, some of these charges sense the electric field. The spacing of positive charges creates a left-handed positively charged spiral (resembling a barbershop pole) along the length of the S4 helix. Three conserved negative charges, two in S2 and one in S3 (Fig. 1A), interact with the charges in S4 [35,36], suggesting that three positive charges of S4 could

reside in the gating canal at one time. Given the gradual pitch of the spiral, three consecutive positive charges would lie on the same face of S4.

S4 Positive Charges Account for the Gating Charge

Wild-type Shaker channels move ~12 to 13 charges per channel (~3 charges per subunit) during activation [28]. If S4 is the voltage sensor, then a mutation that neutralizes a positive charge on S4 should decrease the total gating charge. The maximum reduction expected is 1 charge per subunit for a position that completely traverses the electric field. Of the seven positive charges in the Shaker S4, single neutralizations of R1, R2, R3, or R4 each decrease the total charge by ~1 to 1.7 charges per subunit, whereas neutralization of K7 has no effect [1,32]. This indicates that only the outer S4 charges move through the gating canal. In addition, neutralization of the deep negative charge in S2 also decreases the gating charge by ~1.5 charges per subunit, suggesting that it may move across the electric field in a direction opposite to that of S4 [32]. The fact that some neutralizations decrease the charge by >1 indicates that a neutralization may affect the remaining charges, possibly by changing the pointing angle of their side chains and/or the shape of the electric field in the gating canal.

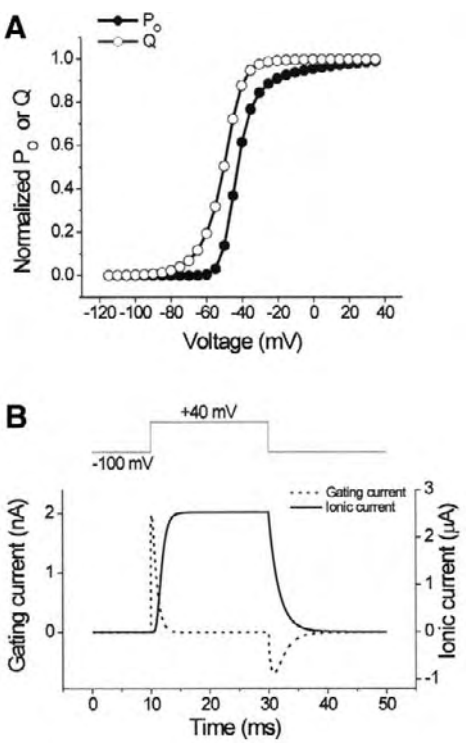


Figure 2 Electrical properties of simulated ion channels. (A) Plot of the movement of the gating charge (Q) and the probability of the channel opening (P_o) at various voltages. Because all voltage sensors must be activated before a channel opens, Q precedes P_o in voltage dependence. (Modeled as in Ledwell and Aldrich [18].) (B) Gating current and ionic current elicited by depolarization. Movement of the voltage sensor generates several nanoamperes of gating current, opens the pore gates, and allows several microamperes of ionic current to flow through the pore.

Residues in S4 Move Fully Across the Membrane

A direct measure of transmembrane motion in S4 has been obtained in Na^+ and K^+ channels by probing the accessibility of engineered single cysteines to internal and external thiol-specific reagents [3,16,37,38,39,40,43]. In the Shaker K^+ channel, the charged and uncharged positions from R1 to R3 are inaccessible to the external solution at negative voltage (resting state) but are accessible to the external solution at positive voltage (activated state). R3 and deeper sites are accessible to the internal solution at negative voltage but inaccessible at positive voltage. The change in accessibility can be accounted for by a rigid body motion of S4 across the membrane (Fig. 4). The motion takes place in multiple steps with at least one intermediate position [3,4]. The model of transmembrane S4 motion is supported by the finding that histidines substituted at positions R2, R3, or R4 can transport protons across the membrane at voltages that allow the voltage sensor to shuttle between resting and activated states [34].

Taking into account all of the accessibility results leads to the following conclusions:

1. S4 moves outward with depolarization in the correct direction to carry the gating charge.

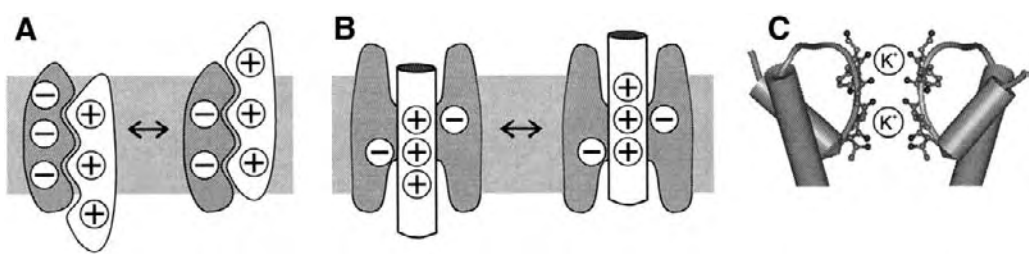


Figure 3 Three ways to move charge through the membrane. (A) Two charged membrane-spanning protein faces slide past each other to move one positive charge across the membrane. (B) A positive membrane-spanning segment slides through adjacent protein and interacts with negative counter charges. Water-filled vestibules formed by the protein shorten the distance required to move one positive charge across the membrane. (C) Movement of K^+ ions through the short KcsA selectivity filter.

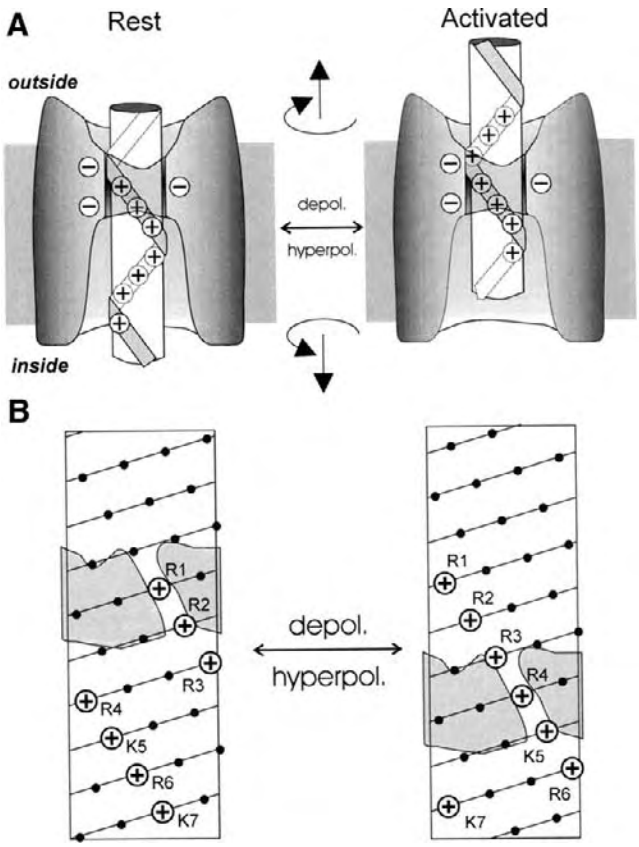


Figure 4 Helical screw motion of S4 activation. (A) Depiction of S4 (the screw) moving through a short gating canal surrounded on either end by two water-filled crevices. Positive charges on S4 interact with negative counter-charges in the adjacent protein (the bolt). The water crevices hydrate S4 charges that do not interact with counter-charges. Depolarization rotates S4 along the stripe of charged positions and in the process moves S4 outwards along its helical axis. (B) Helical net of S4 that illustrates changes in solvent exposure and movement of charge across the gating canal. Positions R1, R2, R3, and R4 move some distance across the gating canal when S4 changes conformation from the resting to activated state.

2. S4 carries the equivalent of 3 charges per subunit across the membrane (R1 to R4 carrying 0.5, 1, 1, and 0.5 charges, respectively), accounting for the total gating charge in wild-type channels.
3. Only a short length of S4 (10 residues, ~13.5 Å) lies in the gating canal at any one time, which means that the canal is considerably shorter than the 35 Å thick core of the membrane and that the electric field is focused on S4.

S4 Moves at the Right Time To Generate the Gating Current

The contribution of S4 charges to the gating charge and the measure of transmembrane S4 motion argue that S4 is the primary voltage sensor. To prove this, it is necessary to show that S4 motion occurs during gating charge movement. The kinetics of protein motion in a channel can be measured optically using voltage-clamp fluorometry (VCF). Fluorophores sensitive to their local environment report local structural rearrangements with a change in fluorescence intensity [24]. The fluorescence of a probe attached at or near S4 changes brightness at voltages where channels do not open but where gating charge moves. The fluorescence and gating charge correlate both kinetically and in steady-state voltage dependence (Fig. 5) [6,9,23,24].

S4 moves in the right direction by a sufficient amount and with the correct kinetics for it to generate the gating charge. But how does it move? Fluorescence resonance energy transfer (FRET) between donor–acceptor fluorescent probes attached to S4s of different subunits provides a clue. An examination of the pattern of voltage-driven distance changes between S4s suggests that S4 twists in an 180° rotation [7,10]. Thus, the motion of S4 appears to involve both outward and rotary components.

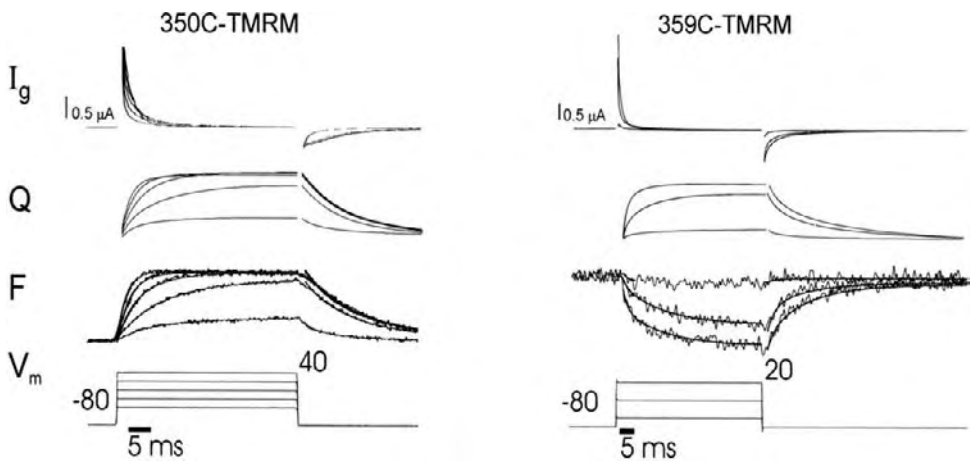


Figure 5 Optical measurements demonstrate that S4 movement generates the gating current. Gating current (I_g), gating charge (Q), and fluorescence (F) were measured simultaneously from a single oocyte expressing non-conducting Shaker channels labeled at either position 350 or 359 with rhodamine maleimide (TMRM). The probe shows voltage-dependent changes in fluorescence intensity for which the kinetics correlate with gating charge movement. The fit to Q is overlaid over the fluorescence trace. For reference, position 359 is located 3 residues before R1 (see Fig. 4).

Physical Models of Activation: Turning a Screw through a Bolt

What kind of physical structure can account for S4 packing and motion? Similar to the short (12 Å) and narrow (~5 Å) ion-selectivity filter that opens to water on either end (see Chapters 34 and 36), the short (13.5 Å) gating canal must also have water-filled crevices at its ends. Unlike the selectivity filter, which is designed for a high rate of flux (~10^7 per second) and so employs loose coordination of permeant ions by carbonyl oxygens, the gating canal probably employs strong electrostatic interactions with counter-charges in S2 and S3 [26,35,36] and nonpolar interactions with S5 and S6 [20] which together form the walls of the canal. These strong interactions result in relatively slow S4 motion (~10^3 per second).

A helical screw motion for S4, originally proposed by Guy and Seetharamulu [11] and Catterall [5], explains how a positive S4 (the screw) would rotate during an outward translation past immobile negative counter-charges (the bolt). As depicted in Figs. 4A and B, a screw motion along the spiral of positive charges translates S4 outward by nine residues during a 180° rotation and accounts for the measured charge movement and exposure changes. The gray cross-section overlapping S4 defines the gating canal or bolt formed by the surrounding protein which includes counter-charges in S2 and S3. The short length of the bolt focuses the electric field on a small portion of S4. On either side of the bolt, water-filled crevices hydrate those S4 charges that do not interact with counter-charges.

The screw clicks through three ratchet steps. Each step moves an S4 charge to the position of the one ahead of it and carries a total charge of ~1. This structural model agrees with a kinetic model based on electrophysiological recordings that proposes that activation involves three sequential steps [29–31].

Although a helical screw motion accounts for the evidence it does not mean that S4 must undergo the full axial translation of nine residues (13.5 Å). The observed rotation of S4 may be accompanied by rearrangements of S1, S2, and S3 that move the negative counter-charges around S4. This would be akin to turning the bolt (S1–S3) around the screw (S4). Also, rearrangements of the other transmembrane segments may move S4 charges simply through S4 rotation [7,10].

Coupling Gating to S4 Voltage-Sensing Motions

Once each S4 has undergone its activation motion, the four subunits undergo cooperative rearrangements to open the internal activation gate and close the slow inactivation gate. How does S4 conformation control these gates? One possibility is that S4 twists open the activation gate by pulling on the internal linker between S4 and S5 [10]. This is intriguing because rotation and tilt of the homologs of S5 and S6 appears to open the activation gate in the bacterial channel KcsA [21, 27] and MthK [14,15]. A separate coupling mechanism controls closure of the slow inactivation gate [9,17,22] via interaction between the activated S4s and the external face of the pore domain. The strength of coupling between S4 and each of the gates sets the duration and voltage dependence of the conductance. This in turn allows voltage-gated channels to shape the electrical signals of the cell.

It will be particularly revealing to learn how protein motions as different as the transmembrane motion of S4 in voltage-gated channels and the conformational rearrangement produced by binding of internal ligands in a C-terminal region near S6 in cyclic nucleotide or Ca^{++} gated channels can actuate the same gates (see Chapter 40). Perhaps voltage- and ligand-dependent channels open their internal gate by a conserved mechanism that is triggered by two different stimuli.

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CHAPTER 37

Ion Permeation: Mechanisms of Ion Selectivity and Block

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Ion channels have been identified in all domains of cellular life—Bacteria, Archaea, and Eukarya—and they are found in every type of cell. They have transport functions—facilitation of net movements of ions and salts—and signaling functions—generation of electrical signals and regulation of cellular free calcium concentration [1]. In each of these roles, ion channels open and close in response to local stimuli and pass a limited subset of ions at high rates. Like enzymes, they are regulated catalysts with high substrate specificity and rapid throughput.

Aqueous Pore

There was much classical biophysical evidence that ion channels have an aqueous pore [1], and the arguments all hinged on measuring fluxes in the channel. The first argument was a technical one. Measurements of forward and backward isotopic K^+ fluxes in K^+ channels showed that K^+ ions move in a coordinated fashion, as if the pore contains a column of several K^+ ions moving in single file in a pore [2]. Similarly, water movement in water channels (aquaporins) has the properties of a continuous column of water molecules moving in correlated fashion [3]. A more intuitive argument for a pore in ion channels is the high throughput rate, which easily reaches values of 10^6 to 10^8 ions per second. This means that all the steps of recognizing and passing each ion across the membrane take place in only 10 to 1000 ns, a time much shorter than any known enzymatic catalysis but perfectly compatible with diffusion in an aqueous pore of atomic dimensions. Another major argument for a pore was the finding that many small channel blockers act like plugs entering a tunnel that becomes too narrow for them to pass all the way through (Fig. 1). Strikingly, the plug could be knocked out of the pore (thus unblocking the channel) by raising the concentration of permeant ions on the *opposite* side of the membrane. The classic example was the block of K^+ channels by intracellular tetraethylammonium ion (TEA) and analogs, which could be relieved by raising the extracellular K^+ concentration [4]. Armstrong [4] argued that the only way for external ions to push out an internal blocker was if they met each other within a pore. Finally, the ion selectivity of several channels (e.g., voltage-gated Na^+ channels, nicotinic acetylcholine receptors, and γ-aminobutyric acid [GABA] receptor channels) could be explained by assuming a rigid pore size that passes ions smaller than the postulated hole but not those larger than the hole [1,5].

Modern confirmation of these older ideas comes from X-ray crystallography. As had been predicted from the functional studies, the crystal structure of the bacterial KcsA K^+ channel shows beautifully a long pore of atomic dimensions with 2 to 4 K^+ ions residing within the narrow tube ([6]; also see Chapter 34). All ion channels related to K^+ channels should have a similar structure. This would include voltage-gated Na^+, K^+, Ca^{2+}, and I_h channels; inward rectifier K^+ channels; and two-P domain K^+ channels, as well as cyclic-nucleotide-gated channels and ionotropic glutamate receptor channels. They all belong to the structural superfamily of ion channels formed by four homologous subunits or by four homologous domains with four P loops (reentrant pore loops) coming together to line the narrowest part of the pore (see Chapter 34).

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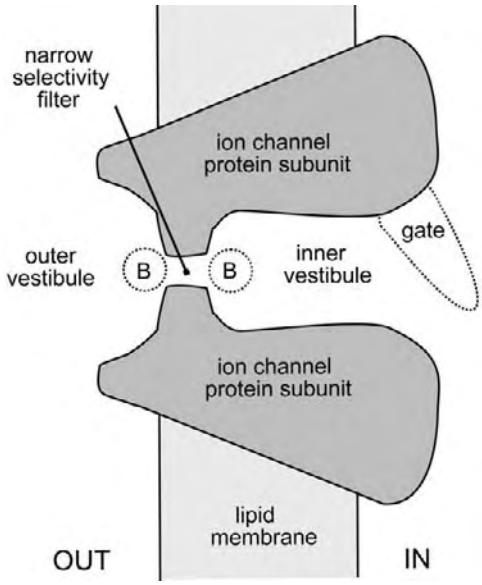


Figure 1 A highly selective ion channel of the voltage-gated family, showing the functional parts discussed in the text. The circles labeled B represent the binding positions of blocking drugs that would act to plug the narrowest part of the pore from the inside or the outside.

Ion Selectivity

Theory

Ion selectivity is necessary for ion channels to play their physiological roles. For electrical signaling it is essential to have two classes of ion channels with different electromotive forces so that alternating opening of one or the other causes the membrane potential to change. The electromotive force generated by a perfectly selective channel is given by the Nernst equation. It depends only on the ratio of the concentrations of the one permeant ion on the two sides and on the absolute temperature. For example, for K^+ ions, the equilibrium potential is

$$E_K = (RT/Fz_K)\ \mathrm{In}\ ([K^+]_o/[K^+]_i) = (59\ \mathrm{mV})\ \log_{10}\ ([K^+]_o/[K^+]_i)$$

where R, T, and F are the usual thermodynamic constants; z_K is the valence of K^+; $[K^+]_o$ and $[K^+]_i$ are the outside and inside K^+ concentrations (more properly, thermodynamic activities); and the right-hand form is a practical formula specifically for 25 °C. For typical excitable cells, the Nernst potential (equilibrium potential) for K^+ ions (E_K) is near –90 mV, and that for Na^+ ions (E_{Na}) is near +50 mV. This means that by opening K^+-selective channels, the cell could achieve a membrane potential of –90 mV (the usual resting condition), and by transiently opening Na^+-selective channels, it can transiently change the membrane potential to +50 mV (a typical strategy during nerve and muscle excitation).

In many ion channels the selectivity is not perfect. If, for example, a channel is permeable to Na^+ and to K^+, then the electromotive force it generates would lie between E_{Na} and E_K. The exact value depends both on the relative permeabilities to each ion (P_{Na}/P_K) and on the relative concentrations of each ion. In the nicotinic acetylcholine receptor, where P_{Na} and P_K are approximately equal, the physiological zero-current potential is near 0 mV. Such nonequilibrium, zero-current potentials are often described by an empirical equation called the Goldman–Hodgkin–Katz (GHK) voltage equation [1]. Indeed the GHK equation provides a useful quantitative definition of the relative permeabilities for ions in real channels.

Examples

Most ligand-gated synaptic ion channels are poorly selective. They select primarily for the *charge* of the ion and will pass a large number of either small cations or small anions ([1]; also see Chapter 38). Good examples are the nicotinic acetylcholine and 5-HT_3 receptor channels, which will pass all monovalent *cations* with diameters up to about 6.5 to 7 Å. Over 65 permeant ions are known for them. Ions such as Na^+, guanidinium, and isopropyl ammonium have nearly equal permeabilities. Small divalent metals and alkaline earths pass also, although usually less well. Analogously, GABA and glycine receptor synaptic channels will pass monovalent *anions* up to diameters of about 5 Å. Thus, anions such as Cl^-, nitrate, thiocyanate, and iodide are highly permeant. These ion channels with large pore diameters and poor selectivity form their pores at the axial point of contact of five homologous protein subunits.

On the other hand, the ion channels formed by four homologous protein subunits or internal repeats have higher ion selectivity and smaller effective pore diameters [1]. Most K^+ channels will pass K^+, NH_4^+, Tl^+, and maybe Rb^+, but they usually do not pass Na^+, Cs^+, or methylammonium measurably. The effective pore diameter is around 3 Å. Voltage-gated Na^+ channels pass 10 cations including Na^+, Li^+, H^+, guanidinium, and even K^+ (with a relative permeability P_{Na}/P_K of about 13). The voltage-gated Ca^{2+} channels select divalent cations over monovalent cations by a factor of 500 to 2000, but they pass Ca^{2+}, Ba^{2+}, and Sr^{2+} with about equal ease. If all divalent cations are removed, Ca^{2+} channels increase their monovalent cation permeability enormously. Among the most selective known channels are some of the epithelial Na^+ channels (ENaC/degenerin) whose K^+ permeability is too low to measure. We are not sure how many homologous protein subunits the ENaC channels contain.

Mechanisms

The mechanisms of ion selectivity are imperfectly understood, but a few principles are clear. The local electrical potential in the general vicinity of the pore and in narrower parts of the pore can strongly bias the selectivity towards cations or anions and tune the preference between monovalents and divalents. All Ca^{2+}-permeable channels have a high density of negative charge in the pore, and neutralizing some of this charge by mutation can make the pore prefer monovalent ions instead of divalents. Similarly, the cation-preferring nicotinic acetylcholine receptor channel can be made permeable to anions by mutations that include neutralizing some negative charges in the pore (see Chapter 38).

Although proteins are not rigid, the concept of a pore size has worked well. The narrowest part of the pore is called the selectivity filter (Fig. 1). Channels usually have a maximum size for permeant ions and are permeable to most ions that are smaller than that size. For channels with a wide pore, simple concepts of friction suffice to describe the greater mobility in the pore of small permeant ions relative to larger permeant ions. In highly selective channels, amino acids of the selectivity filter present polar groups that displace some of the water molecules in contact with the ion. High selectivity cannot be achieved without touching the ion. The relative energies of this electrostatic ion exchange are important determinants of the selectivity sequence in selective channels. The ability to attract ions depends on the relative energy of interaction of water molecules with the ion versus the ligands of the channel wall with the ion. Favorable interaction with the channel can favor permeation up to a point, but an excessive interaction will impede passage by slowing the departure of the ion.

Finally, the mechanics and electrostatics of several ions moving in single file give surprising properties to permeation. The movements of ions become correlated; one ion may interfere with entry of another, and entering ions may help expel other ions electrostatically [2,7].

Block

Many pharmaceutical agents and neurotoxins act by blocking ion channels. For example, local anesthetics and tetrodotoxin block voltage-gated Na^+ channels. Amiloride blocks epithelial Na^+ channels. Dihydropyridines block voltage-gated Ca^{2+} channels. TEA, Cs^+, Ba^{2+}, and some scorpion toxins, related to charybdotoxin [8], block voltage-gated K^+ channels. These agents have in common that they act by physically obstructing the pore near the selectivity filter (Fig. 1) rather than by binding far from the permeation pathway and keeping the channel closed by an allosteric mechanism.

Several biophysical arguments originally led to this conclusion. The first was the finding that many blockers could be driven out of the pore by adding permeant ions to the opposite side of the membrane, as we already described [4]. Another argument was the finding that the block by charged blockers has a voltage dependence. The voltage dependence could be described by two classes of models. In one model, the voltage dependence arose because the charge of the blocker had to move through a portion of the membrane electric field into the pore to reach the blocking position [9]. In the other, the blocker had to displace a column of permeant ions in the pore whose movement through the electric field gave voltage dependence [4,7]. Both models are partially correct, and both require a pore. Finally, measurements of gating currents ([4,10]; also see Chapter 35) showed that voltage-sensitive conformational changes that underlie voltage sensing and normal gating continue to occur when channels are blocked.

Two molecular arguments have greatly strengthened the classical ones. Mutagenesis of amino acid residues of the pore mouth and walls can have large effects on the potency of blockers [1,11,12] and X-ray crystallography of K^+ channels has revealed the blocker sitting within the pore as diagramed in Fig. 1 [13].

How and where does the blocker bind? Classical studies of the voltage dependence of various blockers suggested that both voltage-gated and ligand-gated channels have wider vestibules, on the outside and the inside that taper toward the selectivity filter [1]. Subsequent structural and biochemical work has confirmed these ideas (see Chapters 34 and 38). Larger organic blockers such as tetrodotoxin, local anesthetics, dihydropyridines, amiloride, TEA, or charybdotoxin become lodged in the inner or outer vestibule. Typically, several amino acids of the vestibule or of the beginning of the selectivity filter can be identified by mutagenesis as contact sites (i.e., contributing to the receptor for the blocker). They combine hydrophobic, polar, and charge–charge interactions with the blocker. Paradoxically, some blockers are also permeant. These are ions small enough to enter the selectivity filter but having dwell times in that position that are longer than for the typical permeant ion. Thus, they pass through only slowly. While the filter is occupied by the slowly permeant blocker, other permeant ions have to wait for the channel to clear, so the flux is lowered. Ions in this category include Ba^{2+} and Cs^+ in K^+ channels [14] and H^+ in Na^+ channels [9].

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CHAPTER 38

Agonist Binding Domains of Glutamate Receptors: Structure and Function

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At the majority of excitatory synapses in the brain, the amino acid L-glutamate acts as the neurotransmitter that is released by calcium-dependent exocytosis from presynaptic nerve terminals. After diffusion across the synaptic cleft, molecules of L-glutamate bind to two families of neurotransmitter receptors in the postsynaptic membrane: the ligand-gated ion channels and G-protein-coupled receptors. The architecture of both families of glutamate-activated signaling proteins differs from that for other ligand-gated ion channels, on the one hand, and the majority of G-protein-coupled receptors, on the other. In both cases, a distinguishing feature of glutamate receptors is the structure that generates the agonist binding site. This domain shares homology with a large family of bacterial periplasmic binding proteins, a number of which have been crystallized. In these bacterial proteins, ligands bind in a cleft between two globular domains connected by beta strands. The binding of ligand stabilizes a closed cleft conformation in which the faces of each domain make contact with each other, while in the absence of ligand the domains assume an open clamshell-like conformation [1]. In the case of glutamate receptors, which are thought to assemble as tetramers for the ion channel family and as dimers for the G-protein-coupled family, each subunit contains a complete copy of an agonist binding domain. Thus, there are four agonist binding sites per glutamate receptor ion channel and two agonist binding sites per G-protein receptor dimer. This understanding of the organization of the architecture of glutamate receptor channels emerged only recently and was brought into focus by the solution of crystal structures for the ligand binding domains of two ion channels and a G-protein-coupled glutamate receptor [2–6]. However, the picture is still far from complete, and the mechanisms that couple ligand binding to signal transduction have yet to be defined at atomic resolution.

The domain organization of glutamate receptors is shown in Fig. 1. The simplest architecture is found in GluR0, a prokaryotic glutamate receptor ion channel from the photosynthetic bacterium *Synechocystis* PCC 6803 [7]. In this protein, the ligand binding core is formed by a protein with two globular domains that show structural homology to GlnBP, the glutamine binding protein from *Escherichia coli* [5]. The key differences between GluR0 and GlnBP are that in GluR0 the amino acid sequence that generates the ion channel pore interrupts the S1 and S2 amino acid sequence that encodes the ligand binding domain. This is possible without disrupting the common fold found in GluR0 and GlnBP because the ion channel emerges from the external surface of the second globular domain in GluR0, without occluding the ligand binding site, and thus can be removed by protein engineering for crystallographic studies.

Eukaryotic glutamate receptor ion channels from the AMPA, kainate, and *N*-methyl-D-aspartate (NMDA) subtypes have a more complicated architecture with two differences from GluR0. First, not one but two bacterial periplasmic protein homology domains are present per subunit; second, the ion channel pore contains an additional transmembrane segment followed by a cytoplasmic domain of variable size.

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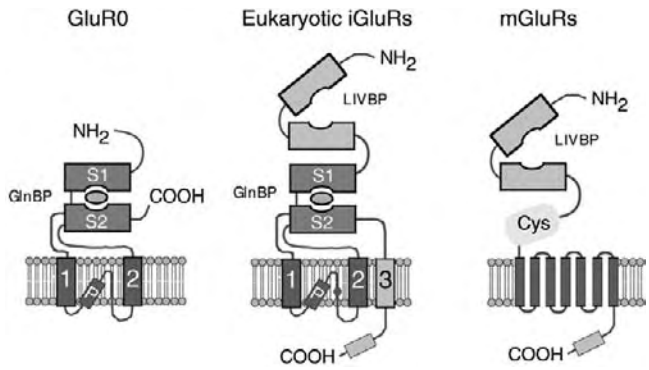


Figure 1 Domain organization of GluR0, a prokaryotic glutamate receptor ion channel, eukaryotic glutamate receptor ion channels (iGluRs), and G-protein-coupled glutamate receptors (mGluRs). The ligand binding domains are shaded to indicate structural homology with two families of bacterial periplasmic binding proteins, glutamine binding protein (GlnBP), and leucine/isoleucine/valine binding protein (LIVBP). The membrane domains of ion channels contain two transmembrane segments (1 and 2) and pore helix (P), combined with a third transmembrane segment for eukaryotic iGluRs; the pore interrupts the GlnBP-homology-domain-creating segments S1 and S2. mGluRs contain a cysteine-rich domain (Cys) that couples the ligand binding core to a seven-segment transmembrane region and intracellular domain.

Starting from the amino terminus, the initial 400 amino acids in eukaryotic glutamate receptor ion channels share weak sequence homology with leucine/isoleucine/valine binding protein (LIVBP). In glutamate receptor ion channels the function of this domain is poorly characterized. The results of functional and biochemical studies show that this region, which is often called the amino terminal domain, plays a major role in subunit assembly [8,9] but does not bind L-glutamate (or in the case of NMDA receptor NR1 subunits glycine) and instead is the site of action of some allosteric modulators [10–12]. Following the amino terminal domain the S1 and S2 agonist binding segments (interrupted by the core of the ion channel pore, similar to the arrangement in GluR0) form the agonist binding domain, as revealed in the structure of the AMPA receptor GluR2 subunit [2,3]. A third transmembrane segment that forms part of the ion channel occurs after segment S2 and precedes the cytoplasmic C-terminal domain. G-protein-coupled glutamate receptors, typified by mGluR1, are also multidomain structures in which each subunit contains a single glutamate binding site that shows structural homology to LIVBP [4,6]. The ligand binding core in G-protein-coupled receptors is followed by a cysteine-rich region, a seven-segment transmembrane region, and a 250-residue intracellular domain. The two subunits are linked together by a disulfide bond between the ligand binding domains.

Despite the fact that L-glutamate is the naturally occurring ligand that activates ion channel and G-protein-coupled glutamate receptors, crystal structures of the ligand binding cores of these proteins reveal distinct mechanisms for the coordination of ligands. In all glutamate receptors, the ligand binds at the interface between the two globular domains that make up an individual subunit. The ligand makes contact with amino acids in domains 1 and 2, but the chemistry of the binding surface differs substantially in individual proteins. This allows the binding of conformationally restricted ligands such as AMPA with exquisite subtype selectivity. The ligand binding cores of GluR0 and GluR2 share a fold similar to glutamine binding protein but have markedly different substrate binding preferences, while the fold of the G-protein-coupled receptor mGluR1 more closely resembles LIVBP. However, in GluR0 and mGluR1 L-glutamate binds in an extended conformation in which the γ-carboxyl group interacts with domain 1, while in GluR2 the torsion angle of the ligand side chain undergoes a 105° rotation, positioning the γ-carboxyl group for interaction with domain 2. The high-resolution structures obtained for GluR0 and GluR2 reveal that in addition to protein–ligand interactions there are multiple solvent-mediated interactions of the glutamate γ-carboxyl group with the ligand binding core. It is believed that L-glutamate first binds to domain 1 and that subsequent domain closure buries the ligand and permits interactions of the alpha and gamma functional groups with domains 1 and 2, stabilizing the ligand-bound, closed-cleft conformation.

How is ligand binding translated into a signal that causes ion channel gating or G-protein signaling? Crystal structures of all three proteins and equilibrium ultracentrifugation experiments for the ligand binding domains of GluR0 and GluR2 reveal the formation of dimers [3–6,13]. While this was to be expected for mGluR1, due to the presence of an inter-subunit disulfide bond, the presence of dimers for GluR0 and GluR2 was a novel finding. In mGluR1, the cysteine residues responsible for dimerization lie in a disordered segment that probably functions to increase the membrane concentration of the dimeric species. Strikingly, for all three proteins the dimer interface is formed exclusively by domain 1 and suggests mechanisms by which ligand binding mediates signal transduction. For GluR2, comparison of the crystal structures of the glutamate-bound and ligand-free forms does not reveal any rearrangement of the dimer interface produced by the binding of ligand [3]. Instead, the distance between the domain 2 surfaces from which the ion channel emerges *increases* in the ligand-bound, closed-cleft conformation. It is thus plausible that agonist-stabilized domain closure causes the linkers leading to the ion channel transmembrane helices to move farther apart and that this forces the ion channel to open (Fig. 2).

A role for the dimer interface in the process of desensitization, which proceeds on a millisecond time scale for glutamate receptor ion channels, also emerges from these structural studies. Experiments with the AMPA receptor GluR2 subunit revealed that stabilization of the intradimer interface by either mutations at the dimer interface or allosteric modulators that bind to the dimer interface and glue the two subunits together reduces desensitization. On the other hand, perturbations that destabilize the interface enhance desensitization. Desensitization almost certainly occurs via rearrangement of the dimer interface, which disengages the agonist-induced conformational change in the ligand-binding core from the ion channel gate.

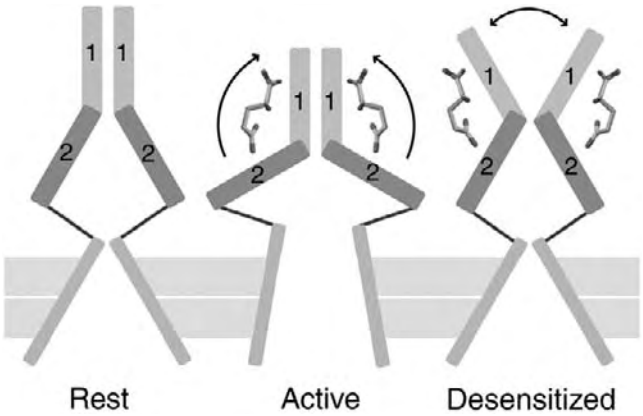


Figure 2 A structural model for iGluR activation and desensitization based on crystallographic and biochemical studies that reveal assembly of the ligand binding cores as dimers via domain 1 surfaces. At rest, the ligand binding domains assume an open conformation and the channel is closed. During activation, the ligand binding cores contract, exerting tension on the ion channel segments and causing the channel to open. During desensitization, the ligand binding cores remain contracted, but movement about the domain 1 dimer interface allows the channel to enter a nonconducting state.

In contrast to the above picture, the binding of agonist for mGluR1 is associated with large changes in the relative orientation of the ligand binding domains which results from a 70° rotation around the dimer interface. This causes the separation between the pair of domain 2 surfaces that leads to the transmembrane segment to decrease in the agonist-bound form, presumably leading to a conformational change that activates G-protein signaling. The different behavior of the dimer interface in response to the binding of agonist in the ion channel and G-protein-coupled glutamate receptors is striking but does not imply that rotations around the dimer interface are absent in the ion channel proteins. Likewise, there may well be additional conformational states available to G-protein-coupled glutamate receptors that do not involve rotations around the dimer interface and which have not yet been crystallized. These structures mark a tremendous advance in our understanding of how glutamate receptors function, but much experimental work remains to be done. There are additional domains and subtypes to crystallize, as well as the full length proteins. There is also the pressing issue of the nature of the conformational states that correspond to the different functional states identified in functional studies.

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CHAPTER 39

Nicotinic Acetylcholine Receptors

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Function

In the 1950s, Katz and his coworkers [1] described the essential functional characteristics of the muscle-type nicotinic acetylcholine receptor in the postsynaptic membrane of the vertebrate neuromuscular junction. This receptor dwells in three types of states: resting, open, and desensitized. In the resting state and the desensitized states, the receptor is nonconducting. In the open state, the receptor conducts Na^+, K^+, and Ca^+, causing depolarization of the postsynaptic membrane. Binding of acetylcholine (ACh) shifts the distribution of states rapidly from resting to open, with the probability of opening being much greater when two AChs are bound than when one is bound. Normally, ACh is removed from the synapse within a few milliseconds by diffusion and by acetylcholinesterase, and the receptor returns directly to the resting state. On longer exposure to ACh, the receptor enters the desensitized state, in which the affinity for ACh is three to four orders of magnitude greater than in the resting state [2], and the receptor returns to the resting state when ACh is removed. Underlying the macroscopic depolarization of the postsynaptic membrane are rapidly fluctuating openings and closings of thousands of receptors [3]. A single open ACh receptor can pass 10^7 cations per second.

Neuronal-type nicotinic ACh receptors are found in peripheral and central neurons. These receptors differ from muscle-type receptors in subunit composition, pharmacology, and channel properties [4,5]. Nicotinic receptors are also found in many invertebrate phyla. At least one invertebrate nicotinic receptor conducts anions rather than cations [6]. Langley [7] observed that muscle contracted on application of the plant alkaloid nicotine and postulated a "receptive substance" for nicotine. Nicotine, known before acetylcholine, is an agonist of the acetylcholine receptor. The archetypical competitive antagonist of the muscle-type ACh receptor is another plant alkaloid, (+)-tubocurarine, which binds to the muscle-type receptor with a dissociation constant of about 100 n*M*. Elapid snake venoms contain positively charged polypeptide toxins (α-neurotoxins) that are also competitive antagonists of the receptor but with dissociation constants in the range of 10 to 100 p*M*. These toxins have been indispensable tools in the exploration of the ACh binding sites and in the assay of muscle-type and some neuronal-type ACh receptors [8].

Structure

Subunit Composition

Muscle-type ACh receptors were first isolated from electric tissue of electric eel (*Electrophorus electricus*) and electric ray (*Torpedo* spp.), which are the richest sources [9]. The receptors in *Torpedo* electric tissue and in fetal muscle are heteropentamers, with the subunit composition $\alpha 2\beta\gamma\delta$ (Fig. 1A) [10]. In adult muscle, the receptor contains an ε subunit in place of the γ subunit and has a greater single-channel conductance and a smaller mean single-channel-open time than fetal receptor [11]. The neuronal-type ACh receptors are also pentamers, but of one, two, or three types of subunits [4,5]. There are nine known neuronal α subunits (α2 to α10) and three known neuronal β subunits (β2 to β4); various combinations of α subunits and β subunits, when expressed heterologously, yield functional complexes with distinct functional properties. Some neuronal α subunits (e.g., α7) form functional homopentamers. Different regions of the peripheral and central nervous system express different combinations of the neuronal subunits.

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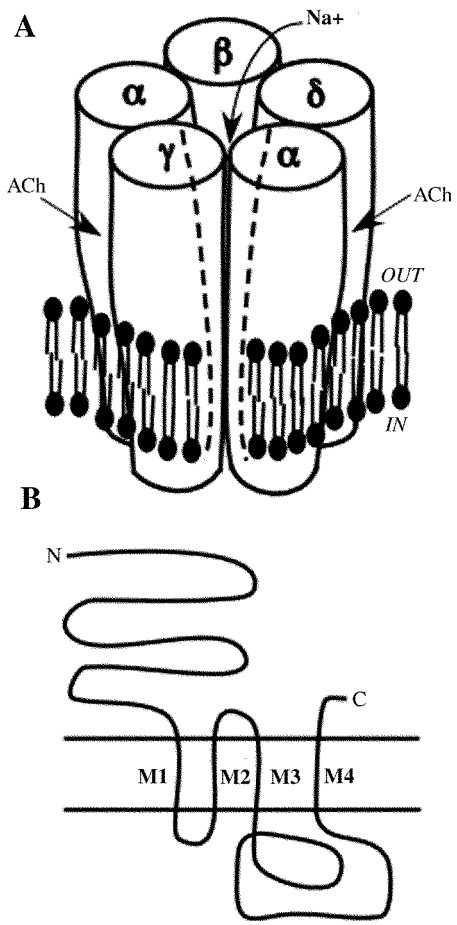


Figure 1 Structure of the nicotinic acetylcholine receptors. (A) Schematic representation of the quaternary structure showing the arrangement of the subunits in the muscle-type receptors, the location of the two ACh binding sites between an α and a γ subunit and an α and a δ subunit and opening to the periphery of the complex, and the axial cation-conducting channel (dashed lines). (B). The threading pattern of the subunits through the membrane.

Primary Structure

The subunits are 450 to 650 residues long. The aligned sequences are sufficiently similar that their common origin is certain; that is, they are all homologous [5,12,13]. One completely conserved characteristic of all of these sequences is a 15-residue loop closed by a disulfide bond between two Cys residues separated by 13 conserved residues. All subunits also contain four hydrophobic segments (M1–M4) sufficiently long to form membrane-spanning α-helices. The subunits of the ionophoric receptors for γ-aminobutyric acid, glycine, 5-hydroxytryptamine, and an invertebrate glutamate receptor are homologous to those of the nicotinic ACh receptors, and these subunits constitute a superfamily of Cys-loop receptors. Conservation of primary structure implies conservation of three-dimensional structure as well.

Secondary and Tertiary Structures

The topology of these subunits with respect to the membrane has been worked out in *Torpedo* ACh receptor (Fig. 1B) [12]. The N-terminal half of each subunit is extracellular. Three membrane-spanning segments (M1, M2, M3) follow. A long cytoplasmic loop connects M3 to a fourth membrane-spanning segment (M4), and a short C-terminal tail is extracellular. Each subunit is glycosylated at one or more extracellular sites.

The structure of the extracellular domain of the ACh receptor can be inferred from the crystal structure of a water-soluble ACh binding protein secreted by snail glial cells [14]. The ACh binding protein is a homopentamer of a subunit that is homologous to the extracellular, N-terminal half of the ACh receptor subunits. The ACh binding protein subunit starts at its N terminus with two short helices; the remaining sequence forms ten β-strands and connecting loops arranged in a modified immunoglobulin fold. There is less certainty about the structures of the membrane-embedded and cytoplasmic domains of the receptor. Patterns of reactivity, infrared spectroscopy, and prediction algorithms indicate that the membrane-spanning segments are largely, but not completely, α-helical [12,15].

Quaternary Structure

The overall shape of the ACh receptor is known from cryo-electron microscopy of two-dimensionally ordered *Torpedo* receptors in membrane [16] and from the X-ray structure of the ACh binding protein [14]. The receptor is a narrow-waisted cylinder, roughly 120 Å in length, of which 65 Å is extracellular, 30 Å spans the lipid bilayer, and 25 Å is intracellular. The extracellular domain is 80 Å in diameter. The five subunits are arranged like thick barrel staves around an axial channel. The channel lumen is about 30 Å in diameter in the extracellular domain and tapers to less than 10 Å in the membrane domain. It is possible that access to the channel on the cytoplasmic side is through gaps between the cytoplasmic domains of neighboring subunits [16].

ACh Binding Sites

The ACh receptor α subunit was originally identified as contributing to the ACh binding site by its specific reaction with a radioactive affinity label, and the two α subunits correspond to the two ACh binding sites per receptor complex. The labeling was mapped to αCys192 and αCys193, and these adjacent Cys were shown to be disulfide-bonded to each other [12], a rare arrangement. Four aromatic residues, well separated in the sequence αTyr93, αTrp149, αTyr190, and αTyr198, were also affinity labeled [17]. These six residues in α are at the interface between the α subunit and a neighboring subunit. They form the principal side of the ACh binding site [14].

In the muscle-type receptor, the complementary side of the first ACh binding site is formed by the γ (or ε) subunit, and the complementary side of the second ACh binding site is formed by the δ subunit. Affinity labeling pointed to γTrp53, γLeu109, γTyr111, γTyr117, and the aligned residues in the δ subunit as contributing to these complementary sides [18].

Mutations of each of the residues from the principal and complementary sides of the site affect agonist binding, gating kinetics, or competitive antagonist binding [17,19]. The location of the ACh binding sites in the interface between subunits is consistent with the idea that binding of ACh promotes relative movement of the subunits that propagates through the bilayer to the gate [12,16].

In the homopentameric ACh binding protein, each of the five binding sites is formed in the interface between opposite sides of adjacent subunits [14]. All of the ACh binding site residues identified in the muscle-type receptor α subunit are on one side of the ACh binding protein subunit, and all of the binding site residues identified in muscle-type γ and δ subunits are on the other side of the ACh binding protein subunit and form the complementary side of the binding site. The ACh binding site is a cage of mostly aromatic residues, the door to which is formed by the adjacent disulfide-bonded cysteines. Based on the ACh binding protein structure, the ACh binding sites in the ACh receptors are about 30 Å above the membrane, opening to the outside of the cylindrical extracellular domain.

Acetylcholine and other specific ligands of the ACh binding site contain at least one quaternary ammonium group or protonated tertiary ammonium group. The preponderance of aromatic residues lining the binding site cavity is consistent with π–cation interaction between aromatic rings and quaternary ammonium groups [20]. In addition, there are conserved negatively charged residues in γ and δ in the vicinity of the binding site that might move toward a bound ammonium group as part of the conformational change leading to channel opening [19].

Channel

The channel has three tasks. It must mitigate the high-energy barrier to the translocation of an ion from one polar aqueous phase to another, through a nonpolar lipid membrane; it must select among ions both by size and by charge; and it must open and close. The energy barrier is partly mitigated by the funnel shape of the channel [16] and by its water content. Only a short section (≈6 Å long) near the cytoplasmic end is narrow enough to force water and a cation to move in single file [21]. It is in this section, where the translocating cations contact the walls, that size selection occurs and where some of the residues determining charge selectivity reside [12,17]. The resting-state gate may also be in this section [22].

The channel-lining residues were identified by photolabeling with channel blockers, by the effects of mutations, and by the accessibility to small, charged sulfhydryl reagents of residues mutated to Cys [12,17]. The pseudo symmetry of the ACh structure, the sequence similarity among subunits, and the labeling within the channel indicate that in heteropentameric complexes the five subunits make similar but not identical contributions to the channel lining. Toward the extracellular side of the membrane where the channel is relatively wide, residues from both M1 and M2 form the lining. Toward the intracellular side of the membrane, where the channel is narrowest, the lining is formed just by M2 and by the residues immediately flanking the cytoplasmic end of M2 in the M1–M2 loop [23]. In the open state, the M2 residues that are exposed in the channel lie within a 100° sector of a helical wheel. By contrast, the M1 residues exposed in the channel do not conform to a regular secondary structure.

It is likely that aligned residues in the sequence are also in register in the channel lining, forming pentameric rings of similar residues exposed at different levels of the channel [17]. The functional roles of some of these rings are clear. Starting from the cytoplasmic end of the channel, in the predicted M1–M2 loop, a ring of four glutamates and one glutamine (five glutamates in homopentameric receptor) strongly determines the magnitude of cation conductance [24] and the intrinsic negative electrostatic potential in the channel [25]. Three steps in the extracellular direction, a ring of four or five threonines and serines strongly determines selectivity among different cations ([26]; also see Chapter 36). These two rings are in the narrowest part of the channel. Rings of hydrophobic residues further in the extracellular direction stabilize the resting state compared to the open state [12].

The structure of the channel is different in the resting, open, and desensitized states. These differences are reflected in electron microscope images of ordered arrays of receptors in membrane [16] and in the binding of channel-blocking aromatic amines, the reactions of channel-lining residues with photolabels, and the reactivity of substituted cysteines with charged sulfhydryl reagents [22]. The reactivity differences and the underlying structural changes are widespread, beyond the immediate vicinity of the gate. One view based on the accessibility of substituted cysteines from the two sides of the membrane is that the resting-state gate is in the same narrow region at the cytoplasmic end of the channel that forms the selectivity filter [22,27]. Another view based on cryo-electron microscopy is that this gate is in the middle of the membrane-spanning portion of the channel [16]. These views could be reconciled if the narrow part of the channel formed by the cytoplasmic end of M2 and the M1–M2 loop led into an antechamber that extended into the plane of the bilayer.

The charge selectivity of a homopentameric ACh receptor [17], as well as of the $5HT_3$ receptor [28], is changed from cationic to anionic by a minimum of three changes in the M1–M2 loop and in M2. Two of the changes are in the narrowest region; the glutamate is changed to glutamine, eliminating the negative charge, and a proline is inserted just before it, lengthening this region by one residue. A third change is two-thirds the distance toward the extracellular end of M2, where a valine is changed to a threonine. The new residues match those in the anion-conducting glycine receptor. The reverse mutations in the glycine receptor change its selectivity from anionic to cationic [29]. The mutation of the ring of glutamates indicates an electrostatic contribution to charge selectivity, but the bases for the effects on charge selectivity of the other two mutations are not known.

Cytoplasmic Domain

The cytoplasmic domain of each subunit consists of a short loop between M1 and M2 and a long loop between M3 and M4. As discussed above, the M1–M2 loop is involved in selectivity and gating. The much larger M3–M4 loop is involved in subunit assembly [30] and in targeting [31], clustering [32], and anchoring [33,34] of the assembled receptor in the postsynaptic membrane. Phosphorylation of sites in this loop modifies the rate of desensitization and may regulate interactions of the receptor with cytoplasmic proteins [35,36].

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CHAPTER 40

Small Conductance Ca^{2+}-Activated K^+ Channels: Mechanism of Ca^{2+} Gating

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Introduction

Small-conductance Ca^{2+}-activated K^+ channels (SK channels) play a fundamentally important role in all excitable cells. They are K^+ selective, voltage independent, and activated by increases in the levels of intracellular Ca^{2+}. SK channels are exquisite Ca^{2+} sensors that couple $[Ca^{2+}]_i$ to membrane potential. SK channels may be activated by a global rise in Ca^{2+} due to entry through voltage-gated Ca^{2+} channels, such as occur during an action potential in nerve cells. They may respond to the discrete localized Ca^{2+} that enters through agonist-induced opening of Ca^{2+}-permeable ionotropic receptors that are close to SK channels, such as acetylcholine (ACh) receptors in inner-ear hair cells. SK channels may also open due to Ca^{2+} released from intracellular stores as occurs in many gland cells, permitting rhythmic hormone release. Centrally important to the physiological roles of SK channels in a wide variety of cell types are their high Ca^{2+} sensitivity and fast gating kinetics. The SK channel family has been cloned, and the mechanism by which Ca^{2+} ions affect channel gating has been investigated. The results reveal a remarkable molecular marriage between SK channels and the ubiquitous Ca^{2+} sensor, calmodulin. They also suggest a novel chemomechanical gating mechanism in which a dimer-of-dimers with two-fold symmetry translates the gating cue of Ca^{2+} binding to a tetrameric ion-conducting pore.

The first report of a Ca^{2+}-dependent K^+ flux was in 1958 from Gardos [1], who observed Ca^{2+}-stimulated K^+ flux across red blood cell membranes. Since then, Ca^{2+}-activated K^+ channels have been recorded from almost every cell type in mammalian organisms as well as many other species. Ca^{2+}-activated K^+ channels fall into two broad classes, both of which have been cloned. BK (big K) channels have large unitary conductance values (≈150–200 pS in symmetrical 140 m*M* K^+) that propelled them to prominence as prototypes for single-channel gating and conduction studies [2]. Under physiological conditions, BK channel gating depends upon voltage as well as intracellular Ca^{2+}. However, recent biophysical studies have shown that BK channels are voltage-dependent channels, and voltage dependence is modulated by Ca^{2+} ions; higher concentrations of Ca^{2+} facilitate voltage gating [3–5]. The molecular mechanism of Ca^{2+}-modulated BK channel gating is not well understood. SK (small K) channels have smaller unit conductance values (10–50 pS) and are gated solely by intracellular Ca^{2+} ions. The mechanism by which Ca^{2+} ions affect SK channel gating is discussed in this chapter.

Clones Encoding SK Channels

SK channel sequences were identified by a virtual screen of the EST database using as probe the amino acid sequences from the pores of K^+ channels that endow exquisite K^+ selectivity, the signature sequence [6]. A short sequence that was related but different from known K^+ channel pore sequences was isolated and used as a probe on

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brain cDNA libraries, yielding three distinct cDNA clones: hSK1 from human and rSK2, and rSK3 from rat [7]. A fourth homologous sequence was subsequently identified, hIK1 (intermediate K; see later discussion) [8,9]. The coding sequences predict polypeptides that share the transmembrane (TM) topology of voltage-dependent K^+ channels and cation-selective cyclic nucleotide-gated channels, with the N- and C-terminal domains within the cytoplasm and a core region of six TM domains. The pore loop resides between TMs 5 and 6, and functional channels are tetrameric assemblies. Amino acid sequences of the three different SK clones are remarkably homologous through the transmembrane core and the proximal segment of the intracellular C-terminal domain. As with other K^+ channel subfamilies, the sequences diverge in their extreme N- and C-terminal cytoplasmic domains. Despite the architectural similarities with voltage-gated K^+ channels, the sequences differ substantially from other K^+ channel subfamilies except for the recognizable deep pore sequence surrounding the selectivity filter, clearly defining SK channels as a distinct branch of the K^+ channel family tree.

Biophysical and Pharmacological Profiles

Heterologous expression of SK cDNAs yields channels with biophysical and pharmacological profiles that recapitulate native SK channel activity. Application of Ca^{2+} to the inside face of inside-out patches activates the channels, and dose–response experiments revealed an EC_{50} for Ca^{2+} of ≈ 0.5 μM. Gating is steeply Ca^{2+} dependent, with little or no channel activity at 0.1 μM and maximal activity at 1 μM, resulting in a Hill coefficient of ≈ 4. This suggests that more than one Ca^{2+} ion is required for SK channel activation and that binding may be cooperative. Rapid application ($\approx$1 ms) of solutions containing saturating Ca^{2+} concentrations (10 μM) resulted in rapid channel activation with current onset occurring as rapidly as the solution was exchanged (activation time constants of 6–13 ms) [10]. The lack of voltage dependence indicated by macroscopic current responses was verified by showing that when the channels were exposed to internal Ca^{2+}, the single-channel-open probability did not differ at various voltages. Single-channel analysis also confirmed the relatively small unit conductance value, $\approx$10 pS in symmetrical K^+ solutions, consistent with measurements of native SK channels [7].

Native SK channels fall into two pharmacological categories based upon block by different toxins. Most SK channels, such as those in central neurons responsible for the medium afterhyperpolarization [11,12], skeletal [13,14] and smooth muscle [15–18], lympocytes [19], and gland cells [20,21], are sensitive to the bee venom peptide toxin apamin and the plant alkyloids *d*-tubocurarine and bicuculline salts. The cloned SK channels are all blocked by apamin ($K_i \approx 25$ pM to 25 nM) [7,22–24], *d*-tubocurarine ($K_i \approx 5$–300 μM) [22,24,25], and bicuculline methylchloride ($K_i \approx 1$–10 μM) [26]. These results strongly suggest that the cloned channels represent the apamin sensitive Ca^{2+}-activated K^+ channels recorded from many different cell types.

Mechanism of Ca^{2+}-gating

CaM Is the Ca^{2+} Sensor

Ca^{2+} might affect SK channel gating either by binding directly to the channel protein or by binding to a separate Ca^{2+} binding protein and subsequent interactions with the SK channel. The rapid activation kinetics of the cloned SK channels are similar to the opening rates for ionotropic receptors such as $GABA_A$ [27] or NMDA-type glutamate receptors [28], where there is a direct interaction between the ligand and the receptor. This suggests that Ca^{2+} ions directly interact with the SK channel protein to affect gating. Moreover, steady-state Ca^{2+} sensitivities of all of the cloned SK channels are indistinguishable, suggesting that conserved residues on the predicted intracellular aspect of the channel mediate Ca^{2+} gating. The cloned SK channels have a half-maximal open probability when exposed to ≈ 0.5 μM Ca^{2+}, consistent with an E–F hand Ca^{2+} binding motif [29]. Examination of the amino acid sequences failed to detect either an E–F hand or C2 motif [30,31]; also, no sequences are similar to the Ca^{2+} bowl implicated in Ca^{2+} binding and BK channel gating [32]. However, if the channel protein directly chelates Ca^{2+} ions, then negatively charged side chains, from glutamates (E) or aspartates (D) are likely to mediate Ca^{2+} binding. Site-directed mutagenesis, neutralizing each of the 21 conserved residues on the intracellular side of the channels, failed to alter Ca^{2+} gating, but results from truncation experiments that progressively deleted regions of the C terminus combined with combinatorial site-directed mutations implicated a region in the proximal C-terminal domain. This highly conserved region of ≈ 100 residues could be modeled as a series of α-helices and, contrary to a domain expected to bind Ca^{2+}, is highly positively charged (net +14).

The seemingly contradictory fast Ca^{2+} activation kinetics (function) and positively charged domain (structure) were reconciled by showing that the implicated domain serves as a binding site for calmodulin (CaM). Yeast 2-hybrid tests and CaM binding experiments showed that CaM bound to the CaM binding domain (CaMBD), a region of the channel extending ≈ 100 amino acids from the cytoplasmic interface with S6. Binding of CaM to the CaMBD occurred in the presence or absence of Ca^{2+}. However, CaM only bound to the C-terminal subdomain of the CaMBD in the presence of Ca^{2+}. Coexpression of SK channels and mutant CaMs with reduced Ca^{2+} affinity yielded SK currents with right-shifted, and shallower Ca^{2+} concentration responses ($EC_{50} \approx 1$ μM; Hill coefficient ≈ 2) [10]. These results support a model for Ca^{2+} gating in which CaM is a constitutively associated subunit of the SK channels. Ca^{2+} binding to CaM induces a conformational rearrangement in CaM that is transduced to the channel subunits and results in opening of the SK channel gate.

Calmodulin is an acidic protein composed of 148 amino acids organized into three domains [33]. The N- and C-terminal globular lobes each contain two E–F-hand Ca^{2+} binding domains. A flexible linker region connects the lobes. Ca^{2+} binding is highly cooperative, with binding first occurring at the C-terminal lobe and then the N-terminal lobe. Contributions of the various E–F hands to SK channel gating were investigated by coexpressing the channel subunits, with CaM containing different combinations of mutant E–F hands. Surprisingly, only the N-terminal E–F hands contribute to SK channel gating; loss of either or both of the C-terminal E–F hands to bind Ca^{2+} did not alter Ca^{2+} responses, while loss of either N-terminal E–F hand shifted the response, and loss of both abolished channel function [34].

Structural Analysis and the Chemomechanical Gating Model

The complex between Ca^{2+}–CaM and the CaMBD was crystallized [35]. The solved structure revealed a symmetrical dimer consisting of two CaMBDs and two CaMs (Fig. 1), with multiple charged and hydrophobic contact points. The initial 17 residues extending from the membrane interface were not resolved. The CaMBDs consist of a relatively short α-helix, followed by a bend and then a long, extended α-helix. The extended helices from the two CaMBDs are in a close antiparallel arrangement and these interactions are the only ones between the channel domains. Two CaMs encase the CaMBDs. Each CaM is in an elongated conformation and touches three helices, two from one CaMBD and one from the other, forming multiple contacts and tying the CaMBDs together. The N-terminal E–F hands are clearly calcified, while the C-terminal E–F hands are Ca^{2+} free, consistent with mutagenesis studies. Closer examination revealed that the Ca^{2+}-dependent reorganization of the N-terminal lobe of CaM is similar to other Ca^{2+}–CaM interactions with substrate proteins, where Ca^{2+} binding to the E–F hand motifs consolidates hydrophobic patches into a larger domain that provides an anchor point for interfacing with the substrate protein [36,37]. These interactions likely underlie the Ca^{2+}-dependent gating process. In contrast, the C-terminal lobe is constrained by multiple interactions with the CaMBD. Several important positions within the E–F hand motifs form strong electrostatic anchoring contacts with the CaMBDs that reorient their side chains so they cannot participate in chelating a Ca^{2+} ion. The strong C-lobe interactions probably form the basis for the constitutive association between the two proteins.

The structure of the Ca^{2+}-free form of the complex has not yet been solved; however, several different biochemical approaches yielded consistent results showing that the CaMBD–CaM complex exists as a monomer in the absence of Ca^{2+}. Taken together, the data support a model in which two monomeric CaMBD–CaM complexes associate to form a dimer upon Ca^{2+} binding to CaM (Fig. 2). The SK channels are tetramers of the pore-forming subunits with a presumed

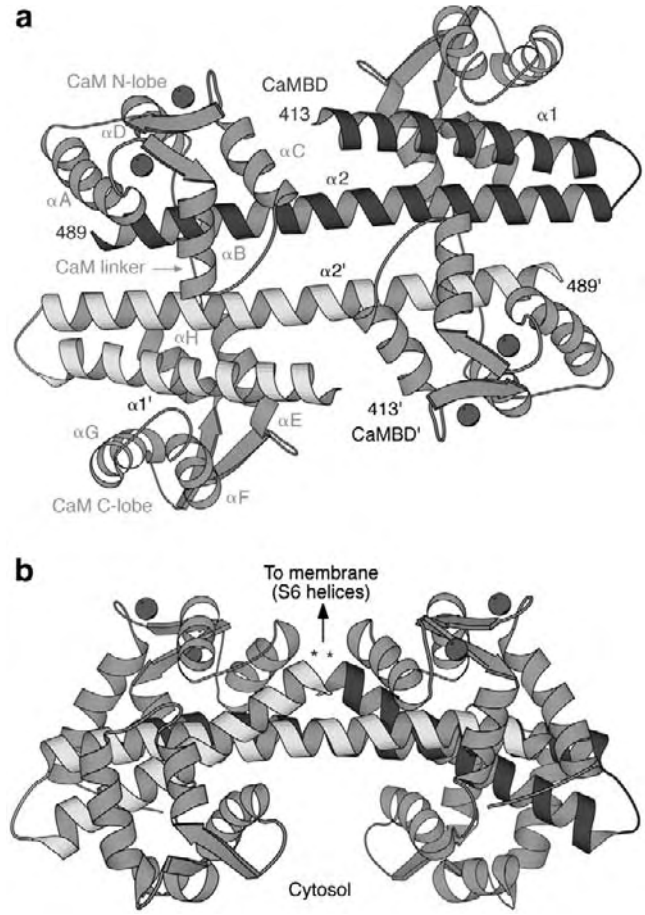


Figure 1 Structure of the CaMBD/Ca^{2+}/CaM complex. (a) Ribbon diagram of the CaMBD/Ca^{2+}/CaM dimeric complex. CaMBD subunits are blue and yellow, CaM molecules are green, and the Ca^{2+} ions are red. Secondary structural elements, the CaM linker, and the first and last observed residues in the CaMBD are labeled. (b) View of part (a) rotated by 90° showing the orientation of the complex relative to the membrane. Arrow indicates the positions of the first observed residue of each of the CaMBD monomers that are linked to the S6 helices.

four-fold symmetry around the pore, at least in the absence of Ca^{2+}. Ca^{2+} binding to CaM induces the formation of a dimer-of-dimers within the gating apparatus, and this may exert a force on the attached S6 helices that results in opening of the ion-conducting pore. This implies that Ca^{2+} gating imposes an asymmetry on the pore structure. Interestingly, the initial 17 residues extending from the membrane interface were not resolved in the crystal structure, and this may be due to the ability of this domain to adopt several conformations reflecting the translation of the gating-dimer subunits to the pore.

Ca^{2+} ions are the gating cue for SK channels, and they are sensed by the CaMBD–CaM complex, analogous to the positively charged residues in the S4 voltage sensor of voltage-dependent K^+ channels. In both cases, the gating cue is transduced to the physical gate of the channel, the structure that occludes ion permeation in the closed state and permits permeation in the open state. For voltage-gated K^+ channels, the gate is composed of residues in the distal portion of

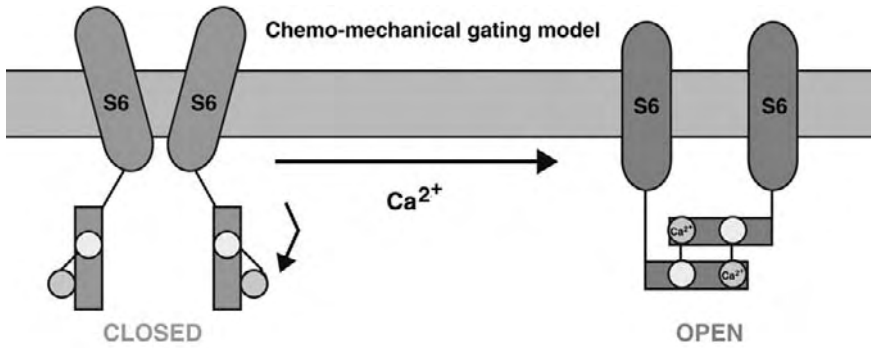


Figure 2 Drawing of the proposed chemomechanical gating model. The cutaway view shows two of the four subunits of the channel (the other two would be in the foreground). For clarity, only the S6 pore helices, the attached CaMBD, and the linker (line) are shown. The CaM C lobe and N lobes are yellow and orange, respectively. In this model, a resulting rotary movement (exaggerated in the figure) would result from formation of the dimeric complex which would drive a rotation between the S6 helices to open the channel gate.

S6 [38–40]. However, for the cyclic nucleotide-gated channels that, like SK channels, are gated by binding of an intracellular ligand, it appears that the selectivity filter of the channel also functions as the gate [41,42]. For SK channels, the location and identity of the gate itself remain to be determined, although studies of SK channels using cysteine-modifying reagents are consistent with a model similar to CNG channels, with the selectivity filter also forming the channel gate.

Pantophobiac After All

Prior to the cloning of Ca^{2+}-activated K^+ channels, Kung and colleagues [43] studied behavioral mutants in *Paramecium* that exhibited an exaggerated response to noxious stimuli. Wild-type animals use the Ca^{2+} that enters during a Ca^{2+}-based action potential to activate two Ca^{2+}-dependent conductances—an inward Na^+ and an outward K^+ conductance—that reverse ciliary motion, allowing the animal to move away from the noxious stimulus. Overreacting mutants, called *pantophobiacs*, lack the K^+ conductance, while underreactors, called *pawns*, lack the Na^+ conductance [44]. The mutations reside in the CaM gene [45]. Remarkably, injection of wild-type CaM into the animals restored the current [46]. The individual mutations segregated to the lobes of CaM, with those in the C lobe affecting the K^+ conductance and those in the N lobe affecting the Na^+ conductance [47]. Interestingly, larvae from *Drosophila* mutants lacking CaM show a similar pantophobiac phenotype [48].

These studies predicted the mechanisms rediscovered and extended by studies of mammalian channels. Moreover, they predicted that CaM might determine the activities of multiple classes of ion channels through direct and lobe-specific interactions. Indeed, recent results have shown direct interactions and in many cases unexpected functional consequences between CaM and voltage-gated Ca^{2+} channels [49], Na^+ channels [50; 51], cyclic nucleotide-gated channels [52], trp (transient receptor potential) channels [53], and skeletal [54] and cardiac [55] muscle calcium-release channels [54]. In addition, a growing list of ionotropic receptors is modulated by direct CaM interactions [56]. Moreover, the consquences of CaM–channel interactions extend beyond biophysical regulation of channel function to nuclear signaling and long-term orchestrated changes in the expression profiles of gene networks [57,58].

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CHAPTER 41

Regulation of Ion Channels by Direct Binding of Cyclic Nucleotides

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Introduction

Certain ion channels are regulated by the direct binding of cAMP or cGMP to a cytoplasmic binding domain. The first such channels to be identified were the cyclic nucleotide-gated (CNG) channels of photoreceptors and olfactory neurons [1,2]. These channels are ligand-gated (i.e., directly opened by cyclic nucleotide) and show only a very weak dependence of gating on voltage. Cyclic nucleotides also modulate the gating of certain channels that open primarily in response to voltage changes, even in the absence of cyclic nucleotides. This review first summarizes the function of cyclic nucleotide-gated channels in visual and olfactory transduction. The molecular bases for these channels and what is known about the structural basis for their function are described next. Finally, the properties of other channels that are regulated by cyclic nucleotides are briefly discussed.

The Cyclic Nucleotide-Gated Channels

The CNG channels of both photoreceptors and olfactory neurons are nonselective cation channels that conduct Na^+, K^+, and Ca^{2+} when activated (opened) by cyclic nucleotide binding. Their single-channel conductance is typically greater than 20 pS when divalent cations are absent, indicating a relatively high rate of Na^+ and K^+ flux. Under physiological ionic conditions, the channels pass an inward current that depolarizes the membrane, but external Mg^{2+} potently blocks current flow through the channels, reducing their conductance to less than 0.1 pS. The smaller conductance makes sensory transduction less noisy because more channels must open to produce a given change in membrane potential.

Physiological Role of CNG Channels in Visual and Olfactory Signal Transduction

In the dark, photoreceptors have a high resting level of cGMP, so CNG channels in the plasma membrane are activated (open). The influx of cations through CNG channels depolarizes the resting membrane of a photoreceptor to around –40 mV and results in an elevated level of Ca^{2+}_i. One important consequence of elevated Ca^{2+}_i is a decrease in sensitivity of the channel to cGMP in the dark, an effect mediated by binding of calcium/calmodulin (Ca^{2+}/CaM) to the channel. Activation of the visual signal transduction cascade upon absorption of a photon by rhodopsin results in stimulation of a phosphodiesterase, leading to hydrolysis of cGMP, closure of the CNG channels, and membrane hyperpolarization. Channel closure also leads to a fall in resting Ca^{2+}_i levels, which produces adaptive responses that enable photoreceptors to respond to subsequent increases in light intensity [3].

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In olfactory neurons, the binding of odorants to their membrane receptors activates adenylate cyclase or guanylate cyclase, depending on the class of odorant receptor that is stimulated. The rise in cAMP or cGMP opens the olfactory neuron CNG channels, causing membrane depolarization and firing of an action potential [4]. The influx of Ca^{2+} during channel activation is important for olfactory adaptation through a Ca^{2+}/CaM-mediated decrease in cAMP sensitivity of the channel (analogous to that found in photoreceptors) as well as other Ca^{2+}-dependent effects [5]. The major functional difference between the CNG channels of photoreceptors and olfactory neurons is in their selectivity for cyclic nucleotides: both rods and cones are activated by cGMP preferentially to cAMP, whereas olfactory CNG channels are nonselective.

Molecular Basis for CNG Channels in Photoreceptors and Olfactory Neurons

Kaupp and colleagues [6] were the first to clone a CNG channel gene (CNGA1, from bovine rod photoreceptors), and subsequent efforts discovered a family of related genes. To date, six mammalian CNG channel genes have been identified in two phylogenetic groups called CNGA and CNGB (see Fig. 1A.). CNGA1 and B1 were initially cloned from rod photoreceptors, CNGA2 and A4 from olfactory neurons, and CNGA3 and B3 from cone photoreceptors. Mutations in human CNG channel genes are responsible for certain degenerative retinal diseases and color vision defects.

Each CNG channel gene encodes one subunit, and a functional CNG channel is made of four such subunits. All CNG channel subunits contain six transmembrane segments (S1–S6) and are homologous to voltage-gated K^+ channel subunits (see Fig. 1B). The latter have a positively charged S4 segment that acts as a voltage sensor and a P region between S5 and S6 containing three conserved amino acids (GYG) that form the potassium selectivity filter (see Chapters 34 and 35). Although CNG channel activation depends on ligand binding and is only weakly sensitive to voltage, the CNG channel subunits all contain a positively charged S4 segment. A P region also is found in CNG channels, but it lacks the first two amino acids of the GYG motif and therefore produces no K^+ selectivity.

Each CNG channel subunit contains in its cytoplasmic C-terminus a highly conserved, 120-residue cyclic nucleotide binding domain (CNBD; Fig. 1B), homologous to the binding domains of cAMP- and cGMP-dependent protein kinases (see volume 2, Chapter 198) and to the catabolite-activating protein (CAP) of bacteria. From the X-ray crystal structures of the cAMP binding domains of CAP and protein kinase A (PKA), the CNG channel CNBD is inferred to consist of a short α-helix (A-helix), followed by a β roll of eight

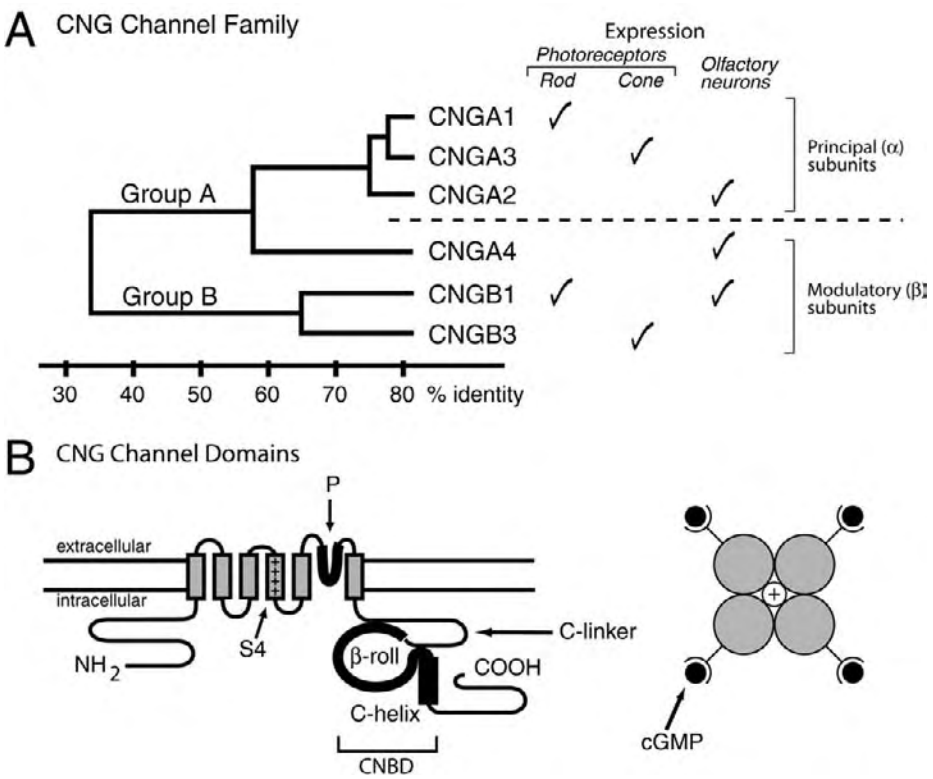


Figure 1 The family of cyclic nucleotide-gated channels. (A) Phylogenetic tree showing relation of six CNG channel genes. (Adapted from Gerstner, A. *et al.*, *J. Neurosci.*, 20, 1324–1332, 2000.) Note the name CNGB2 is not used. (B) Structural features of CNG channels: (left) important domains of a CNG channel subunit; (right) a functional channel composed of four homologous subunits.

antiparallel β-strands, followed by a short B-helix and a long C-helix. A C-linker sequence, connecting the S6 segment to the CNBD, is highly conserved in CNG channels but has no similarity to known sequence motifs.

The CNG channel isoforms exhibit marked diversity in function as well as tissue expression [7]. CNGA1-3 are called principal or α subunits, as they all form functional homomeric channels in heterologous expression systems. The activation properties of these homomeric α channels have been studied by applying cyclic nucleotide to the internal surface of the membrane in cell-free patches. The channels show steep dose–response relations to cyclic nucleotide, with Hill coefficients as high as 2 to 4. Bovine rod CNGA1 channels are highly selective for cGMP relative to cAMP, with 20- to 50-fold higher maximal open probability with cGMP than with cAMP. In contrast cAMP and cGMP activate the rat olfactory CNGA2 channels with equal maximal open probability. The cone CNGA3 channels show an intermediate selectivity for cGMP over cAMP. The structural basis for these differences in selectivity lies, not surprisingly, in the CNBD and is discussed further in the next part.

CNGB1 and B3, as well as CNGA4, are called modulatory or β subunits as they fail to form functional homomers but do coassemble with α subunits to form heteromultimers, whose properties more closely resemble those of native channels than do the homomeric α subunit channels. Thus, the native channels are likely to be heteromultimers of α and β subunits; rods, cones, and olfactory neurons all express different combinations of α and β subunits. Additional diversity arises through differential expression of splice variants of CNGB1 in different tissues. Bovine rods contain predominantly a long 240-kDa form of CNGB1 with a large glutamic acid-rich protein (GARP) domain in its cytoplasmic N terminus. This GARP domain may interact with regulatory proteins in rod signaling cascades. Shorter (100-kDa) CNGB1 splice variants lacking the GARP domain are the predominant forms of CNGB1 in human and rat rods, as well as in olfactory neurons. Different combinations of CNG channel subunits are expressed in other sensory and nonsensory cells; the role of CNG channel-dependent signaling in these tissues remains to be explored.

Structural Basis for Ligand Gating in CNG Channels

CNG channels provide a simple model system for studying allosteric regulation [1,8]. Ligand gating conforms to a cyclic allosteric model in which channels can open spontaneously in the absence of ligand with only low open probability (10^{-3}). Cyclic nucleotides bind more stably to the open state than to the closed state, and this stability difference provides coupling energy, which promotes channel opening. Although binding of ligand to only one or two sites in the tetrameric channel does enhance opening above the spontaneous level, efficient opening requires that all four sites be occupied.

Several domains of the channel important for gating have been identified. In the CNBD, the C-helix is an important determinant of cyclic nucleotide selectivity. An aspartic acid residue in the C-helix (D604 in bovine CNGA1) favors cGMP binding over cAMP binding by forming a pair of hydrogen bonds with the guanine ring of cGMP and a repulsive interaction with an unshared pair of electrons on N6 of cAMP. Moreover, this interaction occurs selectively when the channel is open, contributing large coupling energy for channel activation with cGMP but not cAMP. In CNG channel subunits that are not selective for cGMP, the aspartic acid residue is replaced by an uncharged amino acid.

The conformational change in the CNBD that enhances ligand binding is coupled by the C-linker to the conformational change in the transmembrane domain that opens the pore. A histidine residue in the C-linker can act in a tetrameric channel to chelate Ni^{2+}, and recent experiments (see Flynn *et al.* [8]) showed remarkably that this Ni^{2+} chelation could either increase or decrease the open probability of the channel, depending on the location of the histidine along the length of the C-linker. The results imply that the C-linker forms an α-helix that rotates longitudinally upon channel activation, probably also causing movement of S6. In voltage-gated K^+ channels, S6 forms a movable gate whose translation controls ion entry into the cytoplasmic end of the pore (see Chapter 35). In CNG channels, however, ion entry into the pore is not blocked by the S6 segment, so the role of C-linker and S6 motion during channel activation may be to cause a conformational change of the P region, which would serve as both the selectivity filter and gate.

Other regions of the CNG channel are important in regulating channel opening. For instance, the N terminus of the olfactory CNGA2 subunit can interact favorably either with the C terminus or with Ca^{2+}/CaM. Binding of Ca^{2+}/CaM to the N terminus suppresses the interaction between the N and C termini and inhibits activation of the channel. A similar mechanism works in rod CNG channels, mediated by Ca^{2+}/CaM binding to the CNGB1 subunit.

Other Channels Directly Regulated by Cyclic Nucleotides

The hyperpolarization-activated cation channels (I_h or I_f) are also directly regulated by cyclic nucleotide [9,10]. These channels are voltage gated; they are slowly activated by membrane hyperpolarization (a response opposite to that of most voltage-gated channels) and carry the so-called "pacemaker" current that controls the rhythmic spontaneous firing of action potentials in certain neurons and cardiac muscle cells. Open I_h channels are weakly selective for K^+ over Na^+ (3:1 ratio), and at typically negative resting potentials they pass inward current, which is excitatory (depolarizing the membrane). They contribute to pacemaking because they become activated after the falling phase (repolarization) of an action potential; the resulting excitatory current can depolarize the cell past threshold and cause the firing of

another action potential. DiFrancesco and colleagues [11] first showed that the direct binding of cAMP to these channels enhances the rate of channel opening and thus accelerates the rate of spontaneous firing.

In mammals, the I_h channels are encoded by a family of four closely related genes, termed HCN1–4 (hyperpolarization-activated, cation-nonselective, cyclic-nucleotide-regulated). The HCN subunits are phylogenetically related to the CNG channels, with a six-segment transmembrane domain and a C-terminal CNBD. The HCN channels contain an S4 voltage sensor with a large number of positive charges, more than are present in many depolarization-activated K^+ channels, and the HCN channel P region conserves intact the GYG motif usually associated with K^+ selectivity (although many other P region residues are not conserved). The structural basis for hyperpolarization gating and lack of high K^+ selectivity in HCN channels is not yet known.

Sequence homology searches have identified more distant phylogenetic relatives of CNG and HCN channels. Many of these are voltage-dependent K^+ channels, such as the ether-à-go-go channel (EAG), the related ERG channel (implicated in the LQT congenital cardiac abnormality), and several K^+ channels from flowering plants. Surprisingly, all these examples have putative CNBDs that lack some amino acids known to be important in CNG and HCN channels and in CAP, such as a conserved arginine that contacts the cyclized phosphate moiety. In many cases, the issue as to whether the channels are indeed regulated by direct binding of cyclic nucleotides is still controversial and calls for further study.

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SECTION C

Horizontal Receptors

Robert Stroud, Editor

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CHAPTER 42

Overview of Cytokine Receptors

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Cytokine, and cell adhesion receptors are generally single-crossing receptors that are activated by binding a ligand on one side of the membrane that initiates a response on the other side. The ligands associate with binding sites that may lie within one single receptor molecule or may involve bridging interactions between multiple receptors. The relative disposition of transmembrane segments and their associated domains induces a signal transduction cascade on the other side of the membrane. In all cases, this is mediated by changing lateral associations of receptors by horizontal signaling.

Horizontal receptor signaling is primarily found in multicellular organisms. Downstream signaling pathways inside the cell generally control changes in cell metabolism, such as those that lead to changes in transcription, translation, or replication, or changes that result in apoptosis of the cell. The horizontal receptors are activated by binding a protein ligand that can be monomeric or multimeric and induces a reordering of quaternary interactions between receptors in the cell membrane.

Most cytokines can be grouped into four groups (I to IV) based on the preponderance of α-helical β-sheet, mixed α/β, or mosaic substructures. Reordering of receptor associations as a result of binding is a common theme in horizontal signaling, with enormous diversity in the protein folds, binding sites, and stoichiometries of these signaling complexes. Much less accessible, but of increasing significance, is the evidence that cytokine receptors are maintained in an inactive state by their ordered associations prior to binding the cytokines. The first such inactive but preassociated states have now been established; however, because inactive species are more difficult to detect than activated ones, they have only recently been sought. In some cases, these are poised to bind cytokine, as for members of the tumor necrosis factor (TNF) receptor class. In other cases, such as the erythropoietin (EPO) receptor, the receptors are associated in an "off" state that requires them to dissociate before a productive complex can be made.

In many cases, the intracellular domains of receptors are preassociated with protein kinases, either noncovalently, as is the case with EPO receptors, or by covalent construction on the same gene, as is the case with the epidermal growth factor (EGF) class of receptors. In other cases, the intracellular domains associate with kinases after they bind cytokine, as is the case for human growth hormone (hGH) receptors. Once the receptors are appropriately oriented, the kinases act intermolecularly to phosphorylate each other, regions of the intracellular domains, or other proteins. These in turn act as docking sites for binding and activating other signaling factors. Thus, the membrane surface serves as the nexus for a plethora of pathways within the cell. In their role as the "mailbox" of the cell, they will be the key to intervention by therapeutic drugs as the ability to target protein–protein interfaces reaches maturity.

Cytokine–receptor complexes include those where two identical receptor molecules are dimerized by binding to two different sites on a single cytokine to produce a 2 : 1 complex, as seen for growth hormone and erythropoietin. Other cytokines form complexes where two cytokine molecules bind two identical receptors, such as in the gp130–interleukin-6 (IL-6), granulocyte colony-stimulating factor (GCSF)–GCSF receptor (GCSFR), and fibroblast growth factor (FGF)–FGF receptor (FGFR) complexes to induce allosteric changes that lead to back-to-back associations of the receptors without any contact between cytokines themselves. Still other cytokines act as monomers to bind two different receptors simultaneously such those found in the IL-4 system and gamma-interferon (γ-IFN). Still higher order trimeric complexes are seen for the TNF receptors class.

Overall, our study of the mechanisms of cytokine signaling via single crossing receptors has been driven by defining the

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activated complex structures and their function in recruiting molecular complexes, but this research has also led to determining what constitutes the "off" state of receptors. In the case of erythropoietin receptors, only 50 receptor dimers, oriented by binding erythropoietin on the cell surface, are required to evoke ≈50% signaling. Therefore, our understanding of the reference "off" states is key to understanding the horizontal signaling receptors. The chapters of this section detail some of the most pertinent examples of horizontal signaling mechanisms, which serve to define the pathway to the future.

CHAPTER 43

Growth Hormone and IL-4 Families of Hormones and Receptors: The Structural Basis for Receptor Activation and Regulation

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Introduction

Within the cytokine superfamily, the growth hormone (GH)/prolactin (PRL) and interleukin-4 (IL-4) families of hormones and receptors are arguably the most extensively studied systems focused on structure–function issues and molecular recognition [2–6]. These studies and those of related cytokine systems have been instrumental in defining modes of hormone action and regulation [7–12]. The structure-based mechanisms by which these systems activate are similar [4,6,7]; however, although these mechanisms are conceptually simple (hormone-induced receptor aggregation), the molecular strategies that are employed are complex and hardly predictable [3,8,9,13].

The GH and IL-4 hormones and receptors share many general structure and functional similarities, but there are also important differences that define the details of how these systems initiate and regulate their biological activities. Most noteworthy is the form of the tertiary complexes that compose their respective active signaling complexes. In the case of GH, activity is triggered through a hormone-induced receptor homodimerization. This gives rise to a hormone-to-receptor stoichiometry of 1:2. For IL-4, activity is initiated through a heterodimerization process—the binding of two different receptors produces an organization of 1:1:1 for hormone to receptor 1 to receptor 2. These are fundamentally different processes and involve quite different molecular recognition strategies to regulate biological function.

A comprehensive literature describes the structure–function relationships for these two systems; however, with each additional piece of new information and insight it is clear that we are just beginning to scratch the surface in understanding the strategies under which these systems evolved. A common misconception about these systems is that, because the previous studies have produced such a breadth of important results, all the most critical issues have been resolved. The fact is that these studies have really just laid the foundations for a new generation of investigations that will produce a further level of insight about the subtleties under which molecular recognition processes drive biological function. The goal of this review is to provide a background of our current understanding and suggest areas of future investigation.

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The Growth Hormone Family of Hormones and Receptors

Growth hormone (GH), placental lactogen (PL), and prolactin (PRL) regulate an extensive variety of important physiological functions. While GH biology generally centers around the regulation and differentiation of muscle, cartilage, and bone cells, it is the PRL hormones and receptors that display a much broader spectrum of activities, ranging from their well-known effects in mammalian reproductive biology to osmoregulation in fishes and nesting behavior in birds [1]. Within the cytokine superfamily, the growth hormone (GH)/prolactin (PRL) endocrine family of hormones and receptors is arguably the most extensively studied system focused on structure–function issues and molecular recognition [2–6]. These studies and those of related cytokine systems have been instrumental in defining modes of hormone action and regulation [7–12]. The structure-based mechanisms by which these systems activate are similar [4,6,7]; however, although these mechanisms are conceptually simple (hormone induced receptor aggregation), the molecular strategies that are employed are complex and hardly predictable [3,8,9,13].

The activities of GH, PL, and PRL and their homologs are triggered by hormone-induced homodimerization of their cognate receptors, which produce subsequent signals through a series of phosphorylation events in the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway [14,15] (Fig. 1). The receptors belong to the hematopoietic receptor superfamily [2,16] and have a three-domain organization. It is the cytoplasmic domains of the aggregated receptor complex that bind one or several JAK tyrosine kinases, which then transphosphorylate elements on themselves, the receptors, and associated transcription factors belonging to the STAT family [15].

Structural Basis for Receptor Homodimerization

Tertiary structure plays a role in how the hormone regulates receptor activation. The hormones in this family are long chain four α-helix bundle proteins [4,17]. A notable feature of their tertiary structure is that it contains no symmetry that might support equivalent binding environments for the receptors. How the two receptors bind to the asymmetric hormone was first revealed from the crystal structure of human growth hormone bound to the extracellular domain (ECD) of its receptor (hGH-R) [8]. The structure showed that the two ECDs binding to site 2 and site 2, respectively, use essentially the same set of residues to bind to two sites on opposite faces of the hormone [8] (Fig. 1). An identical model is seen in a prolactin hormone–receptor complex [18]. This binding is characterized by extraordinary local and global plasticity at the binding surfaces. The two binding sites have distinctly different topographies and electrostatic character, leading to different affinities for the receptor ECDs (Fig. 2).

The high-affinity site, site 1, is always occupied first by ECD1 [19]. This sequence of events is required because productive binding of ECD2 at site 2 of the hormone requires additional contacts to a patch of the C-terminal domain

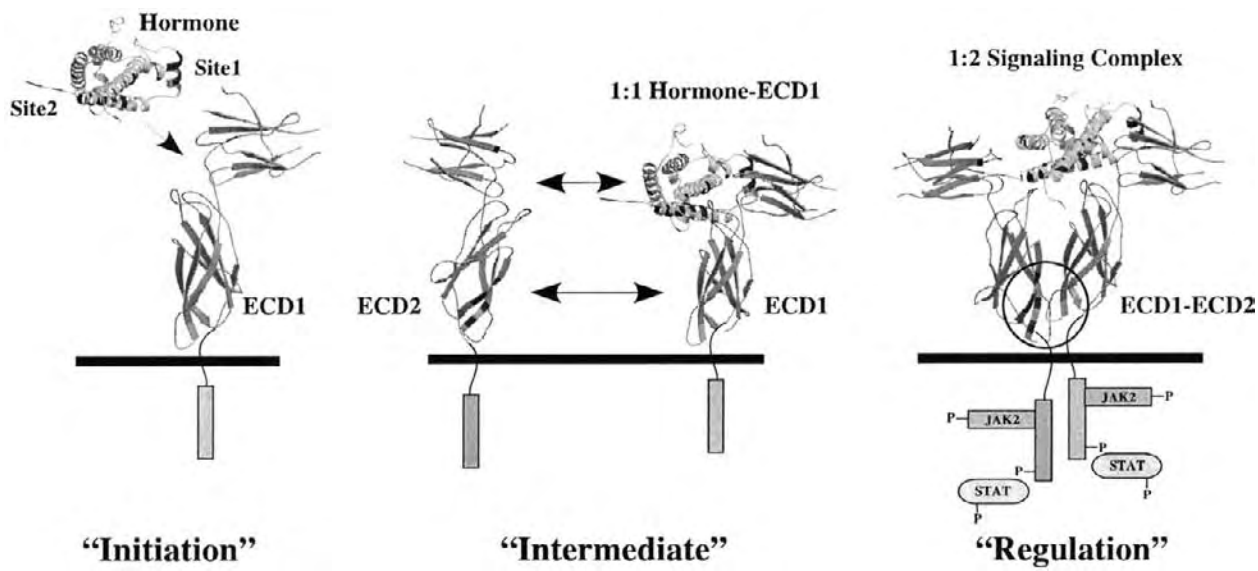


Figure 1 Mechanism of hormone-induced receptor homodimerization. The GH/PRL receptors are three-domain single-pass receptors containing an extracellular domain (ECD), a transmembrane section of about 25 amino acids, and a cytoplasmic domain that forms the binding site for the tyrosine kinase activities. The ECD consists of two fibronectin type III domains (FNIII) connected by a short linker. The hormones are four-helix bundle proteins. The initiation step involves the hormone binding event to the ECD (ECD1). The segments of the molecules that are involved in the contact (site 1) are colored red (hormone) and yellow (ECD1). They form a stable 1:1 intermediate that then recruits a second receptor in the regulation step through two sets of contacts (site 2)—one to the hormone and the other through forming receptor contacts (ECD1–ECD2). This step forms the stable homodimer, which organizes the cytoplasmic components to initiate binding and phosphorylation.

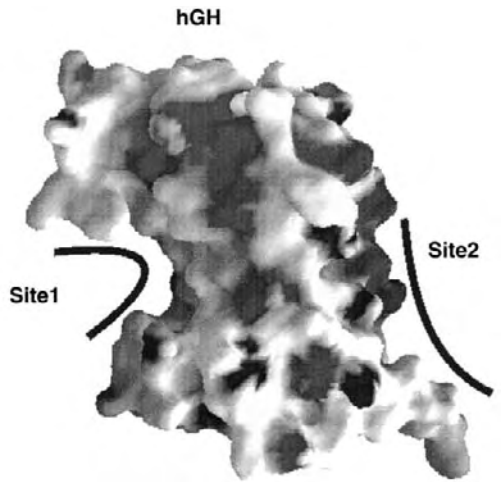


Figure 2 Molecular surface of hGH showing the different topographies of the site 1 and site 2 binding sites. In addition, the two sites possess quite different electrostatic properties (red, negative charge; blue, positive charge) (image rendered between ±10 kT).

of ECD1. The binding of ECD2 is the programmed regulatory step for triggering biological action, and it involves a set of highly tuned interactions among binding interfaces in two spatially distinct binding sites. The energetic relationships between the ECD1–ECD2 contacts and the hormone–ECD2 site 2 interactions are known to be important. However, quantitative data are few in regard to which residues are the main contributors, whether they contribute in an additive or cooperative fashion, and how the binding energy is distributed in the interfaces.

Hormone Specificity and Cross-Reactivity Determine Physiological Roles

Binding to the two structurally distinct sites on the hormone, while using the same binding determinants, requires the receptor binding surfaces to undergo significant local conformational change [8,18]. The structural requirement is further expanded by specificity factors [9]. The biology of PRL and GH is integrated on many levels [20]; however, over the 400 million years since PRL and GH diverged from a common gene parent, evolution has built in different regulating components distinguishing them [21,22]. In primates, the growth hormone receptor (GH-R) is activated solely by homodimerization through its cognate hormone [8,21], but prolactin biology works through regulated cross-reactivity. Most PRL-R receptors are programmed to bind both prolactin and growth hormone [23].

This pattern of specificity and cross-reactivity involves some rather significant molecular recognition challenges as GH and PRL have little (≈25%) sequence conservation even among the residues involved in receptor binding [9,18]. The structure of hGH bound to the prolactin receptor (hPRL-R) showed that in these systems local conformational flexibility of the receptor binding loops, together with rigid-body movements of the receptor domains, facilitates the creation of specific, but different, interactions with the same binding site. The effects of conformational change on altering specificity were also observed in protein engineering studies that "converted" binding site 1 of two PRL-R-specific hormones, hPRL and hPL, into hGH [24–26]. This could be accomplished by substituting the hGH sequence at only five to six places in their sequence. Surprisingly, several of these positions map outside the site 1 hormone–receptor interface. Presumably, they must act as indirect specificity determinants by inducing conformational changes that subtly reorganize the contact residues into productive binding interactions. The implications of this finding are considerable and may open up totally new ways to look at how specificity and cross-reactivity are developed in cytokine systems.

Hormone-Receptor Binding Sites

Binding site 1 of the hormones in the GH-PRL family is formed by residues that are exposed on helix 4 of the helix bindle, together with residues on the connecting loop between helix 1 and 2 [8]. The total surface area buried on the hormone in the hGH–hGH-R and hGH–hPRL-R complexes is about 1300 $Å^2$. In the ovine placental lactogen (oPL)-rat prolactin receptor (rPRL-R) complex (a fully prolactin complex), about 850 $Å^2$ is buried on oPL. Similar surface areas are buried on the respective receptors. The complexes contain approximately the same number of intermolecular H bonds (eight to nine H bonds). The overall packing of the four helices of the hormones is very similar in all the complexes, indicating no global changes of the type seen in the analysis of the structure of an affinity mature hGH mutant [27]. The largest differences in the bound hormones are seen in a small "mini-helix" of two turns (residues 38–47) in the segment connecting helices 1 and 2. In the case of hGH binding to hPRL-R and hPRL-R, the mini-helix differs by about 3 Å between the respective complexes [9].

For both oPL and hGH, the site 2 binding epitope involves residues in helices 1 and 3 (hGH–hPRL-R is a 1:1 complex and thus does not have a site 2 binding site). In contrast to the concave surface of the hormones at site 1, binding site 2 is a relatively flat surface. Upon binding, about 650 $Å^2$ of the oPL surface becomes buried in the interface with rPRL-R2.[1] This compares to about 860 $Å^2$ that is buried in the equivalent hGH–hGH-R2 interface [8]. A noteworthy difference between prolactin and growth hormone complexes is that the oPL–rPRL-R2 interface contains nine intermolecular H bonds, while that of hGH–hGH-R2 contains only four. Thus, although it is somewhat smaller than its hGH counterpart, the oPL site 2 interaction contains over twice the number of H-bonds.

[1]Receptors binding at site 1 are designated by the suffix R1, those binding at site 2 by R2).

The receptor ECDs use essentially the same set of residues to bind to the two distinctly different site 1 and site 2 interfaces on the opposite faces of the hormones [8,18]. The binding surfaces of the receptors are formed by six closely spaced surface loops (L1–L6) that extend from the β-sheet core in a manner somewhat similar to antigen-binding loops in antibodies. Three loops reside in the N-terminal domain (L1–L3), two others in the C-terminal domain (L5–L6). Binding loop L4 serves as the five-residue linker between the domains. The conformation of L4 plays a key role in orientation of the domains with respect to one another. For instance, differences in the conformation of L3 in complexes of hGH binding to hGH-R and hPRL-R result in significant changes in the global positioning of the N- and C-terminal domains of the receptors bound at site 1 [8,9,18]. These differences in the N- and C-terminal domain orientation are an important part of the molecular recognition diversity in these systems.

Receptor–Receptor Interactions

A conserved structural element of the ligand-induced homodimerization of prolactin and growth hormone receptors is a set of extensive contacts between their C-terminal domains. This receptor–receptor interface was described in detail for the hGH:hGH-R 1:hGH-R2 ternary complex [3,8] and modeled for the hGH–hPRL-R ternary complex [3]. Although the topology of the C-terminal domains of the rPRL-R is virtually identical to that of the C-terminal domain of hGH-R, the receptor–receptor interfaces in these two complexes show a marked variation in their orientation and electrostatic character, and different portions of the receptors are involved in the interaction. The surface area buried in the interaction between rPRL-Rs is smaller than that buried between hGH-Rs; the former being ≈ 370 Å^2 compared to ≈ 470 Å^2 for the latter.

Eleven residues are involved in the contact interface on each rPRL-R, while 13 and 17 residues from hGH-R1 and hGH-R2, respectively, make up their site 1 and site 2 interfaces in the hGH–hGH-R ternary complex. In the rPRL-R receptor–receptor interface, there are four H-bonding interactions compared to six H bonds or salt-bridging interactions found between hGH-Rs (Table 1). The receptor–receptor interaction of hGH-R1 and hGH-R2 generally involves the same residues on each receptor [3,8]. In the oPL–rPRL-R complex, about one-third of the interface residues on its rPRL-R1 are also in the interface of rPRL-R2. Although residue 201 in the first receptor H-bonds across the interface in each complex, these interactions are not equivalent interactions because of the differences in orientations of the domains.

Hormone–Receptor Binding Energetics

The energetics of the high-affinity site 1 hGH–hGH-R1 binding that forms the intermediate 1:1 complex has led to a number of important insights into the relationships between binding and specificity determinants and protein–protein interactions, in general [28]. In particular, the seminal work of Wells and colleagues [28–31] demonstrated that the hGH–hGH-R ECD1 protein–protein interactions are characterized by binding "hot-spots" that focus binding energies within a cluster of relatively few residues. Thirty residues of hGH make contact in the site 1 interface with hGH-R1; however, it was shown that about 85% of the binding energy was developed through only eight of the residues, and there were no apparent distinguishing characteristics between the interactions that were energetically important and those of the other side chains that were energetically null [31]. This very efficient use of the binding interface characterized by the concentration of the binding determinants within a relatively small area of the contact surface allows for separate adjacent areas to be used for specificity determinants without compromising the binding.

It has been determined that a similar type of hot-spot also exists for the hGH–hPRL-R1 site 1 interaction and that the interface encompasses the same binding footprint as the hGH–hGH-R1 counterpart [9,31]. The two systems differ, however, in how the energy is distributed within the footprint. For instance, residues such as E174 and R167 make little energetic contribution to binding in the hGH–hGH-R case but play extremely important roles in the hGH–hPRL-R contact surface ($\approx$800-fold difference in binding between the two systems when alanine is substituted at each of these sites). Interestingly, R167 makes a salt bridge to an acidic side chain in both receptors (to E127 in hGH-R and D124 in hPRL-R), yet Ala-scan mutagenesis indicates that only in the case of hGH–hPRL-R does this salt bridge have a positive effect on binding [31]. This is an example of how the energetics of interactions is very context dependent on protein–protein interfaces [3].

The binding properties of ECD2 are intrinsically different than those for ECD1, because they involve the combined effects of two spatially distinct binding surfaces: binding site 2 on the hormone and a contact with the C-terminal domain of the bound ECD1 (Fig. 1). Neither of these surfaces alone supports binding without the interaction of the other. In glaring contrast to the extensive characterization

Table I Receptor–Receptor H-Bonding Interactions (<3.2 Å) for PRL-R and hGH-R Ternary Complexes

rPRL-R1	rPRL-R2	hGH-R1	hGH-R2
I188O	Q196Nε2	S145Oγ	D152Oδ2
Y200N	Q194Oε1	L146N	S201Oγ
D201Oδ1	K198N	T147Oγ	D152Oδ1
E187Oε2	K198N	H150Nε2	N143Oδ1
—	D152Oδ2	Y200Oν	—
—	S201Oγ	Y200Oν	—

of the energetics of site 1 binding, there is considerably less biochemical and biophysical information for site 2 in GH/PRL systems. A study by Cunningham *et al.* [32] showed that site 2 did not contain a well-defined hot-spot, as was the case for site 1. However, there is no information about whether the spatially independent hormone–receptor and receptor–receptor interfaces act in additive or cooperative fashion. This deficiency has greatly limited our understanding of the overall energetics driving the homodimerization process. In this regard, recent data show that the receptor–receptor interface contains a hot-spot in the hGH–hGH-R system and probably plays a more important role than the hormone site 2–receptor contact in stabilizing tertiary forms of the complex (Bernat and Kossiakoff, unpublished data).

Biological Implications of Transient Receptor Dimerization

Functional and structural information suggests that the role of receptor homodimerization is more complicated than simply bringing the cytoplasmic elements of the receptors together. For instance, structural studies of erythropoietin (EPO) and its receptor (EPO-R) indicate that a function of the hormone is to establish a fairly exact receptor alignment, as well as to induce dimerization [33–36]. Based on patterns of cross-hormone and cross-species activities and the known structural differences in the active complexes, exact receptor orientation is probably not as crucial for prolactin and growth hormone systems.

Although strict orientation effects may not be crucial, it appears that the dynamics governing the stability of the aggregated signaling complex are an important regulatory element for the prolactins. Consequently, the inefficient site 2 binding is likely an evolved characteristic of homologous prolactin systems distinguishing their homodimerization process from those of GH and EPO. To explain mutagenesis data influencing site 2 binding, Herman *et al.* [37] have suggested a minimal-time mechanism based on the assumption that signal transduction requires a minimal persistence lifetime for the homodimer to facilitate effective transphosphorylation of the associated JAK2 kinases. Once this goal is achieved, the existence of the dimerized receptors is no longer obligatory. It is proposed that this minimal time is generally shorter for PRL-R than for GH-R, perhaps because the JAK2 kinase is preassociated in the case of PRL-R [38] but not GHR [39]. The minimal-time hypothesis is also supported by a study by Pearce *et al.* [40], who engineered tighter and weaker binding interactions between hGH and hGH-R. They found that increasing affinities of the hGH–hGHR associations at both site 1 and site 2 produced no measurable increases in biological activity. However, reducing affinity at site 1 30-fold marked a point that appeared to correspond to a threshold where activity was affected, suggesting that wild-type hGH–hGH-R affinity is higher at site 1 than it needs to be to sustain full biological activity.

A High-Affinity Variant of hGH (hGH_v) Reveals an Altered Mode for Receptor Homodimerization

Using phage display mutagenesis selections, a variant of hGH that binds >100-fold more tightly to hGH-R at site 1 was produced [41,42]. This variant (hGH_v) has 15 mutations localized in its site 1 binding site and is fully biologically active but is totally specific to hGH-R, losing its ability to bind to hPRL-R. (This is a clinically relevant, second-generation hGH used for treating acromegaly). The recent high-resolution X-ray analysis of the ternary complex of hGH_v bound to two copies of hGH-R [43] indicates global similarity to the *wt*–hGH complex but major important structural differences in the binding interfaces [43]. These changes are exemplified in the finding that of the 17 H bonds that are formed between the hormone and receptors at sites 1 and 2, only two correspond to H bonds in the *wt* complex. This demonstrates the inherent plasticity of the protein–protein interfaces in this system where new contacts can be formed and still remain specific to hGHR in a biologically relevant way.

While it was anticipated that site 1 interactions would be altered as a result of the mutations at this site in the hormone, it was surprising that the largest changes in structural conformation were found in the site 2 interface, where no mutations were made [43]. This interface in hGH_v has only limited structural relationship to its *wt* counterpart. The same sets of hormone and receptor residues are used, but their stereochemical relationships are completely different, with several groups differing by more than 10 Å. Interestingly, this new, reconfigured hGH_v–ECD2 interface has a binding association comparable to that found in the *wt* complex. This structure is an excellent example of the structural cooperativity that exists between binding sites in these systems. Another important aspect of this structure is that the distribution of binding energy among the residues energy at sites 1 and 2 is different than their counterparts in the *wt* ternary complex (Walsh and Kossiakoff, unpublished results).

Site1 and Site2 Are Structurally and Functionally Coupled

It is likely that this new binding solution for hGH_v–hGH-R2 is triggered by a structural mechanism linking site 2 to a subset of the mutations in site 1 introduced in the phage display experiments. It is noteworthy that the structurally distinct conformation of hGH_v at site 2 was under no selection pressure and supports binding of the second receptor as tightly as in the *wt* complex. A specific example of the structural coupling is observed from the altered roles of Asp116 in site 2 of the hormone in the two complexes [43]. Asp116 is located near the center of helix 3, thus the side chain extends off a fairly rigid scaffold. Although Asp116 is adjacent to several important receptor side chains, in the *wt* complex it appears to play a bystander role, making no H bond

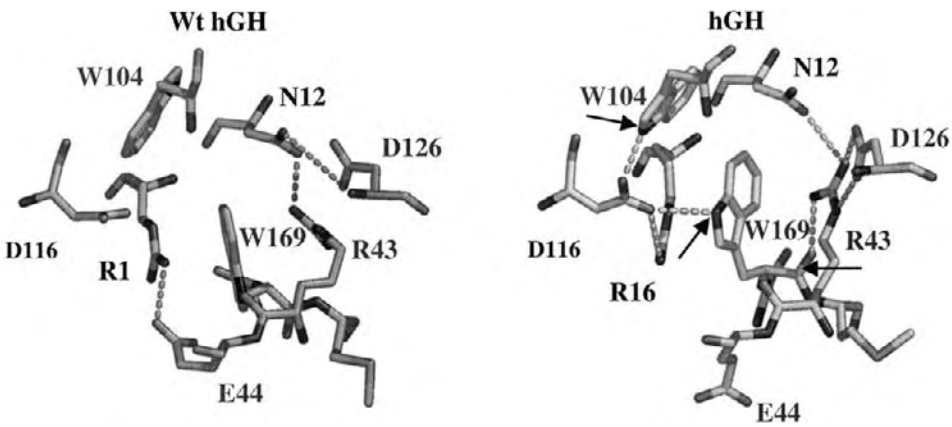


Figure 3 Comparing the structural changes in site 2 of the hGH and hGH_v–hGH-R ternary complexes. Hormone residues are labeled in black and receptor residues in red. Arrows point to the carbonyl oxygen atom of the peptide bond of Trp169 that flips its conformation. The other arrows point to the reorientation of the side chains of Trp169 and Trp104.

to the receptor. It is probable that the small movements of Asp116 that are a consequence of the repacking of the four-helix bundle in hGH_v effectively trigger the new H-bonding scheme where by the carboxylate side chain makes new H bonds to the receptor through the indole nitrogens of the side chains of Trp104 and Trp169, as well as to the side chain of Arg43. As seen in Fig. 3, the Trp side chains undergo a significant reorganization to facilitate the formation of these H bonds. These observations clearly have fundamental importance to our understanding of the inherent efficiencies of these cytokine hormones and receptors as binding entities even outside of evolutionary control.

The concept that the two spatially distinct binding sites on cytokine hormones are structurally and functionally coupled as displayed in the hGH_v complex is novel, and the process whereby new binding surfaces are synthesized by indirect through molecule effects has been termed *functional cooperativity* [43]. In this mechanism, it is not only the mutations in one site that affect the other site. A set of concerted changes also occurs among the hormone and the receptor ECDs. The finding of strong cross-molecular interaction induced during receptor dimerization establishes a new molecular recognition paradigm and opens up fundamental new areas of investigation relating to the mechanisms of biological regulation by protein–protein associations. However, it remains an open question as to how general this is and whether evolution actually uses this strategy to influence the receptor signaling of GH/PRL systems in biologically important ways.

IL-4 Hormone-Induced Receptor Activation

IL-4 is a pleiotropic hormone that mediates several important regulatory responses in the immune system (9A, 10A). The hormone belongs to the short-chain, four-α-helical bundle class of cytokines (4), which is a distinctly different class than the long-chain cytokines represented by GH and PRL. The IL-4 signaling cascade is triggered by a sequential hormone-induced aggregation of two distinct receptor chains (12A, 13A). The so-called α-chain receptor binds first to IL-4 with high affinity (150 p*M*), while the γ-chain binds only weakly to the preassociated IL-4/α-chain complex (8A).

The extracellular parts of these receptors are like those of GH-R/PRL-R consisting of two FNIII domains (11A). Interestingly, neither receptor is specific to IL-4 alone. In contrast to its tight association to IL-4, the α-chain receptor also forms a weak complex with IL-13 (1A). The γ-chain receptor acts as a common receptor element for the IL-2, IL-7, IL-9, and IL-15 signaling complexes (2A). Additionally, the cytoplasmic portions of these receptors differ both in size and function. The cytoplasmic domain of the α-chain receptor is composed of about 600 amino acids and contains the docking sites for JAK1 and STAT6, and the insulin receptor substrate IRS-2, among others (3A, 14A). The γ-chain receptor has a much smaller cytoplasmic domain, which contains a binding site for JAK3. Thus, in contrast to the homodimeric systems GH/PRL, the phosphorylation events that trigger the IL-4 signaling cascade are produced by two different JAK kinases, JAK1 on the α-chain and JAK3 on the γ-chain.

The large difference in binding affinities of the α- and γ-chain receptors to IL-4 results in the active ternary complex being formed in a specific sequential order, similar to GH/PRL (Fig. 1) (see previous discussion). The first step is creating the IL-4–α-chain intermediate, which is followed by the low-affinity binding of the γ-chain. The active ternary complex is short lived because of the weak γ-chain binding. Thus, the transient nature of the ternary complex parallels the dynamics observed in the prolactin system, where formation of the active 1:2 complex was rapidly followed by the dissociation of the second receptor to reform the 1:1 intermediate (22).

Recently, the high-resolution structure of the IL-4: α-chain receptor intermediate complex was reported by Hage *et al.* (4A). Although in many respects this complex is

similar to the 1:1 intermediate complexes formed between hGH and hGH-R/hPRL-R, a notable difference is that the major binding epitope on IL-4 involves helices 1 and 3, rather than 1 and 4, as is the case for the hGH and hPRL receptor binding (5A). This apparent subtle difference actually represents a totally altered site 1 binding stereochemistry between IL-4 and hGH in their respective 1:1 complexes. In fact, the IL-4 orientation is more similar to the low-affinity site 2 of hGH.

A comparison of the structures of the unbound and bound IL-4 molecule indicates that binding not only produces changes in the loops, as expected, but also an adjustment of the four-helix bundle packing (4A). In this respect, it is not known whether a similar packing adjustment happens with hGH binding, as no well-resolved structures of the free wild-type hGH molecule are available. However, the high-affinity hGH variant (hGH_v) [41] shows a similar degree of conformational change on binding [43], albeit the free variant was compared to the bound variant in the context of a 1:2 complex.

IL-4–α-Chain Receptor Interface

The binding interface between IL-4 and the α-chain receptor encompasses slightly over 800 $Å^2$ on each molecule. This compares to about 1100 $Å^2$ for the hGH site 1 interface (8). The binding epitopes are highly discontinuous and in IL-4 are distributed over three helices, with the principal determinants being on helix 1 and 3. The complementary receptor epitope is composed of residues on five loops connecting the β-sheet structure of the FNIII domains (6A).

A binding model has been developed based on the structural information and the functional mapping of the binding energies of specific residues driving the IL-4–α-chain receptor association (6A). The interface has a high degree of electrostatic complementarity between a cluster of positively charged residues on helix 3 of IL-4 that mate with a set of negatively charged groups on the α-chain receptor. The binding energetics on both sides of the interface are organized in an O-ring arrangement. The energetically important residues, which are predominantly hydrophilic, are centralized, surrounded by a shell of hydrophobic side chains in an O-ring configuration. The hydrophobic groups occlude the bulk solvent from the centralized set of hydrophilic, charged interactions, in essence accentuating the electrostatic effects. It is noteworthy that, while in the GH/PRL systems, the energetically important residues were clustered into a single binding hot-spot; the IL-4, the major binding determinants, are a mixed-pair of charged residues that are spatially separated and surrounded by a number of other side chains of lesser importance. This leads to a two-cluster type of epitope rather than a single hot-spot. The kinetic data suggest that the basic side chains on helix 3 forming one of the clusters on the hormone influence the rates of association, presumably through a mutual attraction to the set of negative side chains in the α-chain receptor interface (5A). Based on this complementarity it has been proposed that the molecular recognition event that drives the protein–protein association involves a form of electrostatic steering (5A).

Binding of the γ-Chain Receptor

The γ-chain exhibits no measurable binding to either IL-4 or the α-chain receptor alone (8A) and, as is observed for hGH–hGH-R homodimerization, the binding of the second receptor is facilitated by structural properties generated by formation of the 1:1 intermediate. A major difference, however, is that, whereas the second hGH receptor binds to the intermediate 1:1 complex with high affinity (4 n*M*) (Bernat and Kossiakoff, submitted), the γ-chain binds to the IL-4–α-chain receptor 1:1 complex with an affinity of about 3 μ*M*, resulting in a highly transient signaling complex similar to that of the homodimeric prolactin–prolactin receptor complex (22).

In contrast to the highly charged IL-4–α-chain interface, the binding epitope on the γ-chain receptor is dominated by hydrophobic side chains: Ile100, Leu102, Tyr103, and Leu208 (7A). The loop containing residues 100 to 103 constitutes a focused binding hot-spot that is spatially separated from Leu208. Thus, as was the case for the IL-4–α-chain receptor contact, there is no single concentrated hot-spot in the interface. There are three residues in IL-4 that have been determined to be crucial for γ-chain receptor binding. The side chains of these groups, Ile11, Asn15 on helix 1, and Tyr124 on helix 4 (Fig. 4), form a single extended patch on the surface of site 2 of IL-4 that presumably is positioned to interact with the residues in the two clusters on the γ-chain receptor. Currently, no functional data exist to provide information about the presence or nature of a contact interface between the α-chain and γ-chain receptors. Presumably, such an interface exists because the γ-chain only binds to the 1:1 intermediate complex.

Comparisons of IL-4 with GH (PRL)

Although the active complexes of IL-4 and GH have a number of similar general structure–function features, there are also a number of important differences in how these hormones initiate and regulate their activities. It is noteworthy that, although both hormones are in the four-α-helix bundle family, the differences in the lengths of the helices and the connecting loops in the short-chain versus the long-chain cytokines result in a significantly different overall topography of the molecules. Both molecules have high- and low-affinity binding receptor sites, but because of these topological differences there is no spatially conserved relationship between them.

A principal difference in the biophysics of formation of the high-affinity 1:1 intermediate complexes of both hormones is the electrostatic nature of the hormone–receptor interfaces. In hGH, this interface is relatively neutral with

about the same number of hydrophilic and hydrophobic atoms involved in the overall contact (8). In fact, the hydrophobic side chains of two receptor tryptophans (Trp104 and Trp169) are the dominant players in the interface (30). In contrast, the IL-4–α-receptor interface is highly charged, and the major contributors to binding are involved in the extensive H-bonding network that characterizes the contact (4A). It is most likely that the electrostatic nature of the interfaces influences the observed differences in the kinetics of the binding process in these two systems. The highly charged IL-4 system results in a very fast on rate that is close to being diffusion controlled (5A). This is coupled to a relatively fast off rate. In the GH system, the on rates for forming the 1:1 complex are almost an order of magnitude slower, but so are the off rates (31). Although the resulting equilibrium binding constants are similar, the large differences in the kinetics of the process suggest that formation of the 1:1 intermediate complexes in the two systems has somewhat different requirements for supporting signaling. However, an important caveat to extrapolating *in vitro* binding kinetics to functional relevance in signaling processes is that changes in binding affinities do not track well with altered binding affinities. Although mutations that eliminate binding of the receptor components in solution-based measurements generally eliminate biological activity, in many cases measurable activities are still elicited in systems involving mutations that significantly alter binding, but do not eliminate it (5A, 8A). This suggests that the dynamics of receptor aggregation play a role in triggering activity, but that it is involved in some nonlinear way with other downstream signaling components.

Concluding Remarks

Although the various structures of hormone–receptor complexes provide information that encompasses both the versatility and specificity components inherent in the recognition system that regulates endocrine biology, a general understanding of this process at the molecular level remains challenging to construct, even combining it with the extensive mutational database available to us. One is struck by the extraordinary adaptability of these molecules to synthesize competent binding epitopes for a wide range of large target surfaces. It appears that the binding sites for cytokine receptors using the FNIII scaffold can adapt to binding cytokines via the hormone's one- to four-helix interface (long-chain cytokines such as GH and PRL) or, in the case of a short-chain cytokine motif such as IL-4, through the one- to three-helix interface. These interactions can have quite different affinities and might involve single or multiple binding hot-spots. The binding epitope can be quite specific, as for the hGH receptor, or very promiscuous, as for the γ-chain receptor.

In the case of hGH, the nature of the adjustments required to form the optimum set of interactions between the hormone of each of its two receptors suggests that recognition and binding of the two protein surfaces is directed by an induced-fit mechanism. A relatively large set of structural changes in IL-4 is seen during the transition from being free form to its bound state. This, coupled with the fact that the γ-chain receptor is a common binding element to a number of different hormone-induced signaling systems, suggests that a form of an induced-fit binding process must play a role in these systems. Distinct from the process of molecular recognition associated with the antibody–antigen paradigm, where binding is developed mainly through sequence diversity of the antibody complementarity-determining loops, the cytokine receptors can use essentially a constant set of residues to bind surfaces that are diverse both in sequence and in conformation. This is accomplished by employing conformational diversity, both local and global, and is the unifying hallmark of these systems.

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CHAPTER 44

Erythropoietin Receptor as a Paradigm for Cytokine Signaling

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Introduction

The mechanism of how cytokines and growth factors elicit a signaling response via cell surface receptors remains a major question within the field of cell signaling. It was previously thought that dimerization of the extracellular domains was sufficient to elicit activation of a signaling pathway, and, indeed, a variety of studies have shown that a number of different strategies of dimerization can cause signal activation. However, studies on the erythropoietin receptor (EPOR) have suggested that dimerization alone is not sufficient. Rather, subtle differences in ligand binding to receptor extracellular domains, as shown by various structural and biochemical data, can result in structural deviations that can modulate the signal response.

Signaling of erythropoietin (EPO) through the EPOR promotes the proliferation and differentiation of erythroid progenitor cells and is thus crucial to normal red blood cell development [1,2]. The EPOR is a member of the cytokine receptor superfamily [3], which includes receptors for other long-chain cytokines, such as growth hormone (GH), thrombopoeitin (TPO), and granulocyte cell signaling factor (GCSF), as well as short-chain cytokines, such as interleukins (ILs) 2, 3, and 4. These receptors are single-transmembrane (TM)-spanning proteins that bind their corresponding ligands in their extracellular (EC) domains. A common motif among these receptors is the cytokine homology domain (CHD), which consists of two seven-stranded β-sandwich motifs connected by a proline linker. Signature characteristics within the CHD include interstrand disulfide bonds within the N-terminal domain and a WSXWS-conserved motif located in the C-terminal domain. This WSXWS sequence seems to be essential for productive ligand binding and the resulting activation of the EPOR [4,5], although it is not clear from the structural data how the WSXWS motif carries out any binding or signaling role. The cytokine receptors are coupled to members of the Janus kinase (JAK) family (nonreceptor tyrosine kinases) at a proline-rich sequence in their cytosolic (CT) domain called Box 1. Agonist ligand binding in the EC domain of the receptor leads to a conformational change and reorganization that is permissive for autophosphorylation and activation of the associated JAK, resulting in phosphorylation of the CT domains of the receptors. These phosphorylation events trigger a signaling cascade via the signal transducers and activators of transcription (STATs) that ultimately leads to protein expression and cell proliferation [6]. In the case of EPOR, the associated JAK2 phophorylates many of the eight CT tyrosines, which serve as docking sites for STAT-5. STAT-5, after being activated by JAK2, travels to the nucleus, where it promotes genes that lead to the proliferation and survival of erythroid progenitor cells.

Erythropoietin, similar to GH [7,8], binds to its receptor in a stoichiometry of 1:2. As EPO itself is not a symmetric molecule, two different binding interfaces exist on the cytokine surface which are each capable of interacting with the EPOR. The EPO interaction sites have been named site 1 and site 2, for which the binding affinities are 1 n*M* and 1 μ*M*, respectively [9].

Structural Studies on EPOR

Studies on the binding of different ligand molecules to the EPOR have shown that dimerization of the extracellular

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domains itself is not sufficient for a biological response [10]. Furthermore, structural and biochemical data surprisingly revealed that the EPO receptor exists as a preformed dimer on the cell surface, and that a conformational reorganization of the receptor as a result of ligand binding is necessary to elicit the signal transduction cascade [11,12]. The biological appeal of a preformed dimerized receptor can be understood when one considers the low cell-surface density of the EPOR (<1000 receptors, or 1 μ*M*) *in vivo* [9,12–14]. Clustering of the receptors allows EPO to act efficiently at the cell surface by binding to the high-affinity (nanomolar) site 1 immediately followed by interaction with site 2, despite its low micromolar binding affinity. In the absence of this clustering, monomeric receptor–EPO interactions would be prevalent. As a result, the efficiency of the biological response to EPO would be compromised.

The extensive amount of crystal structure data available on a variety of agonist and antagonist EPOR complexes has enabled a comparison and analysis of different activation states of the EPOR. Four EPOR structures, determined by X-ray crystallography (depicted in Fig. 1), allow for the exploration of the dimerization interfaces involved in various signaling states of the EPOR [10,15–17]. These structures allow us to examine how differences in the nature of the bound ligand lead to changes in the receptor structure and assembly on complex formation.

EMP1, an Agonist, Bound to the EPO Receptor

The crystal structure of an EPO mimetic peptide, EMP1, was determined at 2.8 Å resolution in complex with the extracellular EPO-binding protein domain (EBP) of EPOR [15]. EMP1 is a member of a family of peptide mimetics selected by the phage display method that bind specifically and with high affinity (100–200 n*M*) to the EPOR and have agonist activity [18]. Co-crystals of the EMP1–EBP complex showed that EMP1 binds to two EBP molecules as a peptide dimer, resulting in a 2:2 ligand-to-receptor stoichiometry and generating an almost twofold symmetric assembly of the EMP1–EBP complex [15]. In this arrangement (Fig. 1A), the monomers of the EBP dimer are rotated approximately 180° from each other, and the two domains of each receptor molecule, D1 and D2, are oriented with an elbow angle of approximately 90°. The EPOR dimerization interface in the complex consists almost entirely of interactions with residues from the peptide EMP1 dimer with minimal contact occurring between the receptors themselves.

EMP33, an Antagonist, Bound to EPO Receptor

Studies on the EMP family of mimetics showed the importance of peptide Tyr4 in the peptide-mediated receptor dimerization [19]. A substitution of 3,5-dibromotryosine yielded a biologically inactive peptide, EMP33. Surprisingly, this antagonist was capable of dimerizing the EPO receptor. The crystal structure of EMP33 at 2.7 Å resolution bound to the EBP confirmed that dimerization is indeed not sufficient for signaling [10]. The structure shows an asymmetrically dimerized receptor (Fig. 1B) in which the D1 domain of each monomer is related by a 165° rotation (in contrast to the two-fold (180°) of the EMP1–EBP dimer). Thus, only 15° of rotation distinguishes an active dimer from that of a complex that is incapable of JAK2 activation.

EPO Bound to its Receptor

Crystal structures of a mutant [10,20] of the natural highly potent cytokine EPO with EBP were determined at 1.9 and 2.8 Å resolutions [16]. This EPO–EBP complex binds in a 1:2 stoichiometry, similar to that of the GH–growth hormone receptor (GHR) complexes [8], using two different binding surfaces of the EPO molecule to mediate interactions with each of the EBP monomers (Fig. 1C). These two surfaces, denoted site 1 and site 2, bind the receptor with high ($K_d = 1$ n*M*) and low ($K_d = 1$ μ*M*) binding affinities, respectively [9]. The D1 domain of each monomer is rotated approximately 120° relative to the other. In this complex, the orientations of the D2 domains are approximately in the same plane perpendicular to the membrane, in contrast to the 45° angle of the EMP1–EBP complex. This allows for the C termini of the receptor in the EPO–EBP structure to be in close proximity. Furthermore, this complex confirms the large amount of flexibility associated with receptor assembly, which may explain how a covalently-linked EPO dimer is still capable of binding and activating the EPO receptor, presumably in a 2:2 complex [21,22].

The Unliganded EPO Receptor

An unexpected result surfaced when the crystal structure [17] of the unliganded EPO receptor was determined at 2.4 Å. The asymmetric unit contains an EPOR dimer, such that the dimerization interface is located near the ligand binding site (Fig. 1D). In this unliganded form, the D2 domains are oriented such that their C termini are rotated 135° away from each other [17] and at an angle to the plane of the membrane, placing their expected membrane insertion points approximately 73 Å apart. The equivalent distances of the D2 C termini of the EMP1–EBP and the EPO–EBP dimers are 39 Å and 34 Å, respectively. These values are consistent with the notion that the inter-dimer distance of the unliganded EPO receptor would be too far for the intracellularly associated JAK kinases to interact, thus causing the unbound receptor to be inactive and locked into an off state.

Hot-Spot in EPOR for Ligand Binding

In each of the previous structures examined (Figs. 1A–D), the same binding surface of the EPO receptor is used to interact with the multiple ligands (Fig. 2). Variation of this binding surface is introduced by only small conformational changes in hot-spot hydrophobic residues such as Phe93, Phe205, and Met150 or small adjustments of loops in D1 [23]. This concept of a receptor hot-spot was first introduced by

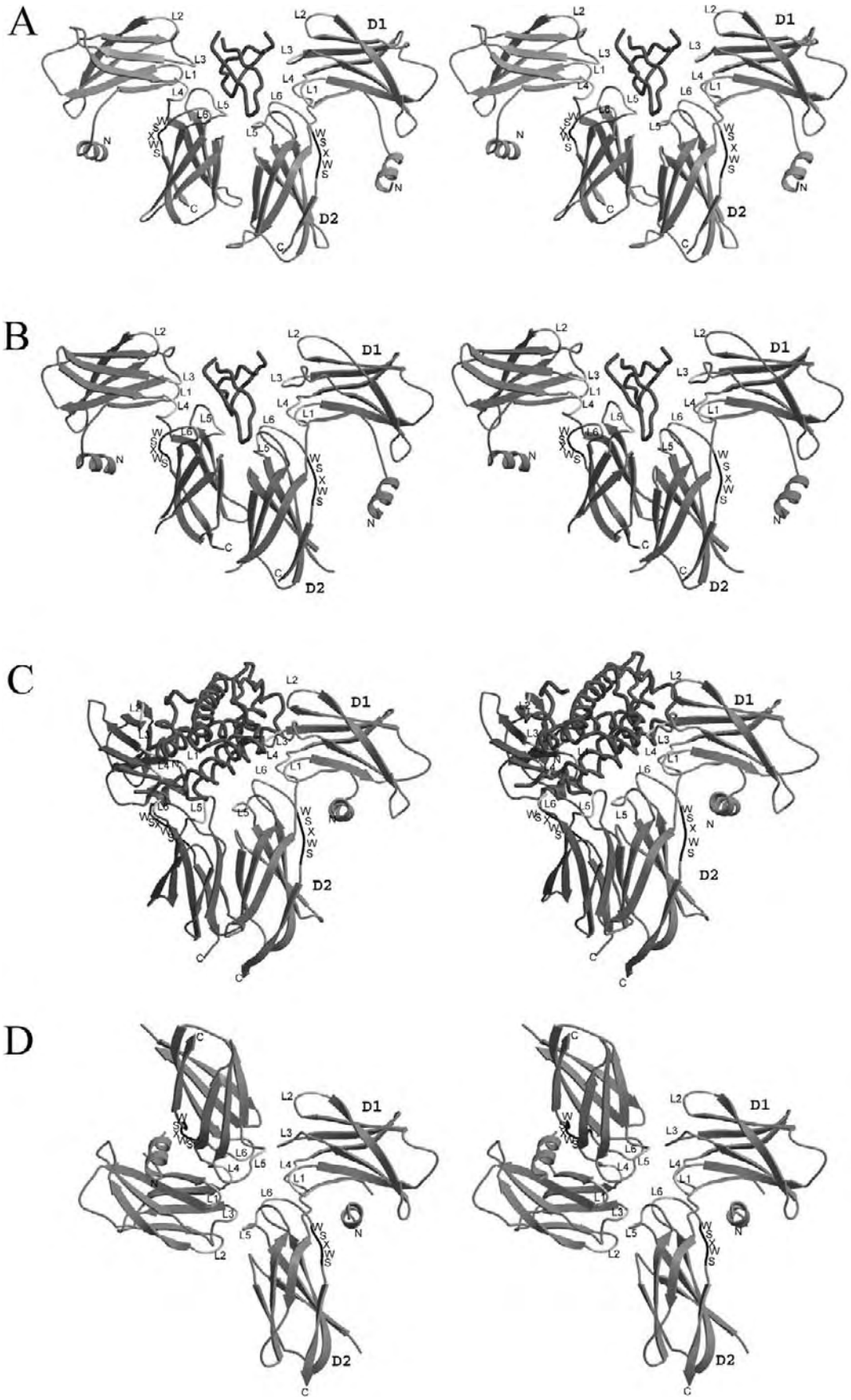


Figure 1 Stereo views of the various crystal structures of the EPOR. In each complex, the receptors are presented as ribbons and the ligands are depicted as tubes. The ligand binding loop tips of the receptor are yellow and labeled L1–L6; the conserved WSXWS signature sequence of the cytokine receptor family is black. The orientation shown for each structure corresponds to superimposing the β-strands of each receptor domain 1 (D1) onto the equivalent D1 domain of the

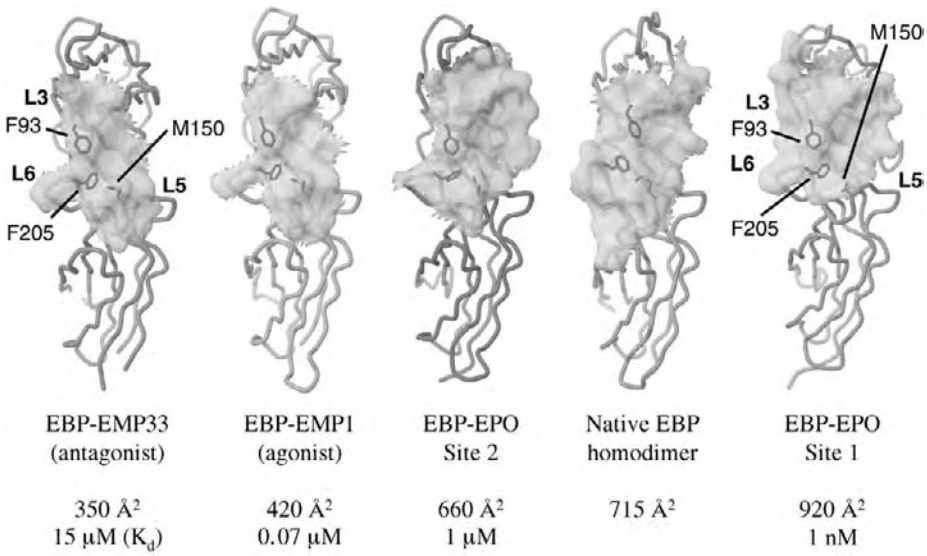


Figure 2 Plasticity of EPOR for ligand binding. EPOR binding surfaces are shown for each ligand receptor interaction, as well as the EBP monomers in the case of the unliganded structure. Molecular surfaces were calculated with GRASP [35], using a probe radius of 1.6 Å. The surface that is within 3.3 Å of the partner ligand is showed. The representation is shown from the smallest binding interface (left) to the largest (right). This buried interface correlates somewhat, but not exactly, with the approximate K_d. The K_d of the antagonist EMP33 is 15 μ*M*, and the K_d of the agonist EMP1 is 0.07 μ*M* [10,15]. Two views of the EPO–EBP structure are shown; one surface represents the high-affinity EPO site 1 (1 n*M*), and the other represents the low-affinity EPO site 2 (1 μ*M*) [9]. Surfaces were converted to MOLSCRIPT [34] objects and rendered in Raster3D [33]. (Adapted from Wilson, I. A. and Jolliffe, L. K., *Curr. Opin. Struct. Biol.* **9**, 696–704, 1999.)

Clackson and Wells for the human growth hormone [24]. Differences in K_d approximately correlate with the amount of surface area buried in each of the binding interfaces for the liganded complexes (Fig. 2). Thus, the EPOR is capable of presenting a number of the same interaction surfaces in different contexts for different ligands, each of which correlates with different activation states of the receptor. This example demonstrates that slight differences in the contact surface interaction can lead to a significant shift in receptor orientation, in turn propagating a much larger effect on the signaling response.

Biochemical Studies Supporting Preformed Dimers

Remy *et al.* [11] used an *in vivo* complementation assay to demonstrate that a ligand-induced conformational change of the EPOR dimer is required for activation. In these studies, chimeras that contained the extracellular and transmembrane domains of EPOR were fused to two complementary fragments of murine dihydrofolate reductase (DHFR) through flexible linkers of different lengths. Cells transfected with these chimeras express the receptors at the cell surface. In this experiment, DHFR activity is restored if the two complementary fragments are brought into close proximity. Chimeras containing a short 5-residue linker could not restore DHFR activity in the absence of EPO. In contrast, chimeras that contained the 30-residue linker allowed complementation both in the presence and absence of EPO. This linker-length correlation agrees well with the crystallographic distances between the C termini of the two EBPs in their free and bound states. Thus, taken with the unliganded EPOR structure, these observations suggest a ligand-induced reorganization of the dimer that results in activation of the signal cascade.

Recent biochemical studies of the transmembrane and juxtamembrane domains of the EPO receptor further support preformed EPOR dimers and the importance of receptor orientation for productive signaling. Constantinescu *et al.* [25] have shown that the transmembrane domains of the EPO receptor interact *in vivo* using antibody-mediated immunoflouresence copatching assays. These oligomerized receptors are not constitutively active, but rather require EPO binding to induce signaling. Furthermore, experiments that swapped the transmembrane domains of the EPO receptor for the strongly dimerizing transmembrane domain of glycophorin A still showed a dependence on EPO for JAK2 activation.

Further studies demonstrate the importance of the relative orientation of a region in the cytosolic juxtamembrane domains of the EPOR in signaling [26]. Alanine scanning has indicated that three residues, Leu253, Ile257, and

EMP1–EBP complex. (a) Weak agonist EMP1–EBP complex (EBP, cyan; EMP1, red; PDB code 1ebp) [15]. (b) Antagonist EMP33–EBP complex (EBP, salmon red; EMP33, red; PDB code 1eba) [10]. (c) Strong agonist EPO–EBP complex (receptor site 1, green; receptor site 2, purple; EPO, red; PDB code 1cn4) [16]. (d) Self-dimer EBP–EBP native complex (EBP, orange [33]; PDB code 1ern) [17]. Molecules were made using MOLSCRIPT [34] and rendered in Raster3D [33]. For color figures, see CD-ROM version of *Handbook of Cell Signaling*. (Adapted from Wilson, I. A. and Jolliffe, L. K., *Curr. Opin. Struct. Biol.* **9**, 696–704, 1999.)

Trp258 (LIW), in this region were necessary for EPO-induced phosphorylation, but not for binding of JAK2. Accordingly, these residues are likely to be involved in a switch mechanism propagated from EPO binding to the EC that positions JAK2 correctly for appropriate activation (Fig. 3a). These three residues are likely to occur on the same face of the protein surface, as the secondary structure analysis predicts an α-helix continuing from the TM through this region. Experiments in which additional alanine residues were inserted into this juxtamembrane region were performed in order to assess the affect of changing the relative "register" of these regions and, hence the effect transmitted to the intermolecular domains (Figs. 3b–d). Each alanine insertion rotates the register of the predicted α-helix by 109°. A single insertion greatly diminishes signaling by EPO, whereas a three-residue alanine insertion restores signaling

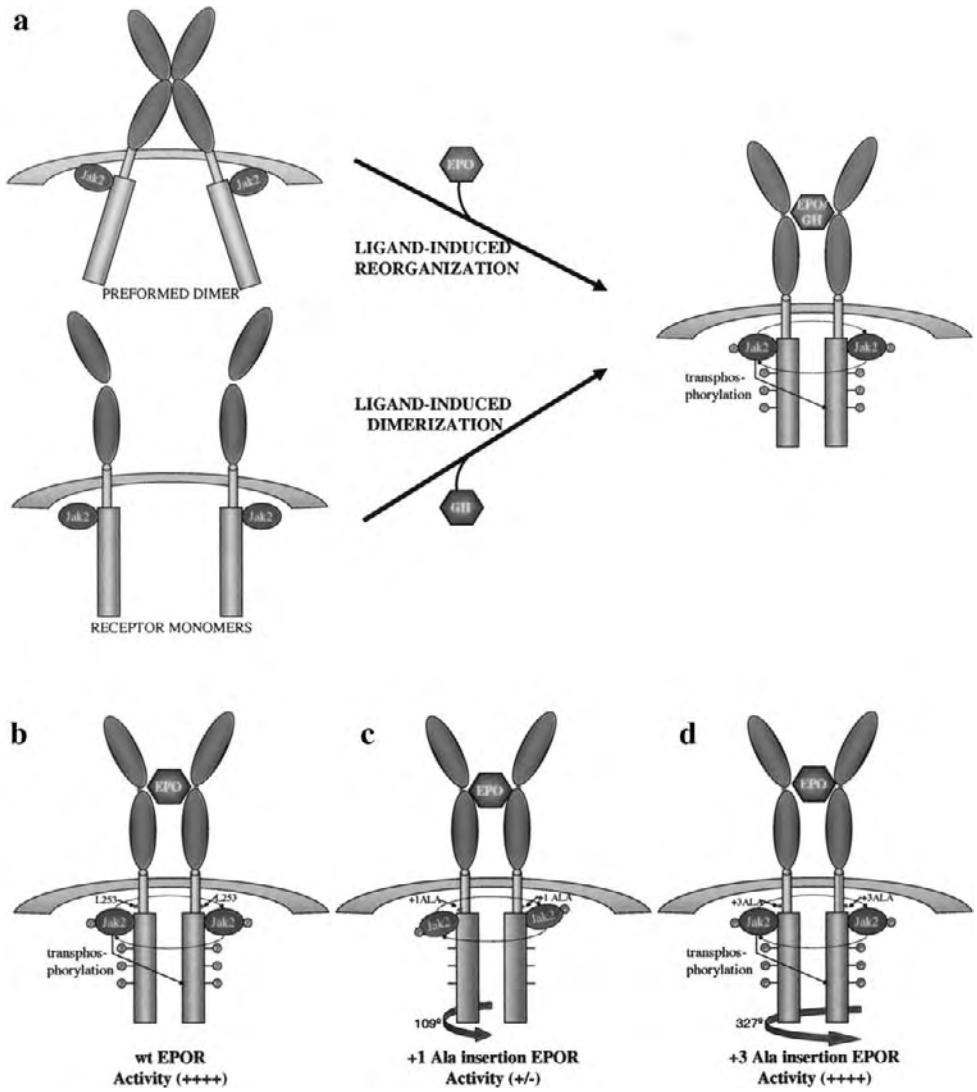


Figure 3 A model of EPO-induced dimerization and activation of the EPO receptor, JAK2 transphosphorylation, and tyrosine phosphorylation of the EPO cytosolic (CT) domain. (a) The schematic depicts contrasting views of ligand-induced signal activation. Top panel depicts a preformed dimer whereby the binding of cytokine (e.g., EPO) induces a structural reorganization, leading to active signaling. Bottom panel (b, c, d) depicts receptor monomers on the cell surface in which the binding of cytokine (e.g., GH) leads to dimerization, resulting in active signaling. EPO receptors are dimerized by EPO. The transmembrane (TM) domain is shown as a yellow cylinder and continues as a rigid helix into the CT domain through residue W258. L253, I257, and W258 comprise a hydrophobic patch expected to be at one face of this α-helix. L253 is shown in this helix. Upon activation (b), JAK2 transphosphorylates its partner JAK2 molecule, which in turn phosphorylates the partner EPOR CT domain tyrosine residues. Phosphorylation sites are marked as red circles. Insertion of one alanine residue prior to L253 (c) rotates the putative transmembrane α-helix by 109°, causing a change in the "register" of the hydrophobic patch. In this case, EPO is capable of inducing JAK2 phosphorylation; however, tyrosine phosphorylation of the EPO CT domains does not occur. Insertion of three alanine residues prior to L253 (d) restores wild-type activity to the EPOR. A three-alanine insertion restores the "register" of the hydrophobic patch, as the helix is rotated by 327° [26].

to the same level as the wild-type protein. Furthermore, it is not the increase in distance (≈5 Å) from the transmembrane domain (corresponding to one turn of helix) that alters JAK2 activity; instead, variation in the relative orientation of the LIW patch is crucial for proper signal response to EPO. Further insertion of two alanine residues in the TM region confirmed the predicted secondary structure of a continuous rigid helix from the TM through the Trp258, as a two amino-acid insertion restores the register of the LIW hydrophobic patch (Fig. 3b). Sequence alignments reveal that the hydrophobic nature of these three residues is conserved throughout nine other members of the cytokine receptor superfamily. Furthermore, the spacing of these residues relative to each other and their distance from Box 1 are strictly conserved, even though their distance from the membrane varies [26].

Two alternative models can explain the possible mechanisms of activation by cytokines and growth factors (Fig. 3a). For EPOR, the evidence is consistent with the unliganded EPOR existing as a preformed dimer on the cell surface in an "inactive" state and that binding of EPO *in vivo* causes a conformational change that is propagated through the membrane to the cytosolic domain. In addition, the relative orientation of the receptor is critical for signal generation, again consistent with the distinctly different agonist and antagonist structures. This ligand-dependent structural reorganization allows for interaction of JAK2, thereby eliciting a signaling cascade.

Other Cytokine Receptor Superfamily Members

While all cytokines exert their initial signaling response by way of forming active transmembrane receptor complexes on the cell surface, it is unclear whether preformed dimers are the prototype for all members of the cytokine receptor superfamily. The crystal structures of GH–GHR [8] and the ovine placental lactogen (oPL) bound to the extracellular domain of the prolactin receptor (PRLR) [27] reveal a 1:2 stoichiometry similar to that of the EPO–EBP structure. To date, no structures are available for the extracellular domains of these receptors in their unliganded form. Studies of GH mutants infer that GH binds to a monomeric GHR and then forms a stable complex with a second GHR [7] (Fig. 3a); however, the possibility that GH binds to a preformed GHR dimer and alters its conformation is also not inconsistent with these data. The nonsymmetrical nature of the PRLR receptors in the dimerized complex reveals a large amount of flexibility between the N- and C-terminal receptor domains, suggesting a possibility of domain orientation change in response to binding.

The crystal structure of GCSF bound to the cytokine receptor homologous region of the GCSF receptor, denoted gs-CRH, has been determined [28]. This structure shows two 1:1 complexes in the asymmetric unit related by a pseudo two-fold axis of symmetry, resulting in a 2:2 stoichiometry in the crystal structure. The GCSF ligand binds in a different mode than that of the previously described structures. Although the individual components in each of the 1:1 complexes are essentially identical, differences in the ligand receptor interfaces, as well as the relative orientations between the N- and C-terminal domains, are substantial. Thus, it is possible to extrapolate that the two different interfaces between GCSF and the N-terminal gs-CRH induce a slight difference in the orientation angle with the C-terminal domain, which may propagate an altered signal response. This structure may reveal one stage in the sequential formation of an active complex [28].

The notion of preformed dimers on the cell surface is not solely associated with the cytokine receptor super-family. The epidermal growth factor (EGF) plays important roles throughout development including cell proliferation, differentiation, and survival of multicellular organisms [29]. The EGF receptor (EGFR) is a member of the growth factor receptor tyrosine kinase family. Using chemical cross-linking experiments and sucrose density-gradient centrifugation, experiments have demonstrated that EGFR forms a dimer in the absence of bound ligand [30]. A "flexible rotation" model was proposed for EGFR activation in which the binding of EGF induces rotation of the juxtamembrane domain and, accordingly, the transmembrane domain. Consequently, the dimeric intracellular domains dissociate, allowing for the catalytic kinase domains to become accessible to their substrate tyrosine residues.

Earlier studies on the tumor necrosis factor receptor (TNFR), a member of the nerve growth factor receptor family, also revealed the potential existence of preformed dimers. The unliganded TNFR crystal structure, determined at 2.25 Å resolution, reveals that two types of dimers, parallel and anti-parallel, exist in the crystal lattice. Both dimer interfaces bury a high amount of surface area and are stabilized by large numbers of van der Waals and ionic interactions and, therefore, are thought to be biologically relevant [31]. These studies propose a model in which the TNFR, in its unliganded form, exists as the anti-parallel dimer with the cytoplasmic domains of each receptor in the pair separated by over 100 Å. Although the ligand-bound TNFR structure shows a 3:3 stoichiometry [32], in light of the EPOR studies discussed here, the unliganded TNFR dimer may indeed be biologically relevant.

Conclusions

Members of the cytokine receptor superfamily undergo major conformational reorganizations in order to respond to ligand binding and elicit appropriate signal activation. In each member of the family, strict regulation is important for specific response to the signaling molecule, and aberrant signaling can have considerable medical consequences. Studies on EPOR have led to a greater appreciation of the subtleties of receptor activation. The variety of ligands capable of binding to a single interface of the receptor is a consequence of slight variations of loops and side chains on the receptor surface. Furthermore, each of these bound ligands is capable of eliciting a different signal response by propagating slight changes in the orientations of the cytoplasmic domains of

the receptors through the membrane. The knowledge gained from the structural data permits the further design of peptide mimetics and small molecules. These future advances not only may help to further our understanding of the intricacies of the cytokine receptor signaling but also may have direct applications for clinical use.

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CHAPTER 45

A New Paradigm of Cytokine Action Revealed by Viral IL-6 Complexed to gp130: Implications for GCSF Interaction with GCSFR

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Introduction

The gp130–cytokine system has been the subject of extensive protein structure–function studies aimed at elucidating the basis of ligand recognition and receptor activation. A longstanding question has been the architecture of the higher order signaling assembly. It is clear from functional studies that the paradigm of gp130–cytokine recognition will differ substantially from the classical homodimeric systems typified by human growth hormone and its receptor. Recently, the crystal structure of a viral interleukin-6 (IL-6) complexed with the D1, D2, and D3 domains of the gp130 extracellular domain has reconciled much of the functional and mutagenesis data that exist for a variety of gp130–cytokine systems. The topology of the viral IL-6–gp130 assembly also appears to satisfy the structural requirements of an analogous signaling complex of granulocyte colony-stimulating factor (GCSF) and its receptor. A previous crystal structure of an "inactive" form of this complex can be supplanted by a new model incorporating known functional data implicating the N-terminal immunoglobulin (Ig)-like domain of the GCSF receptor as the key to assembling an interlocking tetramer, as seen in gp130–cytokines.

Receptor/Ligand Interactions

Although many different cytokine systems exist, a relatively restricted set of topological solutions is utilized by the different families to assemble higher order signaling complexes. The most basic building block is the interaction of the cytokine helical faces with the receptor cytokine-binding homology regions (CHRs), as typified by the human growth hormone (hGH) and erythropoeitin (EPO) examples, among others [1–5]. This interaction is a universally conserved recognition module that is then utilized in different geometries by various cytokine systems. The majority of cytokines require hetero-oligomerization of cytokine-specific receptors with shared signal-transducing receptor(s) [6], so the structural basis of receptor activation is quite different from the simpler homodimeric systems.

The gp130 System

The gp130–cytokines, also referred to as the IL-6 family, encompass the largest group of cytokines, which utilize shared receptors in hetero-oligomeric signaling complexes [7].

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gp130 is a signal-transducing receptor shared by IL-6, Kaposi's sarcoma herpesvirus (KSHV) (viral) IL-6, IL-11, ciliary neurotrophic factor (CNTF), cardiotrophin (CT-1), leukemia inhibitory factor (LIF), oncostatin (OSM), and NNT-1/BSF3. The extracellular domain of gp130 is approximately 597 amino acids, which is divided into five fibronectin type III (FN-III) domains plus a sixth N-terminal domain that appears to be Ig-like. Cytokine engagement by the gp130 extracellular region occurs through three membrane-distal, β-sheet sandwich domains (D1, D2, D3)[8]. The gp130 CHR resides at D2/D3, but gp130 requires an additional N-terminal (D1) Ig-like activation domain (IGD) in order to be functionally responsive to cytokine. The structure, role, and disposition of this IGD in recognition and activation were not known until recently [9]. Exhaustive mutagenesis of gp130–cytokines (particularly IL-6) indicates that the locations for sites 1 and 2 are analogous to those of the hGH and EPO examples, except that site 1 (located on the B and D helices) is recognized by the α-receptor, and site 2 (located on the A and C helices) is recognized by the gp130 CHR to form the heterotrimeric IL-6–Rα–gp130 complex (1:1:1) [10].

A transition to a higher order, hexameric (IL-6–Rα–gp130; 2:2:2) signaling assembly is a defining feature of gp130–cytokines and is mediated through a functional interaction between the unique third receptor-binding epitope (site 3, located at the tip of the four-helix bundle on the A/B loop and end of D helix) and the gp130 activation domain (D1 or IGD) [11–15].

Viral Interleukin-6

Kaposi's sarcoma herpesvirus (KSHV, or HHV8) is a recently discovered γ herpesvirus that is a likely causative factor for the development of Kaposi's sarcoma, as well as other neoplastic diseases associated with Kaposi's sarcoma [16]. KSHV encodes a functional homolog of interleukin-6 (vIL-6) that has an ≈22% sequence homology to human IL-6. A striking feature of vIL-6 is that it has bypassed the human IL-6 (huIL-6) requirement for an α-receptor (Rα) to activate gp130; vIL-6 directly activates gp130 on cells that do not express an Rα [17]. Therefore, vIL-6 is an Rα-independent version of IL-6 and would then be predicted to form a tetrameric complex (2:2, 2 vIL-6 to 2 gp130) [9].

GCSF and GCSFR

Granulocyte colony-stimulating factor is a cytokine of the long-chain, class I type that is a growth factor for the differentiation and maturation of neutrophilic granulocytes. Like gp130, GCSF receptor (GCSFR) is composed of six domains subdivided into an N-terminal IgD and five FN-III domains that show an overall sequence homology of 46% [18]. The GCSFR CHR is located at the D2 and D3 domains. A site 2 has been mapped for GCSF interaction with the GCSFR CHR that is analogous to the gp130–cytokine site 2 that is located on the A and C helices [19,20]. However, like gp130–cytokines, the GCSFR N-terminal IgD is required for activation [18], but the identity of a *bona fide* GCSF site 3 has been elusive [21]. The stoichiometry of the GCSF–GCSFR complex has been experimentally determined to be 2:2. Hence, because GCSF does not have an Rα as found for many gp130–cyotkines, it has strong analogy to viral IL-6, which also lacks an Rα and forms a 2:2 complex.

Structure of the Viral IL-6–gp130 Complex

The structure of the complex assumes a novel "hammock-like" configuration containing a tetrameric arrangement of two vIL-6 molecules and two human gp130 receptors (approximate dimensions 95×56×65 Å) (Fig. 1A) [9]. The complex is tethered together through the interaction of one face of vIL-6 (site 2) with a gp130 CHR (D2 and D3) and by a second epitope (site 3) at the tip of the vIL-6 four-helix bundle interacting with the IGD (D1) domain of a gp130 different from the other half of the tetramer (Fig. 1). It is clear from this structure that the D1 domain is essential for the formation of the activated, interlocking, higher order assembly. As seen from the top, the overall configuration results in a large hole in the middle of the complex (≈16×45 Å), a spacing likely critical for the gp130 extracellular domains to orient the intracellular domains correctly for signal propagation (Fig. 1B). The macromolecular architecture of the vIL-6–gp130 tetramer is novel for cytokine–receptor complexes and represents a previously unseen mode of receptor activation mediated by the IGD [9].

Site 1

The classical site of interaction of α-receptors with gp130–cytokines is known as site 1 and has been mapped to the faces of the B and D helices [10,22]. Because vIL-6 action can be independent of Rα, it is not required for crystallization of the complex with gp130 [9]. However, by analogy with human IL-6, the location of site 1 is clear. The outward helical face of vIL-6 (B and D helices), where huIL-6 would interact with Rα (site 1), is unoccupied (Figs. 1A and B) but openly accessible in the complex.

The Site 2 Interface

The site 2 contact surface between vIL-6 and the gp130 CHR is primarily composed of hydrophobic interactions between the vIL-6 A and C helices and the C/D and E/F loops of the gp130 D2 domain and B/C loop of the D3 domain (Fig. 1A). An exquisite shape complementarity exists between the cytokine and receptor, in which the protruding gp130 "elbow" fits into a slot in a diagonal

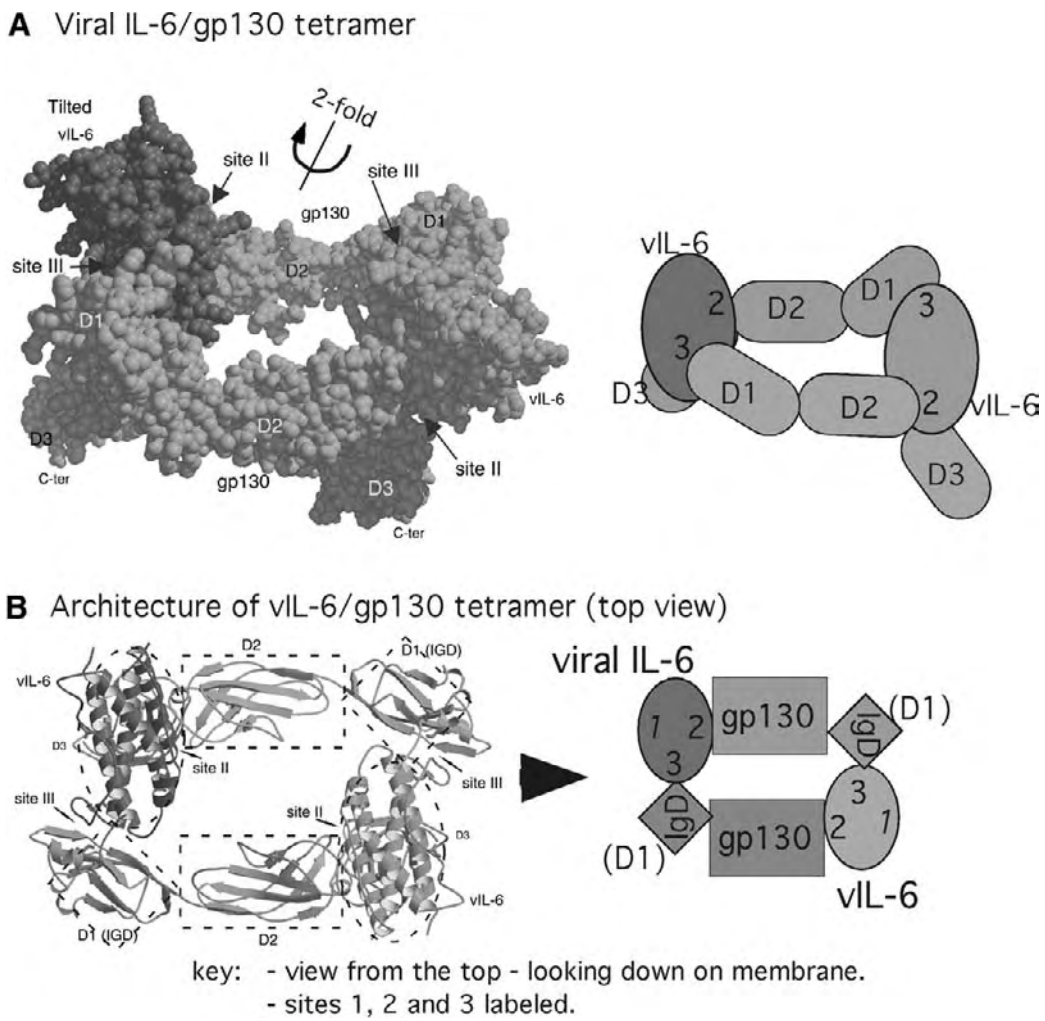


Figure 1 Crystal structure of viral IL-6 in complex with the gp130–D1, D2, D3 [9]. (A) Tilted space-filling view where all domains are in view. Note the large hole in the middle of the tetramer. For clarity, a schematic drawing of this orientation is shown to the right. (B) Ribbon drawing of a top view of this complex, looking down on the membrane. A schematic drawing of the top view, with identical domain coloring as the ribbon figure, is shown to right. In this orientation, the D3 domains are underneath the cytokines and therefore are not visible.

groove on a concave face of vIL-6 created by the crossing angles of the A and C helices. This knob-in-hole packing likely underlies the site 2 recognition mode across all gp130–cytokines.

The Site 3 Interface

The gp130–cytokines possess a third, or site 3, functional epitope that is necessary for receptor activation, but the nature of its interaction with gp130 is unclear [22]. In the structure of the vIL-6–gp130 complex, the site 3 interaction is comprised of an extensive interface between the tips of the vIL-6 four-helix bundle (A/B loop and start of the D-helix) and the edge (GF strands) of the upper three-stranded β-sheet of the gp130 IGD (D1) (Fig. 1B). The complementary shape of the interface is formed by the convex torpedo-like tip of vIL-6 resting in a concave depression formed by the curvature of the upper D1 β-sheet.

Implications of the vIL-6–gp130 Tetramer Structure for the Active GCSF–GCSFR Extracellular Signaling Complex

A crystal structure has been reported for GCSF in complex with the CHR (domains 2 and 3; also called the BN and BC domains, respectively) of the GCSFR (Fig. 2A) [2]. The structure revealed both expected and unexpected results. The complex is a 2:2 tetramer, with each GCSF molecule interacting with each receptor through the canonical site 2 epitope (also called major interface) on the face of the A and C helices (Fig. 2A) [2]. However, the unexpected result was that the two GCSF/GCSFR complexes formed a "side-by-side" dimer mediated by an interaction between the D3 (BC) domain and the N terminus of GCSF (or, minor interface) (Fig. 2A). The minor interface was not expected, as this interaction was not detected in previous structure–function studies. However, because the overall complex in the crystal was 2:2, a case was

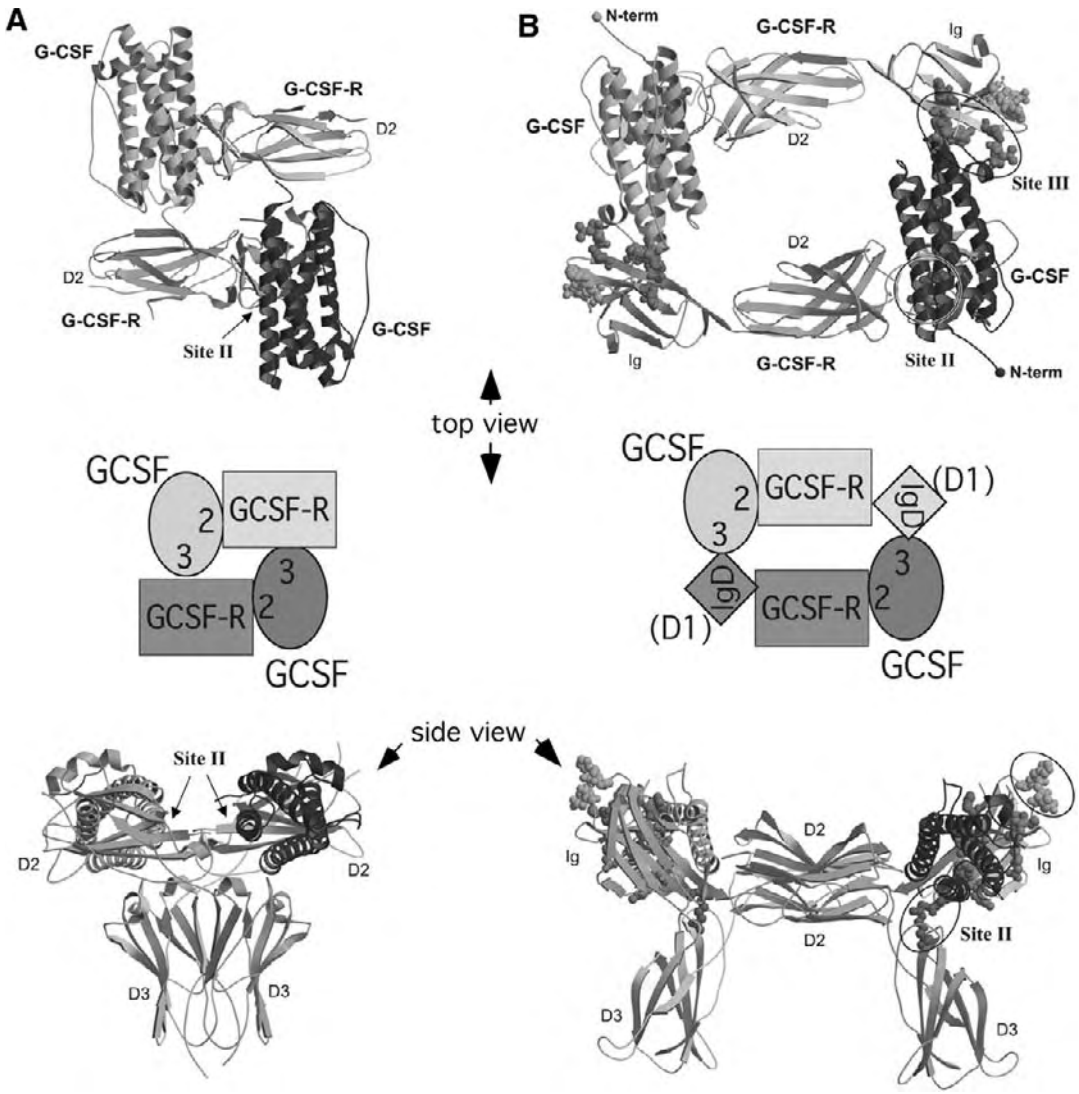


Figure 2 Inactive and active models of GCSF complexed to GCSFR. (A) Top view of the crystal structure of GCSF complexed to the GCSFR D2 and D3 domains as reported by Aritomi *et al.* [2]. Underneath the ribbon drawing is a schematic showing the assembly in the same orientation as the top view. At the bottom is a ribbon diagram of this structure shown from the side. (B) Hypothetical model of the active GCSF–GCSFR complex based on the viral IL-6–gp130 tetramer [9,21]. The D1 domain of GCSFR is included in the model and is shown interacting with site 3 of GCSF from epitope mapping studies (residues in space filling) [21]. A schematic in the middle shows the assembly in the same top-view orientation as in part (A). At the bottom is a ribbon drawing of a side view of the GCSF–GCSFR tetramer model.

made for this assembly representing the active signaling complex even though the GCSFR was missing the N-terminal domain known to be required for signaling[2].

Layton *et al.* [21] undertook an epitope mapping study that placed the elusive GCSF site 3 at the identical location found for gp130–cytokines (Fig. 2B). Further, these authors constructed a molecular model for the active GCSFR signaling complex (D1, D2, D3) based on the structure of the viral IL-6–gp130 tetramer (Fig. 2B). The model satisfies functional data for GCSF and is essentially identical to the vIL-6–gp130 framework, where site 2 on the A/C face and site 3 at the end of the D-helix enables formation of an interlocking tetramer (Fig. 2B). Thus, the utility of spatially distinct functional epitopes on both cytokine and receptor for assembling an interlocking dimer has been repeated in a distinct cytokine system.

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CHAPTER 46

The Fibroblast Growth Factor (FGF) Signaling Complex

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Introduction

The fibroblast growth factor (FGF) signaling system is a ubiquitous cellular sensor of local environmental changes and mediator of cell-to-cell communication with broad roles in development and organ homeostasis in the adult. Through the interaction of heparan sulfate (HS) with both activating FGF polypeptides and transmembrane FGF receptor (FGFR) tyrosine kinases, the system is rigorously modulated by tissue architecture. Diversity and cell and tissue specificity of signaling result from the combinatorial oligomerization of a family of 23 FGF homologs, diverse oligosaccharide motifs within HS chains of proteoglycans, and a plethora of ectodomains resulting from splice variations from four genes coding for four intracellular tyrosine kinases.

FGF Polypeptides

Fibroblast growth factor homologs are single heparin-binding polypeptides sharing a homologous core split by areas of less homology [1,2]. The heparin-binding property reflects the intimate association of the polypeptides with HS chains of proteoglycans in the extracellular matrix. The core sequence is flanked by unique N- and C-terminal sequences. N and C termini of the homologous core are important in receptor and HS interactions, and the unique N terminus outside the core is important in the fine structural arrangements of the oligomeric FGFR signaling complex [3,4]. Most FGFs are translated with a conventional secretory signal peptide at the N terminus; however, FGF1, FGF2, and several other homologs are not, although they appear in the external environment. This has fueled interest in novel mechanisms of FGF exit from cells as well as intracellular signaling [5]. Of the 23 homologs, the structures of FGF1, FGF2, FGF4, FGF7, and FGF9 have been determined. Crystal structures indicate that the FGFs share a remarkably similar three-dimensional structure characterized by a conserved hydrophobic patch that interacts with FGFR and a unique heparin-binding surface that differs dramatically among FGFs [6]. Functional analysis by site-directed mutagenesis indicates that heparin binding, FGFR binding, and biological activity are intimately associated [2,6,7]. The specificity of an individual FGF is likely determined by a composite of the unique heparin-binding domain and side-chain interactions with the ectodomain of a specific oligomeric HS–FGFR tyrosine kinase complex.

FGFR Tyrosine Kinases

FGFs elicit activity through activation of transmembrane FGF receptor tyrosine kinases in partnership with HS proteoglycans. The FGFR monomer is composed of a single polypeptide chain that has a glycosylated extracellular ligand-binding domain, a transmembrane domain flanked by juxtamembrane sequences, and an intracellular tyrosine kinase followed by a C-terminal domain (Fig. 1). The intracellular juxtamembrane region may be a determinant of conformational activation and interaction of the intracellular domain

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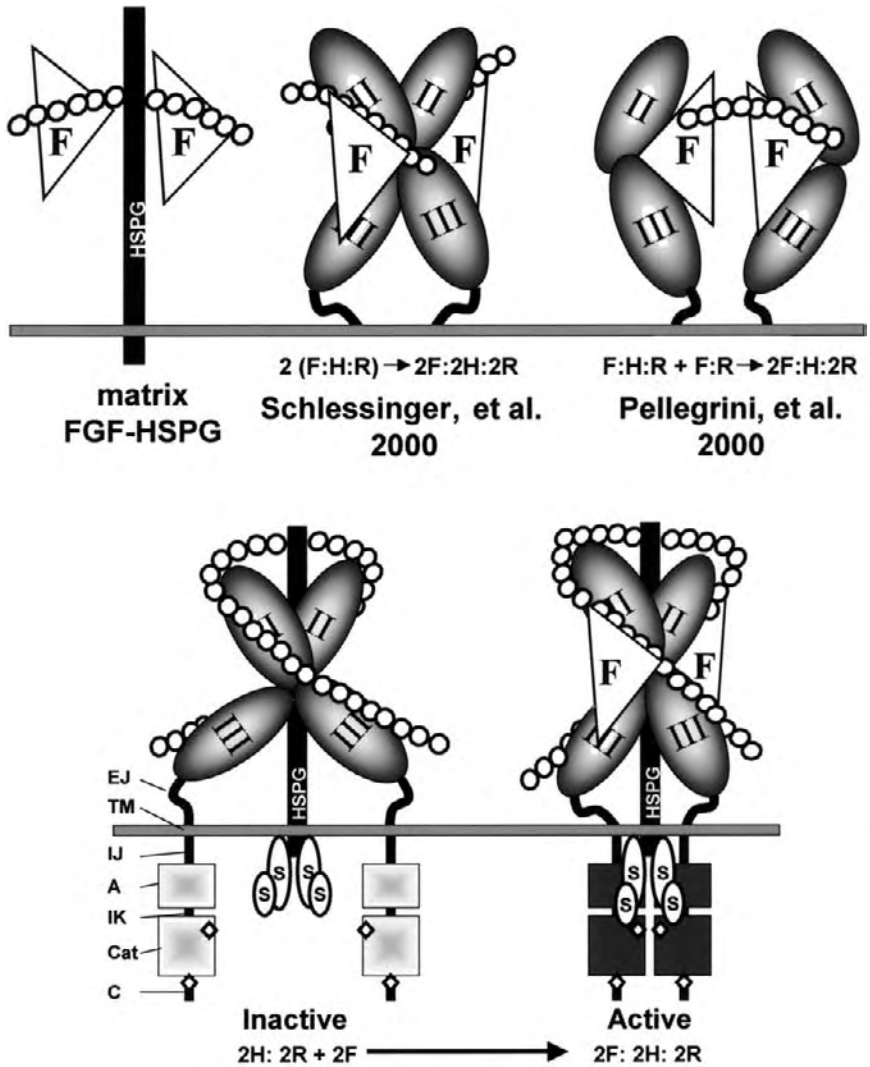


Figure 1 Models of the FGFR signaling complex. Independent binding of FGF to matrix heparan sulfate chains of a membrane-anchored proteoglycan (HSPG) in both orientations confirmed by crystal structures of heparin–FGF complexes is indicated. Also shown are the two divergent models of active dimeric complexes of heparin oligosaccharide (H) (open circles), FGFR (R) comprised of Ig modules II and III, and FGF (F), with the order of assembly proposed by the authors. At the bottom, an alternative conformational model in membrane context is depicted. EJ, extracellular juxtamembrane; TM, transmembrane; A, kinase ATP binding domain; IK, interkinase domain sequence; Cat, kinase catalytic domain; C, C-terminal sequence; S, membrane-anchored FGFR kinase substrates. Functional tyrosine phosphorylation sites are indicated by diamonds.

with membrane-bound substrates [4]. C-terminal to the intracellular juxtamembrane domain is the relatively conserved tyrosine kinase sequence domain comprised of adenosine triphosphate (ATP) binding and catalytic subdomains. Within the kinase domain is a kinase repression/derepression regulatory sequence containing two of the three functional tyrosine autophosphorylation sites [2,8,9]. Extending from the C terminus of the kinase domain is a less-conserved region among the four FGFRs containing a third functional tyrosine autophosphorylation site.

The extracellular domain of the transmembrane FGFR kinase consists of two or three immunoglobulin (Ig)-like modules. Ig module II or III of FGFR independently binds heparin or HS, and, in complex with affinity-selected heparin or HS, independently binds an FGF [2,10,11]. Heparin–module II complexes bind a wider spectrum of FGFs than Ig module III–heparin complexes [10,11]. However, only one FGF binds to a monomeric ectodomain composed of Ig modules II and III [3,4,10,12]. Ig modules II and III cooperate both within monomers and across dimers with cellular HS to confer specificity for FGF [2,3,12]. The FGFR forms ligand-independent dimers or higher order oligomers on the cell surface that are dependent on the sequence between Ig modules II and III [13]. A highly conserved sequence domain rich in basic amino acids within the N terminus of Ig module II is required for the interaction with heparin and HS [3,14]. Mutations in this domain significantly affect both heparin and FGF binding

and activities of the FGFR complex [14]. N-terminal to Ig module II is an alternatively spliced sequence rich in serines and acidic residues called the *acidic box*. When present, alternatively spliced Ig module I modifies the affinity for both heparin and FGF [15]. The exon coding for the acidic box sequence is always included whenever Ig module I is present. The acidic box sequence between Ig modules I and II may be a structural requirement as well as a functional element that contributes to reduction of affinity for both HS and FGF by Ig module I [3,15].

Heparan Sulfate

Pericellular matrix HS participates in rigorous control of the FGFR signaling system at multiple levels exceeding anticoagulation in complexity and impact on biological processes. The independent interaction of matrix HS with FGFs affects location and trafficking within tissues, access to the FGFR signaling complex, and lifetime and stability by protection against proteases [2,6]. Structure–function analyses and co-crystal structures of FGF1 and FGF2 with heparin oligosaccharides indicate that the heparin-binding domain of FGF is a composite domain contributed by distal sequence residues and formed by secondary and three-dimensional structures [6,16]. Of the 23 FGF homologs, only co-crystal structures of FGF1 and FGF2 with heparin oligosaccharides have been determined. Although a variety of carbohydrate-like electrolytes interact, affinity purification and structural analysis indicate that a hexameric heparin oligosaccharide exhibiting at least 2-O sulfation and both 2-O and 6-O sulfation is required for highest affinity interaction of heparin oligosaccharides to FGF2 and FGF1, respectively [2,6,17]. Crystal structures of FGF4, FGF7, and FGF9 have also been determined, but attempts at co-crystallization with simple 2-O and 6-O sulfated 6- to 12-mer oligosaccharides have failed [6]. Differing heparin-binding domains suggest specific requirements in respect to composition and length of interactive oligosaccharide [6]. As predicted by the unique heparin-binding domain of FGF7 relative to FGF1 and FGF2, a longer heparin oligosaccharide that exhibits anticoagulant activity and the presence of a 3-O sulfate is required to interact with and protect FGF7 against protease [6].

In the absence of FGF, the FGFR ectodomain forms a binary complex with heparin with a K_d of 10 n*M* that is competent to bind FGF in the absence of additional heparin or cellular HS [2,10,18–20]. The strict dependence of FGF binding on heparin/HS and the high-affinity formation of a functional, specific complex of FGFR with heparin/HS requires the presence of extracellular concentrations of divalent cations [18]. Similar to FGF7, only the fraction of heparin or HS that binds to antithrombin and has anti-Factor Xa activity (anticoagulant heparin/HS) exhibited functional high-affinity binding to FGFR in the absence of FGF [6,20]. An antithrombin-binding species of syndecan-1 from premalignant prostate epithelial cells, for which the HS chains form a binary complex with isolated recombinant FGFR1 Ig module II, is rare and present at an estimated 10,000 molecules per cell [21]. The independent and specific interaction of heparin and HS with the FGFR ectodomain protects it against proteolysis, stabilizes it, and may represent the composite complex into which FGF binds [2,14,18,19]. A partnership between the FGFR isotype and the rare and specific HS that binds FGFR from mixtures determines the specificity of a binary complex for a particular FGF [2,10,19,22].

Oligomeric FGF–FGFR–HS Signaling Complex

It is generally agreed that the activity, or access to substrates, of the FGFR tyrosine kinase is repressed by a flexible structural domain for which tyrosine phosphorylation by a neighboring FGFR kinase within a dimer or higher order oligomer releases the repression [2,9]. Still unclear are the order of assembly; the stoichiometry; the conformation of the inactive and active complexes of FGFR ectodomains, HS chains, and FGF; and how the interactions are transmitted to promote intracellular kinase–substrate interactions. A monomeric unit of the FGFR complex is generally agreed to be a composite ternary complex of one FGF, one FGFR, and one heparin/HS chain in which the sugar chain in a specific orientation concurrently interacts with heparin-binding domains from both FGFR and FGF [2,3,12]. However, prediction of how monomeric units come together to transactivate the intracellular kinases is complicated by models from dimeric crystal structures derived from the same FGF, FGFR ectodomain, and artificial heparin-derived oligosaccharide in which the second unit of the dimer is fundamentally different [3,12] (Fig. 1). One structure suggests a symmetrical complex that arises from back-to-back interaction of two identical ternary complexes of FGF, FGFR, and heparin/HS [3]. This dimer, consisting of FGF, FGFR, and heparin/HS with stoichiometry of 2:2:2, is stabilized by secondary interactions between FGF in one ternary unit and FGFR in the other unit, FGFR–FGFR contacts, and each heparin chain that spans both FGFRs. Another structure [12] suggests that the ternary complex of FGF, FGFR, and heparin/HS recruits a second complex of FGF and FGFR through the single chain of heparin/HS. The heparin/HS chain binds asymmetrically to an FGF bound independent of heparin/HS to a second FGFR. The net result is a heteropentameric complex with overall stoichiometry of two FGFs to two FGFRs to one heparin/HS chain, with minimal direct contacts between the two face-to-face FGFRs. The dimeric complex is stabilized simply by the sugar chain bridge between one ternary unit and FGF bound to the second unit. In both models, sustained derepression of the active sites of the monomeric FGFR kinases by transphosphorylation was proposed to be limited by the concentration and distance of both ecto- and intracellular domains (full-length FGFR), which can be overcome in the two-step dimerization reaction stabilized by both HS and FGF. The basis for the widely divergent crystal structures among the same three subunits is unclear. The differences are likely a consequence of the artificial and simple

sulfated heparin oligosaccharides available for crystallization studies, sensitivity of specific heparin interactions with FGF and FGFR to electrolyte conditions, and difficulty in dissection of specific and nonspecific interactions among heparin, FGFR, and FGF at high concentrations of the heparin polyelectrolyte.

An alternative model of the oligomeric FGFR signaling complex that unifies biochemical, structural and functional data to date has been proposed [2,4,10,18–20]. This model proposes a preexisting, inactive, unliganded complex of two FGFR ectodomains interacting back-to-back upstream of Ig module III while anchored to a proteoglycan core through two HS chains. HS chains interact with a primary heparin-binding domain on Ig module II and extend across the dimer to interact with Ig module III on the adjacent partner (Fig. 1). Specific functional HS binding to FGFR in contrast to nonspecific electrolyte interaction is divalent cation dependent and requires rare oligosaccharide motifs properly spaced within the HS chain. Divalent cations and HS cooperate in the ectodomain to conformationally restrict the kinase–substrate relationship between intracellular domains and maintain dependence of activity of the complex on the binding of FGF or other perturbations of the external matrix HS–FGFR relationship [2,18]. Docking of activating FGF into composite sugar-protein sites formed by Ig modules II and III of the preexisting inactive complex or other perturbations of the FGFR–HS relationship is transmitted to the intracellular kinase domains to overcome conformational restrictions that limit activity and access to substrates. Transmission of conformational change across dimers and to the intracellular juxtamembrane and ATP binding site of the kinase is enhanced by the bivalent contact of FGF with both FGFRs within the dimer [4]. In this model, HS chains of matrix proteoglycans play a central role in negative control of preexisting unliganded kinase complexes, the requirement and specificity for FGF, the cell context specificity of FGF signaling, and integration of signaling with tissue matrix remodeling.

Intracellular Signal Transduction by the FGFR Complex

Despite its ubiquity and broad spectrum of biological activities, there are large gaps in our knowledge of how the FGFR kinases access substrates, activate them, and transmit extracellular changes to intracellular signal pathways. Up to seven phosphorylated tyrosines in the FGFR intracellular domain have been reported, but only three or possibly as few as two sites that are conserved among the four FGFR have been clearly linked to repression/derepression of kinase activity and interaction with a substrate [2,8]. Among the autophosphorylation sites, tyrosine 653 and possibly 654 in concert are important for derepression of the FGFR1 kinase. Autophosphorylated Tyr766 in the COOH terminus interacts directly with phospholipase Cγ (PLCγ) through its SH2 domains and is required for PLCγ phosphorylation and pathways connected to it [8]. However, Tyr766 and the activation of PLCγ appear to be dispensable for FGFR1-elicited cellular responses, including mitogenesis, neuronal differentiation, mesoderm induction, induction of urokinase-type plasminogen activator, and chemotaxis. Although Tyr766 is not essential for the mitogenic activity of the FGFR, it is required for the age-dependent acquisition of the proliferative response to FGFR1 and FGFR1-dependent activation of the MAP kinase signaling pathway in premalignant epithelial cells [23].

The activation of the MAP kinase signaling pathway has been implicated in most FGFR1 responses studied to date. Phosphorylation of one or more membrane-anchored SUC1-associated neurotrophic factor (SNT) (also called FRS2, for FGF receptor substrate 2) proteins by the FGFR1 kinase recruits and activates the GRB2/SOS1 complex that then interacts with *ras* to activate the MAP kinase signaling pathway [24,25]. The quantity and quality of phosphorylation of SNT1 are both FGFR isotype and cell type specific [26]. Similar to FGF and HS specificity and specific structural arrangements within the extracellular domain of FGFR oligomers [2,4,10,13], the differential phosphorylation of SNT1 by FGFR appears only to occur in intact cells and is dependent on cell membrane context [26]. The differential phosphorylation of SNT1, both in quality and quantity, may be involved in determination of the signaling specificity of FGFR isotypes. Cell membrane and cytoskeletal context likely determine FGFR isotype- and cell-type-specific conformational relationships between the FGFR kinases and intracellular membrane-anchored substrates, just as they determine relationships among the FGFR ectodomain, matrix HS, and FGF.

The kinase domains of the four FGFR isotypes exhibit greater than 80% homology [2]. In some systems, the four FGFR isotypes elicit similar and redundant effects on cell responses and activate similar downstream signal transducers [27–30]. In others, individual isotypes exhibit dramatically different effects on cell phenotype, some of which are in opposition. The quantity of or sustained signaling from a single isotype can also affect quality of the response. For example, although FGFR1 and FGFR3 intracellular domains appear redundant in eliciting neurite outgrowth in PC12 neural cells [30], only the FGFR1 kinase elicits neurite outgrowth when the FGFR ectodomain is utilized [31]. The FGFR3 kinase failed to sustain outgrowth and Ras-dependent gene expression, but instead induced neural-specific gene expression pathways that were Ras independent [32]. In bladder [33], prostate [34,35], and salivary tumor epithelial cells [36], the resident FGFR2 kinase promotes homeostasis and suppresses the tumor phenotype. This is in contrast to ectopic FGFR1 that appears in malignant tumors. The appearance of FGFR1 in premalignant epithelial cells initially does not drive malignancy and activate signaling pathways associated with tumor phenotype. However, chronic activity over time causes activation of pathways that promote tumor progression and support the malignant state [23,34]. For more details on the role of the FGF family in specific biological systems and the diverse

signaling pathways perturbed by the FGF signaling system, readers are referred to specialized reviews [1,2,24–25,37,38].

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CHAPTER 47

Structure of IFN-γ and Its Receptors

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Interferon-γ (IFN-γ) is a pleotropic cytokine that induces antiviral, antiproliferative, and immunomodulatory effects on numerous target cells [1]. These diverse biological activities are initiated by IFN-γ-mediated aggregation of at least two different cell surface receptors: IFN-γR1 and IFN-γR2. X-ray crystallographic studies of IFN-γ and its receptors have been undertaken to delineate the molecular architecture of the receptor complexes and to understand the detailed recognition mechanisms that are ultimately responsible for IFN-γ biological responses. Here, these structures are summarized in the context of current biochemical and bioactivity data.

Natural forms of human IFN-γ are comprised of two 143-amino-acid peptide chains that are posttranslationally modified to contain an N-terminal pyroglutamic acid residue, N-linked glycosylation at two positions, and a heterogeneous C terminus containing the positively charged sequence KTGKRKR (residues 125–131). The crystal structure of human IFN-γ has revealed the tight association of two peptide chains (comprised of six α-helices, labeled A to F, from the N to C terminus) into a remarkable intertwined helix topology to form a symmetric dimer (Fig. 1) [2]. As a result, the two-fold related domains of IFN-γ are formed from the first four helices of one chain (A–D) and the last two helices (E′ and F′) from the other. Despite almost no sequence identity, the identical intertwined topology is also observed in the crystal structure of IL-10 [3]. The α-helices that form each domain are 9 to 21 residues long and are essentially linear (with the exception of helix F, which displays an ≈ 50° bend). The helices are connected by short loops of 3 to 5 residues, except for the 13-residue AB loop that encircles helix F. C-terminal residues 124 to 143 extend away from the core of the molecule and are presumed to be flexible.

IFN-γR1 and IFN-γR2 are both type I membrane proteins that contain extracellular and cytoplasmic domains connected by a hydrophobic membrane spanning helix. The extracellular domain of IFN-γR1 binds IFN-γ with high affinity (≈1 n*M*), while IFN-γR2 exhibits essentially no affinity for IFN-γ. Coexpression of IFN-γR1 and IFN-γR2 on cells results in a fourfold increase in affinity for IFN-γ compared to cells expressing IFN-γR1 alone, suggesting that the IFN-γR2 binding site is formed from residues on IFN-γ and IFN-γR1 as presented in the IFN-γ/IFN-γR1 complex. IFN-γ-induced formation of the biologically active complex of IFN-γ, IFN-γR1, and IFN-γR2 activates the Janus kinases (JAK1 and JAK2) that are associated with the cytoplasmic domains. JAK-dependent phosphorylation of the intracellular domain of IFN-γR1 results in the recruitment of the nuclear transcription factor STAT1 and subsequent expression of IFN-γ-inducible genes [1].

At this time, structural information is only available for the extracellular domain of IFN-γR1 (sIFN-γR1) as it exists in complex with IFN-γ (Fig. 1) [4–6]. Based on this work, sIFN-γR1 is comprised of two fibronectin type III domains (FnIII). The FnIII modules consist of a sandwich of two antiparallel β-sheets made up of seven β-strands: A, B, E, G, F, C, and C′. The N-terminal domain (D1) and the membrane-proximal or C-terminal domain (D2) are oriented at ≈120° to one another. The IFN-γ binding site is located at the crevice between D1 and D2. Receptor residues that contact IFN-γ are located on five different segments (labeled L2 to L6) that correspond to CC′ and EF loops of D1, the domain linker, and BC and FG loops in D2.

In agreement with solution and cell-surface binding studies, the structure of the IFN-γ/IFN-γR1 complex revealed the

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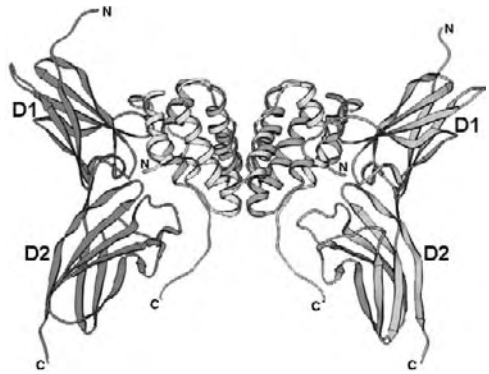


Figure 1 Ribbon diagram of the IFN-γ/IFN-γR1 receptor complex. The putative position of the cell membrane is at the bottom of the figure. The N and C termini of each molecule are labeled, as well as the D1 and D2 domains of the receptor.

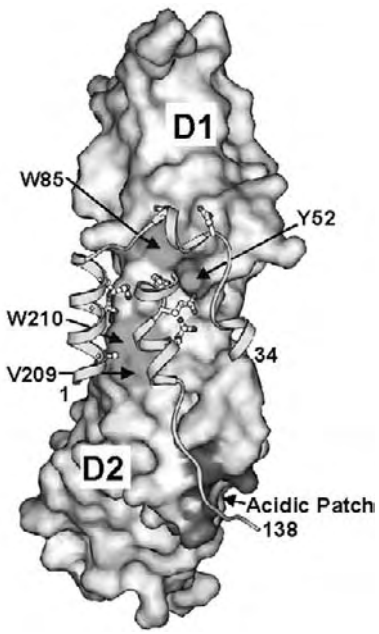


Figure 2 The IFN-γ/IFN-γR1 interface. IFN-γR1 is shown as a molecular surface while IFN-γ segments (residues 1–34 and 108–138) that bind IFN-γR1 are represented by a yellow ribbon. Several IFN-γR1 residues that participating in important interactions in the interface are labeled by arrows. The sidechains of IFN-γ residues Val-5, Glu-9, Arg-12, Ser-20, Asp-24, His-111, Glu-112, and Gln-115 are shown. The acidic patch located on IFN-γR1 is labeled, and the predicted interaction with a modeled C terminus of IFN-γ is shown.

symmetric binding of two IFN-γR1s to one IFN-γ dimer (Fig. 1). The two receptors in the complex do not interact with one another and are separated by about 100 Å at the putative position of the cell membrane. The large distance between the IFN-γR1s is consistent with the 1:2 complex being an intermediate that is dependent on IFN-γR2 binding and JAK2 recruitment to initiate the phosphorylation cascade. The two-fold symmetry of the IFN-γ/IFN-γR1 complex suggests that it contains two binding sites for IFN-γR2.

The IFN-γR1 binding site is comprised of IFN-γ residues 1 to 34 from one chain and residues 108 to 123 on the other (Fig. 2). These residues form a continuous binding site that includes helix A, the AB loop, and helix B from one chain and helix F from the other. More recently, a 2-Å structure of a mutant IFN-γ/IFN-γR1 complex has revealed additional details of the binding site, including the participation of five ordered waters in the interface and the reassignment of IFN-γR1 Val-206 to an unfavorable phi-psi value to optimize its interactions with IFN-γ [6]. In all free and bound structures reported to date, the C terminus of IFN-γ that is important for binding and biological activity has not been observed; however, an acidic patch was identified on the receptor that may provide a "Velcro-like" interaction site for the basic C terminus of IFN-γ [4].

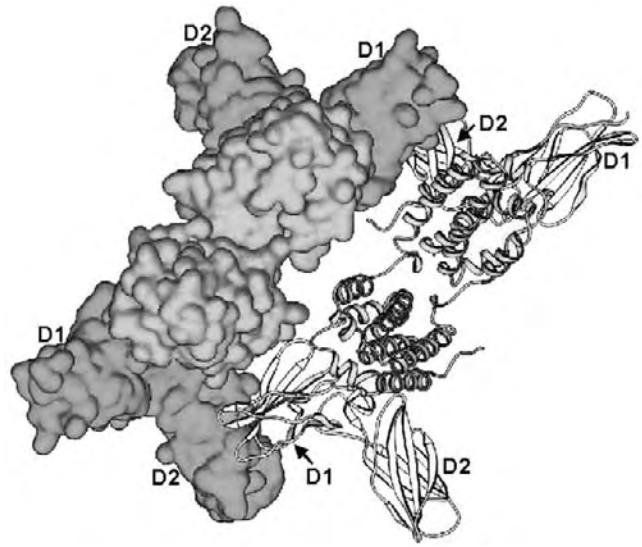


Figure 3 Crystal structure of the 2:4 IL-10/IL-10R1 complex [7]. The view is looking down the two-fold axis of IL-10. The 1:2 IL-10/IL-10R1 complex represented by molecular surfaces is very similar to the IFN-γ/IFN-γR1 complex shown in Fig. 1. The predicted positions of the IL-10R2s are shown as ribbons. The D1 and D2 domains for each receptor are labeled. This structure provides a possible model for the biologically active IFN-γ/IFN-γR1/IFN-γR2 complex.

Limited sequence identity confirms that IFN-γR2 is structurally similar to IFN-γR1; however, the inter-domain angle, receptor binding loops, and binding site cannot be accurately predicted. Currently, the most intriguing model for IFN-γR2 binding has been proposed from the analysis of the crystal structure of the IL-10/IL-10R1 complex [7]. In solution and the crystals, IL-10 and soluble IL-10R1 form a complex consisting of 2 IL-10 dimers and 4 IL-10R1s (Fig. 3). Structure and sequence comparisons of IL-10 and IFN-γ receptors suggest that high-affinity (IL-10R1 and IFN-γR1) and low-affinity (IL-10R2 and IFN-γR2) receptors may share a common binding site on their respective cytokines. The 2:4 IL-10 receptor complex suggests a model for how the low-affinity IFN-γR2 may simultaneously interact with IFN-γ and IFN-γR1. This structural model is supported by limited homolog scanning on IFN-γR1 and radiation inactivation data showing that IFN-γ biological activity requires two IFN-γ dimers [1]. Confirmation of this model will require the structure determination of the IFN-γ/IFN-γR1/IFN-γR2 complex.

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CHAPTER 48

Structure and Function of Tumor Necrosis Factor at the Cell Surface

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Introduction

Among all the members of the tumor necrosis factor (TNF) family, TNF-α and TNF-β are the most pluripotent. TNF-α was first discovered as a factor generated by endotoxin-stimulated macrophages that induces hemorrhagic necrosis of transplanted tumors [1]. TNF-β (lymphotoxin, or LTα) was identified as a lymphocyte-derived cytotoxin [2]. Cachectin, a mediator of fever and cachexia, was shown to be identical to TNF-α (hereafter, TNF refers to both cytokines). Although the systemic toxicity of TNF-α precludes its use in cancer chemotherapy, inhibitors of TNF-α have become important agents in the treatment of certain inflammatory diseases such as rheumatoid arthritis and Crohn's disease [3,4]. The two cell-surface receptors for TNF—TNF-R1 (p55, CD120a) and TNF-R2 (p75, CD120b)—are coexpressed by many types of cells. Stimulation of TNF-R1 induces apoptosis by activation of procaspase 8. The same receptor can induce proinflammatory cell proliferation by activating nuclear factor κB (NF-κB) and possibly mitogen-activated protein (MAP) kinases such as p38. TNF-R2 induces proliferation, but not cell death, and appears to be the less potent of the two receptors. The cellular physiology of TNF signaling is discussed in several recent reviews [5–9], and the reader is directed to these for primary references to the TNF literature. (Membrane-proximal events mediated by the TNF receptor (TNF-R) are also described in Chapter 54 of this Handbook.) This chapter focuses on the structural chemistry of TNF–receptor interactions at the cell surface, the state of the receptor before and after engagement with TNF, and the immediate structural consequences of TNF–TNF-R interactions. Even with this limitation in scope, the author regrets that many significant contributions to the literature could not be acknowledged within this short review.

TNF-α is widely expressed and is the prototypical member of a family that includes at least 19 members. Family members have diverged such that they are only 25 to 30% identical to each other in amino acid sequence [5,8,10]. Like many of its homologs, TNF-α is a type II membrane protein; the human ortholog has a 76-residue N-terminal propeptide composed of a 25-residue intra-cytoplasmic domain followed by a transmembrane sequence. TNF-α is released from the cell surface after cleavage by an extracellular, ADAM family metalloprotease [11]. Secreted and cell-surface TNF-α may play distinct roles in signaling [12]. TNF-β, which is expressed predominantly in lymphocytes, has a normal signal sequence and is secreted.

Structure of Tumor Necrosis Factor

Tumor necrosis factor α, like most of its homologs, is a homotrimer, with the exception of LTβ, which is heterotrimer composed of two distinct subunits ($LT\alpha_2$ and $LT\beta_1$) [13]. Trimers of TNF-α are stable and do not dissociate *in vivo*. The three-dimensional structure of TNF-α was the first of its family to be determined [14,15], followed by that of TNF-β [16], CD40L [17], TRAIL [18,19], and most recently sTall [20]. All have a distinctive "Greek key jellyroll" β-sandwich fold, consisting of two five-stranded β-sheets in direct opposition (Fig. 1). The same fold is characteristic of icosahedral virus coat proteins, although the latter have no sequence

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Figure 1 Schematic of a trimer of TNF-β in which the polypeptide trace of β-strands are depicted as arrows and loop segments as tubes. Residues that bind to TNF-R1 are red. The membrane-proximal surface of the trimer, as bound to TNF-R1, is located at the bottom of the diagram.

identity to TNF homologs that would establish an evolutionary relationship (although sTall does assemble into an icosahedral cage). On the other hand, TNF family members have distinct sequence similarity and obvious structural homology to trimeric, globular C1q domains [10] of the heteromeric molecules that function in the complement pathway. Hence, the TNF and gC1q domains may share an ancient kinship in the early development of the immune response.

Tumor necrosis factor monomers associate through a conserved girdle of hydrophobic or aromatic amino acid residues that encircle a trimeric axis of symmetry. Beta strands at the edge of the outer β-sheet of each monomer contact an extensive surface of the inner sheet of the adjoining molecule (Fig. 1). TNF-α and other homologs possess a disulfide cross-link between connecting loops that may confer stability to the molecule in the extracellular environment but is unlikely to serve a role in receptor recognition.

TNF Receptors

Nearly 30 TNF-R homologs have been identified [8,10,21]. Most are type I membrane proteins with cytoplasmic C-terminal domains and single transmembrane segments. Extracellular domains are composed of two or more cysteine-rich modules that contain binding sites for TNF family ligands. Both TNF-R1 and TNF-R2 are N-glycosylated, and the former is also O-glycosylated [22,23]. TNF-R1 and TNF-R2 have cytoplasmic domains of 221 and 174 residues, respectively. Those of other family members range from 30 to over 300 residues in length. None are known to exhibit enzymatic (kinase or phosphatase) activity. The cytoplasmic regions of TNF-R1, Fas, DR-3, and others that mediate apoptotic responses include ≈80-residue protein modules known as death domains (DDs). Structural studies reveal these to be six-α-helix bundles [24]. Death domains participate in specific homotypic interactions with DDs in other proteins. Death domains belong to a superfamily that includes death effector domains (DEDs) and caspase recruitment domains (CARDs), to which they have obvious structural but little sequence identity [24]. Mutagenic and structural studies indicate that DDs and their cousins can interact with each other through at least three distinct molecular surfaces [25–27], suggesting possible mechanisms by which single domains could interact simultaneously with different partners.

Soluble ligand binding proteins can be generated by proteolytic cleavage of extracellular domains from membrane-bound receptors, as is the case for TNF-R1 and TNF-R2, or by alternative exon splicing of sequences encoding the transmembrane domain, as is the case for the transcript-encoding Fas (see Bodmer *et al.* [10] and references within). Together with virally encoded receptor extracellular domain homologs, these molecules may serve as ligand scavengers and receptor competitors.

Extracellular (Ligand Binding) Domains of TNF Family Receptors

The extracellular domain (ECD) of TNF-R1 is an elongated chain of four interleaved, 40-residue, cysteine-rich domains (CRDs), each of which is stabilized by three disulfide cross-links (Fig. 2a) [28]. High-resolution structural analyses of the TNF-R1 ECD [29,30], together with sequence analysis of homologous domains [31] indicated that CRDs are constructed of binary combinations of three topologically distinct modules of type A, B, and C (Fig. 2a). More recent analyses confirm that this scheme, with modifications, is generally applicable to the TNF-R family [10]. Thus, the four CRDs of the TNF-R1 receptor can be described as A1-B2/A1-B2/A1-B2/A1-C2, while TNF-R2 is A1-B2/A1-B2/A1-B1/A1-B1 (numerals indicate the number of disulfide bridges within the module). Few residues, beside the cysteine pairs that serve as structural scaffolds, are conserved among homologous modules.

Ligand–Receptor Complexes

Tumor necrosis factor α binds tightly to ECD domains of TNF-R1 and TNF-R2 with dissociation constants in the range of 0.1 to 1 n*M* [22,32]. The structure of the TNF-β–TNF-R1 complex, which contains three receptor ECDs and one TNF-β trimer, immediately suggests a mechanism for the proximal events of signal transduction [28] (Fig. 3). Within the complex, each ECD binds to the crevice formed at the interface between two neighboring, triad-related TNF-β subunits. The receptors do not contact each other, yet all are tethered together by virtue of their mutual interaction with

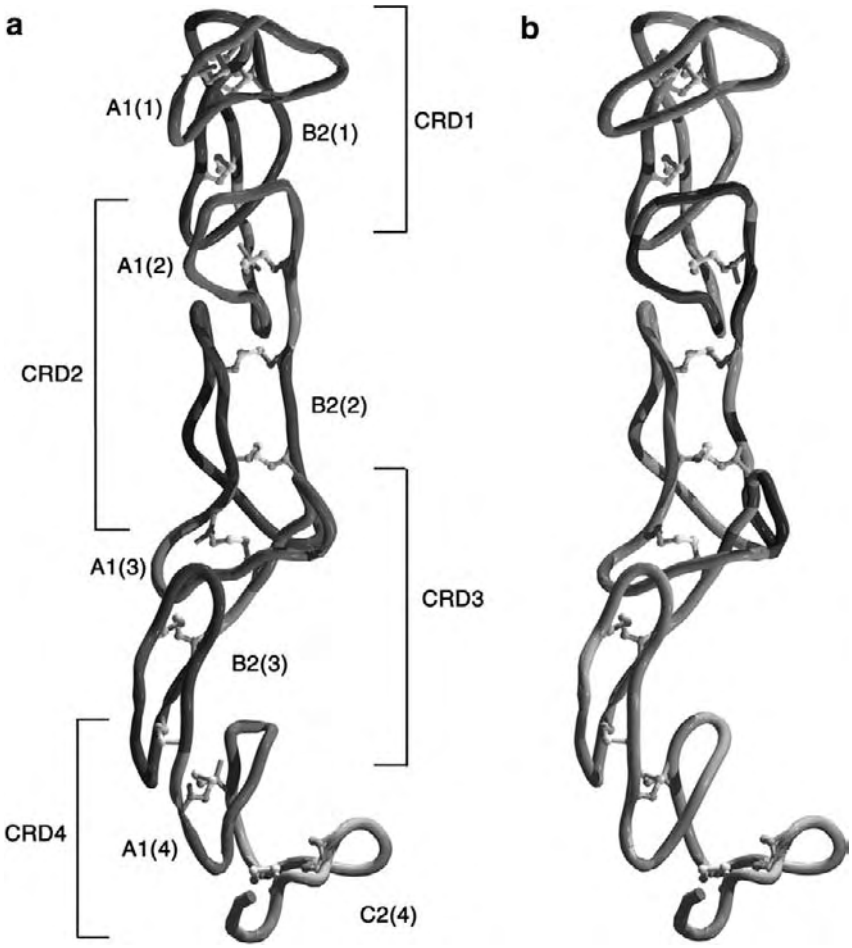


Figure 2 Diagram of the extracellular domain of TNF-R1. In panel (a), brackets show the boundaries of individual CRDs, and modules of type A, B, and C are shown as red, blue, and gray, respectively. In panel (b), residues in CRD2 and CRD3 that bind to TNF-β are blue; residues that mediate parallel dimerization in the crystal structure of unliganded TNF-R1 are red. These residues are located in CRD1 and are located within the PLAD domain. The ECD is oriented with the N terminus of the molecule at the top of the diagram.

the same trimeric molecule of TNF. The cytoplasmic domains of the three receptors are thus juxtaposed, although the mechanism by which they interact is yet unknown.

The TNF-β–TNF-R1 interaction involves two separate contact regions (Fig. 2b) corresponding to the second and third CRDs of TNF-R1 which bind to protruding polypeptide loops of TNF-β. The recently determined structure of the TRAIL receptor bound to its ligand reveals a similar mechanism of engagement [18,19]. Complexes of other members of the TNF-R family with their ligands are likely to exhibit a similar mode of binding. However, the putative ligand (and receptor) binding regions of homologous receptors and ligands share no apparent sequence identity. This is true even of TNF-R1 and TNF-R2, which recognize the same ligands, and of TNF-α and TNF-β, which bind to the same receptors [10]; hence, the same binding surfaces must perform two different recognition functions. Human growth hormone receptor binds both prolactin and growth hormone, and the structures of its complexes with the two ligands reveals one solution to the dual specificity problem [33].

Consequences of Ligand–Receptor Complex Formation

The death domain of TNF-R1 is capable of self-association and subsequent apoptotic signaling, as demonstrated by over-expression of DD constructs for TNF-R and Fas [34]. It is probable that, upon formation of the TNF–TNF-R complex, interactions among receptor DDs generate interdomain binding sites for adaptor proteins such as the TNF-receptor-associated death domain protein (TRADD) or Fas-associated death domain protein (FADD) that are not present in unliganded receptor molecules. The crystal structure of a death domain complex (involving DDs from the *Drosophila* proteins Pelle and Tube) [35] and that between the CARD domains of Apaf1 and Caspase 9 [36] reveal two non-overlapping interfaces that, it has been postulated [25], could be exploited to form a heterotrimeric DD complex.

Upon its formation, TRADD is recruited to the TNF–TNF-R1 complex via homotypic DD interactions. The C-terminal DD of TRADD binds other DD-containing

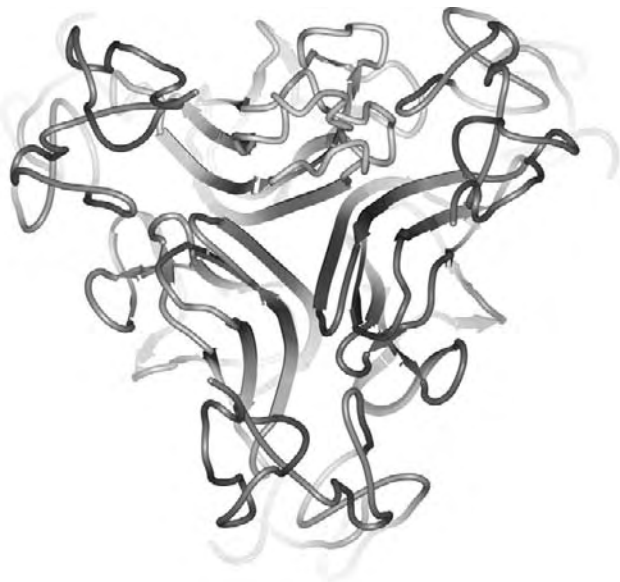


Figure 3 A view of the complex between TNF-β and three molecules of the TNF-R1 ECD [28]. The complex is viewed along the three-fold axis of the TNF-β trimer, looking toward the plasma membrane. Subunits of TNF-β are red, green, and gray, and the polypeptide backbone of the TNF-R1 molecules is gold.



Figure 4 A model showing how TNF-β–TNF-R1 complexes could form aggregates on the cell surface through homodimeric interactions between TNF-bound receptor molecules. The complexes are viewed resting on the surface of the membrane. The receptor dimer (center) that mediates the contact between the two complexes is as observed in crystals of unliganded TNF-R [29]. The dimer interface is formed by residues in CRD1 domains of the interacting ECDs (circled) that correspond roughly to the PLAD [46]. The structure of the TNF-β–TNF-R1 complex is as determined by Banner *et al.* [28].

partners, including the adaptor protein FADD and the serine/threonine kinase RIP1 [9,10]. The amino terminal DED of FADD can form a complex with the corresponding DED of procaspase 8, thereby increasing the local concentration of apoptotic proteases at the plasma membrane [37]. TNF–TNF-R1 can also elicit cell proliferation by recruiting TNF-R-associated factors (TRAFs), which mediate the activation of NF-κB [38]. The C-terminal domain of TRAF-2 trimerizes through a coiled-coil segment and possesses an ≈150-residue β-sandwich domain that binds to the N-terminal α/β sandwich module of TRADD [39]. TNF-R2 contains no DD and therefore cannot elicit an apoptotic response. It does, however, contain a short ≈10-residue cytosolic sequence to which TRAF domains bind [40,41]. The affinity of TRAF-2 for TNF-R2 is considerably lower than that for TRADD, suggesting that TNF-R2 is the less potent signaling receptor. TNF and its receptor complex are ultimately cleared from the cell surface through a clathrin-mediated endocytic pathway [42,43].

Receptor Preassociation

The structure of the ECD of TNF-R1 [29] provided the first indication that TNF receptors can associate in the absence of ligand. Two-fold symmetric receptor dimers of two distinct types, both involving substantial intersubunit contact areas, were discovered. So-called anti-parallel dimers involved extensive isologous contacts between receptor CRDs 2 and 3, at an interface that overlaps substantially with the TNF binding site. Although there is no evidence that such interactions occur *in vivo*, such dimers would prevent TNF binding and could also serve an autoinhibitory role in the absence of ligand.

In a different arrangement observed in the same crystals, the ECDs are parallel and packed back-to-back. In this case, the dimerization surface is located in CRD1, which is not involved in TNF binding (Fig, 2b). Indeed, the TNF binding sites are diametrically opposed to the dimerization surface. It was suggested that TNF–TNF-R complexes could be assembled into clusters on the plasma membrane by virtue of homotypic interactions between receptor CRD1 domains (Fig. 4). Recently, two research groups [44–46] demonstrated that, even in the absence of ligand, both the Fas and TNF-R1 exist in a preassociated state in the plasma membrane. Residues responsible for their interaction were found to be located within a contiguous, pre-ligand-binding assembly domain (PLAD). The PLAD domain is located within CRD1 and appears to correspond to the surface defined earlier in the structure of the unliganded receptor [29] (Fig. 2b). Several mutations observed to abrogate preassociation of TNF-R1 correspond to residues at the dimerization interface observed in the crystal structure of unliganded TNF-R1. PLAD domains are receptor specific. Transplantation of the PLAD/CDR1 domain of TNF-R2 onto TNF-R1 allows the latter to associate with other TNF-R2 molecules. Ligand binding and preassociation appear to be cooperative phenomena, in that mutations of certain residues within the PLAD also interfere with TNF binding [46]. Accordingly, the TNF-R1 self-association domain partially overlaps that of the TNF binding domain [28,29]. Whether preassociated receptors form dimers, as observed in crystals, or trimers, as suggested by Chan *et al.* [46] remains to be determined.

Stimulation of certain receptors in the TNF-R family causes large-scale redistribution and clustering of signaling complexes on the cell surface, followed by internalization of

receptor complexes through endocytic pathways. Engagement of Fas by its ligand is facilitated by receptor preassociation and results in the formation of receptor–ligand microaggregates. The structural basis of this process is unknown but may involve associations between ligand-bound receptors that occur cooperatively with ligand binding, as suggested by the model depicted in Fig. 4. Subsequent recruitment of FADD and procaspase-8 to Fas–Fas ligand microaggregates produces the death-inducing signaling complex (DISC) [47], which itself assembles into higher order clusters. This process is amplified by procaspase activation. Actin and actin-binding proteins have been implicated in both the initial recruitment of FADD and the clearance of receptor clusters through endocytic pathways [48].

Conclusion

Signaling through TNF receptors is probably paradigmatic of that which occurs through many of its homologs. The action of TNF is pleotropic in that it can initiate both apoptotic and proliferative pathways. The mechanisms of signal integration that commit the cell to one or the other fate have yet to be fully elucidated. Receptor-proximal events in both cases appear to be mediated through protein-interaction domains in receptors and cytosolic adaptor proteins. Crystallographic analysis of the TNF-R1 receptor and its complex with TNF-β suggests that ligands and their receptors assemble into complexes with two- and three-fold symmetry and that higher order lattice-like structures could be generated though the operation of this symmetry. TRAF proteins are homotrimeric, as are their complexes with TNF-R2 peptides and with TRADD, suggesting that the symmetry of extracellular components is recapitulated in the intracellular signaling complexes formed upon receptor ligation. On the other hand, death domains have not been found to assemble into homotypic complexes with three-fold symmetry. The organization of TNF signaling complexes is likely to be key to the mechanism of signal transduction and propagation. Thus, elucidation of the structures of TNF signaling complexes, however transient, will represent a significant achievement in our understanding of TNF signaling mechanisms.

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CHAPTER 49

The Mechanism of NGF Suggested by the NGF–TrkA-D5 Complex

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Introduction

Nerve growth factor (NGF) is the founding member of the neurotrophins, a family of growth factors responsible for the formation and maintenance (survival or apoptosis) of neuronal populations in the peripheral and central nervous system. The neurotrophins are of potential therapeutic interest in a number of neurological disorders such as Alzheimer's and Parkinson's diseases (reviewed in Siegel and Chauhan [1]). In addition to NGF, the neurotrophin family includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and neurotrophin-6 (NT-6). These molecules exert their biological activities through binding to two unrelated classes of receptors. One of these, the p75 neurotrophin receptor ($p75^{NTR}$), also referred to as the low-affinity receptor, binds to all neurotrophins with nanomolar affinity and is responsible for their apoptotic activities. The Trks, or so-called high-affinity receptors, are more specific and mediate the trophic effects of neurotrophins through dimerization-induced tyrosine kinase activity. TrkA binds to NGF (and weakly to NT-3), TrkB to BDNF and NT-4/5 (and weakly to NT-3), and TrkC binds to NT-3. The presence on the same cell of both $p75^{NTR}$ and TrkA results in high-affinity binding sites with picomolar affinities for NGF [2].

Neurotrophins

The neurotrophins share a pairwise sequence identity of about 50% and belong to the family of cystine-knot-containing growth factors [3]. They adopt very similar three-dimensional structures, containing a monomer core of a pair of irregular, anti-parallel, two-stranded β-sheets that carry the cystine-knot motif as well as both termini on one end of the molecule and three hairpin loops on the other [4] (Fig. 1A). Two monomers are assembled in a parallel fashion to yield a non-covalent but tightly packed homodimer with a dumbbell-like shape (Fig. 1B). The conserved residues are clustered in distinct segments that cover about half of the molecule and share a local sequence identity among all neurotrophins of about 70%. Three of these segments map onto three of the β-strands that form the "handle" of the dumbbell as well as a large portion of the dimer interface. To date, crystal structures have been reported for the uncomplexed NGF, NT-3, and NT-4/5 homodimers as well as the BDNF/NT-3 and BDNF/NT-4/5 heterodimers. These structures, in combination with a wealth of mutagenesis studies and truncation experiments, have led to a good understanding of how affinity and specificity between neurotrophins and the Trk receptors are achieved, as demonstrated by the successful creation of multi- and pan-specific neurotrophin variants (reviewed in Wiesmann and de Vos[5]; also see Robinson *et al.* [6]).

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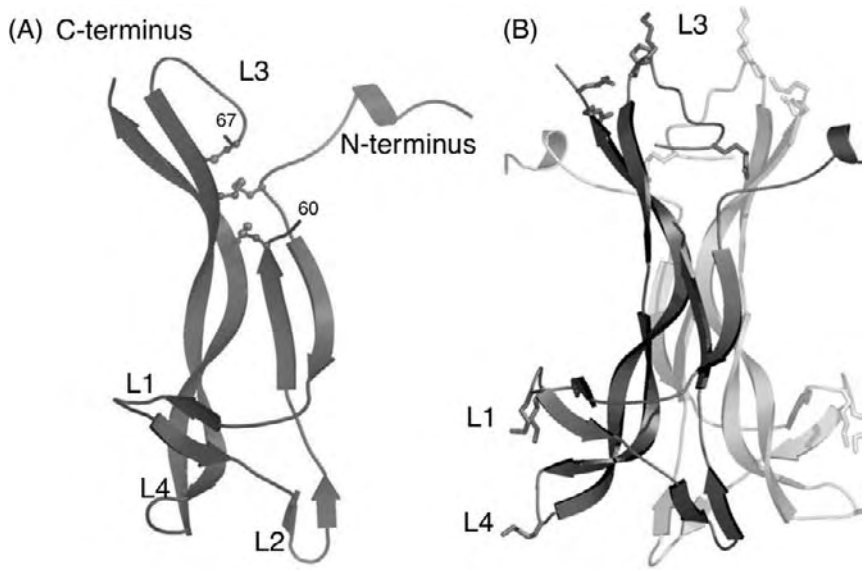


Figure 1 The neurotrophin fold, as exemplified by NGF (taken from the NGF–TrkA-D5 complex [11]); all subsequent figures display NGF in orientations similar to that shown in part (B). One loop, bounded by residues 60 and 67, is poorly ordered or disordered in most neurotrophin structures. (A) Ribbon rendering of the backbone of the NGF monomer. The cystine knot is shown as a ball and stick. (B) Ribbon rendering of the backbone of the NGF dimer.

Trks

The Trks are receptor tyrosine kinases with a molecular weight of about 85 kDa. Their extracellular portions share a sequence identity of about 35% and have five distinct domains [7]. Domain 2 (D2) consists of three leucine-rich repeats and is sandwiched between two cysteine-rich domains (D1 and D3). D4 and D5 have an immunoglobulin (Ig)-like fold, and are followed by a 40-residue-long linker segment connecting the extracellular portion to the transmembrane helix. Domain deletion studies, experiments with truncated and chimeric versions of the Trks, and alanine-scanning mutagenesis experiments have identified D5 as being most critical for high-affinity neurotrophin binding (see references in Wiesmann and de Vos [5]). While some studies indicate that D4 enhances complex formation between the neurotrophins and the Trks, others indicate that D4 is required (at least *in vitro*) for proper folding of the receptor. D4 has also been suggested to be necessary to prevent ligand-independent formation of receptor complexes. The roles of D1, D2, and D3 are even less clear. In contrast to earlier studies indicating that the leucine-rich repeats are involved in neurotrophin binding, the crystal structure of NGF in complex with TrkA-D5 (see below) shows that extensive contacts with NGF are unlikely [5]. Instead, the leucine-rich repeats may be involved in intra- or intermolecular Trk interactions, possibly stabilizing the signaling complex [8]. Finally, D1 through D4 may play a role in complex formation between the Trks and $p75^{NTR}$ in a receptor–receptor assembly that provides high-affinity binding sites for the neurotrophins or, alternatively, in influencing the architecture of a possible ternary complex between NGF, TrkA, and $p75^{NTR}$.

The crystal structures of D5 of TrkA (TrkA-D5), TrkB (TrkB-D5), and TrkC (TrkC-D5) are all domain-swapped dimers, where strand A of each molecule is replaced by the same strand of a symmetry-related neighbor [9,10]. This domain swapping is believed to be a folding artifact caused by the absence of D4, but the correctly folded structures can be modeled with minor changes. The structures contain two β-sheets in a β-sandwich fold, and belong to the I-set of the Ig superfamily; however, they possess a number of distinct and unusual features that deviate from the canonical I-set fold [9]. For example, the usual buried disulfide bridge, connecting both β-sheets, is absent in D5; instead, D5 possesses a solvent-exposed disulfide bond formed between cysteine residues in two neighboring β-strands. Another noteworthy difference concerns the loop connecting strands A and B which is three residues shorter than in other I-set members. It is likely that the shortness of this loop increases the propensity of these domains to misfold when expressed as single domains.

NGF–TrkA-D5 Complex

The crystal structure of the complex between NGF and TrkA-D5 [11] substantiated the ligand-binding function of D5, revealed the structural basis for specificity and cross-reactivity among the neurotrophins and their Trks, and defined the orientation of the complex with respect to the membrane. The overall shape of the complex resembles a bat, with the wings formed by the two copies of TrkA-D5 arranged symmetrically around the NGF body in the center (Fig. 2). Because the C terminus of D5 is expected to point toward the membrane, NGF is oriented with its two-fold

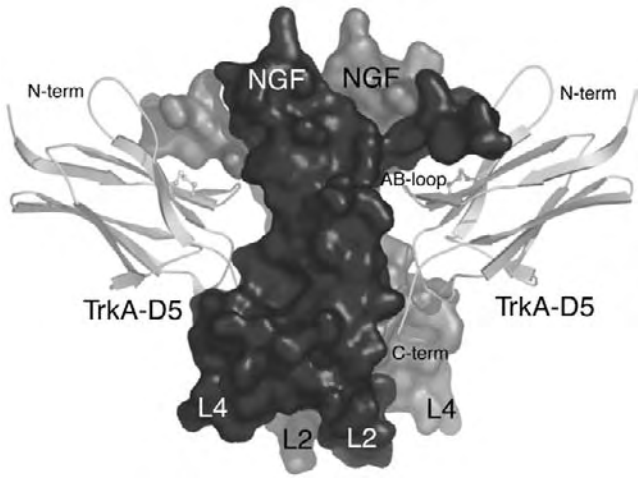


Figure 2 Crystal structure of the NGF–TrkA-D5 complex. NGF is rendered as a solvent-accessible surfaces (dark and light gray) and TrkA-D5 as a gray ribbon with the disulfide bond buried in the interface; NGF is shown as a ball and stick. The first and last ordered D5 residues are labeled "N-term" and "C-term", respectively. In this figure and in Fig. 3, the cell membrane would be at the bottom.

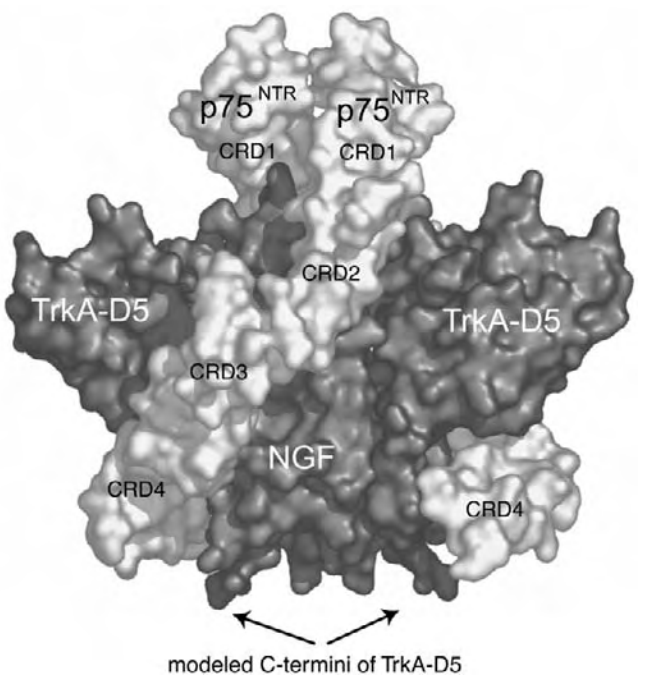


Figure 3 Model of a ternary NGF–TrkA-D5–p75^{NTR} complex. All components are rendered as solvent-accessible surface, with NGF in medium gray, TrkA-D5 in black, and p75^{NTR} in light gray. The C-terminus of TrkA-D5 was modeled to interact with hairpin loops L2 and L4 at the bottom of NGF; in this model, it also interacts with CRD4 of p75^{NTR}.

axis perpendicular to the membrane, with its termini and the cystine-knot motif at the top and the three hairpin loops L1, L2, and L4 facing the membrane (Figs. 1B, 2). In this crystal structure, D5 is correctly folded, and the A/B loop is tightly packed against NGF. While ligand binding does not induce any significant differences in D5, NGF undergoes an important change. The N-terminal residues of NGF, which are known to be critical for receptor binding and are flexible in all crystal structures of neurotrophins in their unbound state, become well-ordered in the NGF–TrkA-D5 complex. The NGF N terminus now forms a helical turn (Fig. 1), which packs tightly into a hydrophobic pocket on the surface of D5; interestingly, the unusual disulfide bridge found in D5 but not in other I-set members forms the bottom of this pocket. A comparison of the D5 structures of TrkA, TrkB, and TrkC reveals that the residues lining the walls of the pocket are not conserved; therefore, the interactions in this patch are likely to confer specificity between the ligands and their receptors [9]. A second binding patch involves residues from the central portion of the NGF dimer. Most of the residues involved in this part of the interface are conserved among both the neurotrophins and the receptors, and similar interactions were predicted for all neurotrophin–Trk complexes [11]. Finally, loops L2 and L4 at the bottom of the NGF dimer (Fig. 1B) share very little sequence conservation and are known from swapping experiments to be important for specificity [12]. Neither of these loops is in contact with D5, but the C terminus of D5 projects toward the groove between them. Therefore, in the native complex with intact TrkA, a portion of the linker segment connecting D5 to the transmembrane helix could interact with these loops, consistent with the observation that deletions or certain point mutations in this segment result in decreases in neurotrophin binding.

p75^{NTR}

p75^{NTR} enhances survival signals induced by NGF when in the presence of TrkA, while it activates apoptotic signals in a TrkA-negative background (reviewed in Barker [13]). p75^{NTR} lacks intrinsic catalytic activity and is a member of the tumor necrosis factor receptor (TNF-R) superfamily. Members of this superfamily contain between two and six cysteine-rich domains (CRDs) in their extracellular portion (reviewed in Locksley *et al.* [14]), which usually have six cysteine residues shown from structural studies to form a ladder of three disulfide bridges. These domains are fairly rigid building blocks, and stacking of multiple repeats produces the elongated, rod-like shape of the receptor.

p75^{NTR} has four cysteine-rich repeats. Crystal structures of complexes in this superfamily have shown that CRD2 and CRD3 are most intimately involved in the ligand interaction [15]. Similarly, while all four domains of p75^{NTR} are required for NGF binding, CRD2 appears to be most critical (see references in Wiesmann and de Vos [5]). In contrast to the Trks, the 60-residue-long linker region that connects the CRDs to the transmembrane helix is not required for binding. The binding site for p75^{NTR} on the neurotrophins has been mapped through mutational analyses onto two spatially separated regions. One patch at the top of NGF (see Fig. 1B) involves a number of positively charged residues in loop L3 and the C-terminal region. The second patch, at the bottom of NGF, is defined by positively charged residues from loops L1 and L4. The crystal structure of the NGF–TrkA-D5 complex shows

that neither of these patches is buried by TrkA-D5, allowing for the formation of ternary NGF–TrkA–$p75^{NTR}$ complexes at the cell surface. Based on these observations, a possible model for such a ternary complex is shown in Fig. 3 [5].

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CHAPTER 50

The Mechanism of VEGFR Activation Suggested by the Complex of VEGF–flt1-D2

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Introduction

Vascular endothelial growth factor (VEGF or VEGF-A), also known as vascular permeability factor, is a potent mitogen that is highly specific for endothelial cells. VEGF plays a central role in the development of the vascular system and the processes involved in angiogenesis and is also implicated in diseases that require the formation of new blood vessels such as diabetic retinopathy, age-related macular degeneration, psoriasis, rheumatoid arthritis, and tumorigenesis [1,2]. Additional members of the still expanding VEGF family currently include VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PlGF) [3]. These molecules share a sequence identity of approximately 30 to 50%, are generally active as glycosylated homodimers, and bind to one or more of the three VEGF receptors VEGFR-1 (also known as flt1), VEGFR-2 (KDR) and VEGFR-3 (flt4). The biological responses triggered by VEGF are modulated through a number of different isoforms generated through alternative splicing events, the most prominent forms being comprised of 121, 165, 189, and 206 amino acids, respectively. Cleavage of $VEGF_{165}$ with plasmin yields two fragments [4]. The longer fragment includes the N-terminal residues 1 to 110 and remains dimeric in solution. This receptor-binding domain is about as active as the short splice variant $VEGF_{121}$. The shorter, monomeric fragment is the heparin-binding domain, which is also present in all the longer VEGF isoforms.

Heparin-Binding Domain of VEGF

The enhanced biological activity of the longer VEGF isoforms has recently been attributed to the formation of ternary complexes with VEGFR-2 and neuropilin [5]. The neuropilins are receptors for the class 3 semaphorins and participate in the guidance of growing axons to their targets. Their coreceptor function for VEGF (and PlGF) is mediated through the heparin-binding domain of these isoforms [6], resulting in enhanced affinity of VEGF for VEGFR-2 [7]. Neuropilin-1 also binds directly to flt1, in an interaction that antagonizes binding of VEGF, suggesting a role for flt1 as a negative regulator of angiogenesis [7].

The three-dimensional structure of the 55-residue heparin-binding domain of the longer VEGF isoforms consists of a novel heparin-binding fold that can be divided into two smaller subdomains, each having a short, anti-parallel β-sheet and two disulfide bonds [8] (Fig. 1A). The C-terminal

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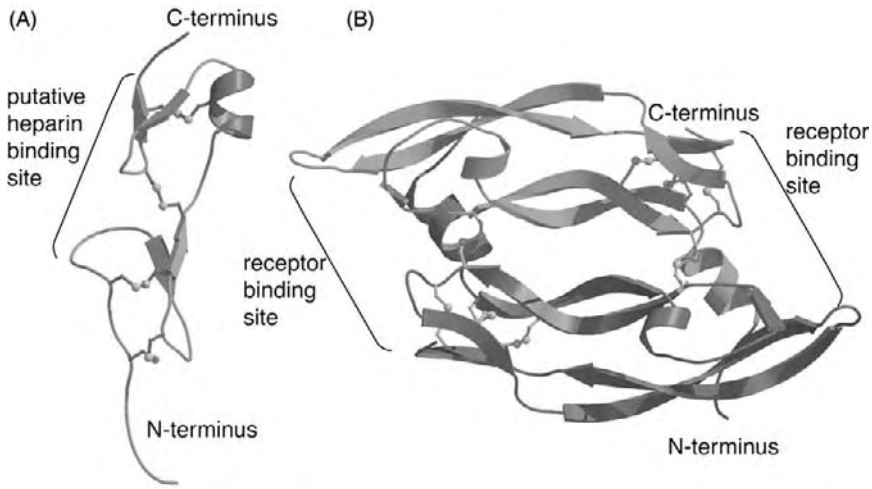


Figure 1 Three-dimensional structure of the domains of $VEGF_{165}$, with the backbone rendered as ribbons and disulfide bonds as a ball and stick. (A) NMR structure of heparin-binding domain [8]. (B) Crystal structure of the receptor-binding domain [9].

subdomain contains a short helical segment that packs against the β-sheet. One side of this subdomain together with an adjacent loop stemming from the N-terminal half carries the majority of positively charged residues, and the resulting positively charged surface is believed to be responsible for the heparin-binding functionality [8].

Receptor-Binding Domain of VEGF

Crystallographic analysis of the receptor-binding domain of VEGF [9] confirmed that VEGF is a member of the cystine-knot-containing family of growth-factors [10] and resembles platelet-derived growth factor (PDGF) in overall structure and quaternary arrangement. Each monomer has two pairs of anti-parallel, two-stranded β-sheets in its core with the N terminus and the characteristic cystine-knot motif at one end of the sheet (Fig. 1B). Two monomers are covalently linked by two disulfide bonds and arranged in an anti-parallel manner, with their central β-sheets lying side-by-side and the N-terminal region packing on top of the other monomer. The resulting VEGF homodimer has a flat shape and is about 65 Å long, 40 Å wide, and 10 Å thick in its central portion. One of the more prominent differences between VEGF and PDGF involves the N-terminal segment, which adopts a helical conformation in VEGF but is extended in PDGF. Interestingly, several residues important for receptor binding are located in this region. A recent crystal structure of the receptor-binding domain of PlGF is very similar to that of VEGF, supporting the notion that all members of the VEGF family have very similar overall structures [11].

VEGF Receptors

Given the similarities in overall architecture between VEGF and PDGF, it is not surprising that their receptors, too, share common features. Both the VEGF receptors and the PDGF receptor family members—PDGFR-α, PDGFR-β, colony-stimulating factor 1 (CSF-1) receptor, stem cell factor (SCF) receptor (or c-kit), and flt3)—have a number of immunoglobulin (Ig)-like repeats in their ectodomain, a single transmembrane helix, and a split tyrosine kinase domain in the cytoplasmic portion. Sequence alignments and functional studies suggest that the five Ig-like repeats of the PDGF receptors correspond to the N-terminal five of the seven Ig-like domains of VEGF receptors [12]. Domain swapping and deletion studies identified the three N-terminal repeats as the growth-factor-binding regions (for references to individual studies, see Robinson and Stringer [3]). The function of the first domain, which is not involved in the interaction with the ligand, may be to regulate binding indirectly, as its deletion from VEGFR-2 enhances the VEGF association rate. The ligand binding determinants are contained in domains 2 and 3, as tandem constructs of these domains of flt1 and of VEGFR-2 bind to VEGF with near wild-type affinity. The isolated second domain of flt1 (but not VEGFR-2) still binds with an affinity of about 1 n*M*. Its structure shows that this domain is a member of the I-set of the Ig superfamily, but that it contains a number of rare and characteristic features that are conserved in domain 2 of the other receptors of the PDGF and VEGF families, indicating that the structure and function of this domain are conserved [12,13]. The function of the fourth domain has been addressed through ligand-induced cross-linking studies on flt1 and by using dimerization-inhibitory antibodies to SCFR (see references in Robinson and Stringer [3]). For both receptors, a dimerization site was identified within this domain, suggesting that the two copies of domain 4 in the signaling complex may be in direct contact. The function of domains 5 to 7 of the VEGF receptors is less clear, but it has been suggested for VEGFR-2 that they may have a role in inhibiting signaling in the absence of ligand [14].

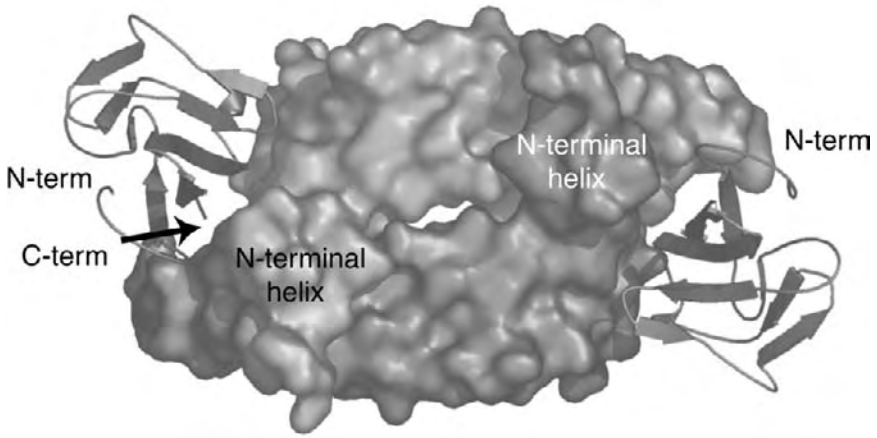


Figure 2 Crystal structure of the VEGF–flt1-D2 complex, viewed toward the membrane. VEGF is rendered as accessible surface, and D2 of flt1 as ribbon [12].

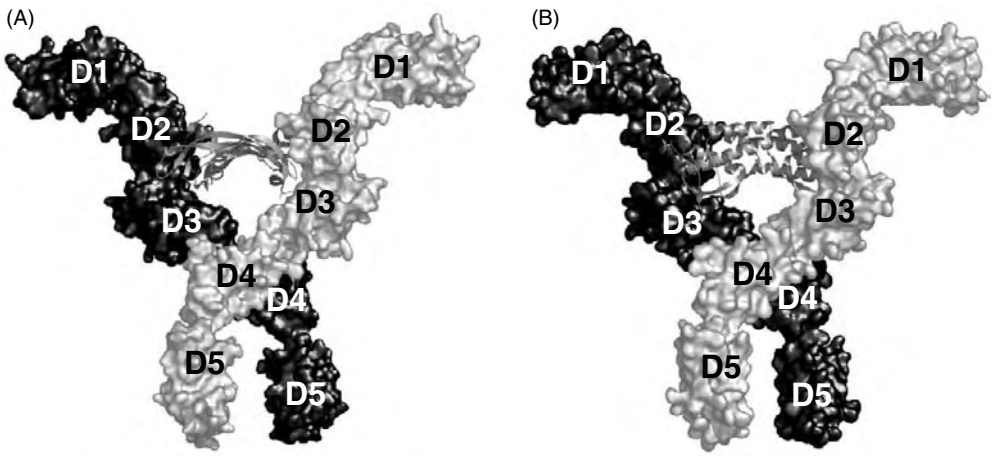


Figure 3 Models of signaling complexes, with receptors rendered as accessible surfaces and ligands as backbone ribbons. The membrane would be at the bottom of the figure. (A) Receptor complex of cystine-knot ligands VEGF, PlGF, or PDGF. (B) Receptor complex of four-helix-bundle ligands SCF, Flt3L, or M-CSF.

VEGF–flt1-D2 Complex

The crystal structure of the complex between the receptor-binding domain of VEGF and the second Ig-like domain (D2) of flt1 revealed the mode of ligand–receptor interaction, suggested how the third Ig-like domain would contribute to enhance binding between the growth factor and the intact receptor, and defined the overall geometry of the signaling complex. In the structure [12], two copies of flt1-D2 bind symmetrically at the diametrically opposed ends of the VEGF dimer (Fig. 2). Each copy is in contact with both VEGF monomers, forming a flat interface lacking any major knob-into-hole interactions. Approximately 70% of the buried surface is of a hydrophobic nature, with only a single direct polar interaction. From this structure, the identification of residues in contact with receptor made possible the generation of receptor-selective VEGF variants [15] that have proved useful in elucidating the specific biological roles of flt1 and VEGFR-2 [16]. Overall, the binding site on VEGF as seen in the crystal structure is in good agreement with alanine-scanning experiments mapping residues important for VEGFR-2 binding. However, a number of additional binding determinants that are located at the bottom (membrane-facing) side of VEGF are not in contact with flt1-D2. The C terminus of this domain projects toward a groove at the bottom of the VEGF, suggesting that in the intact receptor the third domain would be positioned in contact with these residues. Based on these data, a model was proposed for the overall geometry of the complex between VEGF and the first four receptor domains (Fig. 3A) [12]. Given the structural homology between VEGF and PDGF, on the one hand, and the similarities in domain structure between their receptors, on the other, all the growth factors in these families are expected to assemble their receptors into signaling complexes of a similar architecture. It is striking, however, that some ligands for members of the PDGF receptor family are structurally unrelated to VEGF and PDGF. Flt3 ligand (Flt3L), SCF, and CSF-1 are helical proteins resembling the class 1 four-helix-bundle cytokines, a family of usually monomeric ligands that interact with members of a distinct

receptor family, namely the hematopoietic receptors [17]. The crystal structures of Flt3L [18], SCF [19,20], and CSF-1 [21] show that these molecules form dimers with overall dimensions that are very similar to those of VEGF and PDGF. Given these similarities and taking into account mutagenesis data mapping the receptor-binding sites, it appears that these three four-helix-bundle ligands interact with their receptor tyrosine kinases in a manner that results in an assembly of signaling complexes of architecture and dimensions similar to those of VEGF (Fig. 3B) [18,19]. Thus, the geometric requirements for productive signaling through the receptors in this family, first deduced for the VEGF–flt1 complex, now appear to be conserved even for a class of ligands with a completely unrelated fold.

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CHAPTER 51

Receptor–Ligand Recognition in the TGFβ Family as Suggested by the Crystal Structures of BMP-2–BR-IA$_{ec}$ and TGFβ3–TR-II$_{ec}$

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Introduction

The transforming growth factor β (TGFβ) superfamily is a large group of soluble, dimeric factors that initiate and control proliferation and differentiation of many cell types in animals, thus playing important roles in embryonal development and adult tissue homeostasis. Malfunctioning of this tightly controlled signaling system leads to developmental disorders, and severe defects in organ function and is the cause of several diseases, including various types of cancer [1,2].

Signaling by these factors requires binding of the ligand to two homologous but functionally distinct types of transmembrane serine/threonine receptor kinases, designated type I and type II. Upon ligand-complex formation, the constitutively active type II receptor kinases activate the type I receptor kinases through phosphorylation in a conserved juxtamembrane region (GS-box). Activated type I receptors are then able to phosphorylate targets further downstream of the signaling pathways [1,3,4]. Despite the large number of ligands in the TGFβ superfamily, the number of receptors is comparatively small. Also, in contrast to other highly specific receptor–ligand systems, the members of the TGFβ superfamily display some promiscuity, in that several ligands are able to recognize different receptors and most of the receptors can bind different ligands. With a focus on BMP-2 and TGFβ, this chapter summarizes current knowledge about the initiating events in signal transduction within the TGFβ superfamily based on crystal and solution structures of ligands, receptor ectodomains, and receptor–ligand complexes.

Ligand and Receptor Structures

Currently, more than 30 different ligands within the TGFβ superfamily have been described. They can be categorized into the subfamilies of the name-giving TGFβs; growth and differentiation factors (GDFs); activins/inhibins; bone morphogenetic proteins (BMPs), which is the largest of the subgroups; and a few others [5]. A comparison of the three-dimensional structures of TGFβ1 [6], TGFβ2 [7,8],

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TGFβ3 [9], BMP-7 [10], GDNF [11], and BMP-2 [12] shows that the ligands share a common fold, which is characterized by an extended nine-stranded β-sheet formation and one α-helix per monomer. Significant differences are only observed in loops and at the N termini. The overall fold of a monomer has been described as a curled hand, in which the extended β-sheets represent the fingers [7] and the α-helix the heel or wrist [13] region of the hand. The monomer fold is stabilized by a network of disulfide bridges forming the so-called cystine knot, a motif that is also found in a number of other growth factors [14,15]. The functionally competent molecule is formed by a covalent connection of two monomers through a disulfide bridge, forming a dimer (usually, but not necessarily, a homodimer) in anti-parallel, face-to-face orientation. This relative orientation of the subunits distinguishes the TGFβ superfamily members from those of the vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) families, which have similar monomer structures but dimerize in different ways [16].

The receptor chains are composed of a small extracellular (represented by the subscript "ec") ligand binding domain, typically composed of around 120 amino acid residues, one short transmembrane fragment, and a cytoplasmic part, which differs in size between type I and type II and contains the catalytic kinase domain. Recently, three crystal structures of the ligand binding domains became available, one in the unbound form (ActR-II_{ec} [17]) and two in complex with their ligands (BMPR-IA_{ec} [18] and TGFβR-II_{ec} [19]). Despite the low sequence similarity of less than 20% between type I and type II receptor ectodomains, the overall structure of both groups is very similar (Fig. 1). They fold into single, compact domains with a rigid core formed by a central anti-parallel β-sheet whose architecture is maintained by five partially conserved disulfide bridges. Structural variations occur mainly at the edges of the sheet and in loop regions. This fold, which has previously been described for a number of snake venom toxins, can be compared to an open left hand and was named the *three-finger toxin fold* [20]; however, no functional similarities to the receptors have been reported.

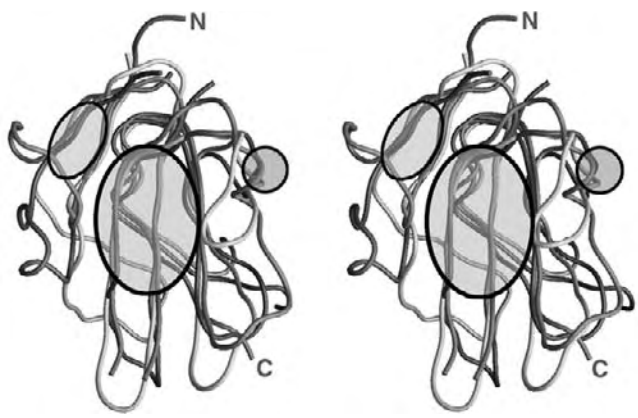


Figure 1 Superposition (stereo) of the receptor chains mActR-II_{ec} (blue), BR-IA_{ec} (red), and TGFβR-II_{ec} (green). The view is looking down onto the central part of the common ligand binding epitopes of BR-IA_{ec} and ActR-II_{ec} (the palm of the receptor hand, shaded magenta). The region shaded in red marks marks the position of the highly conserved hydrophobic residue of type I receptors (Phe85 in BR-IA) that fits into a conserved hydrophobic pocket of the ligand. The ligand binding area of TGFβR-II_{ec} is shaded in green and is clearly distinct from the ligand binding area in BR-IA_{ec}. The orientation is such that for BR-IA_{ec} the membrane proximal side is at the bottom. The N and C termini are indicated for BR-IA_{ec}.

Receptor–Ligand Complexes

Although BMPs and TGFβs are very similar in structure, there are significant differences in the molecular mechanism of recognition and signal activation. In TGFβ signaling, complex formation is initiated by high-affinity interactions between the ligand and the type II receptor TβR-II. This primary complex subsequently recruits the low-affinity type I receptor (TβR-I), which is not able to bind to the ligands in the absence of TβR-II [21,22]. The sequence of events is likely to be reversed in the case of BMPs, as exemplified by studies with BMP-2: BMP-2 has high affinity for the type I receptors BR-IA and BR-IB (and possibly ActR-I), but lower affinity for BR-II and ActR-II. Affinity for type II receptors is slightly increased in the presence of type I receptor; however, even in the absence of the latter, BMP-2 is able to interact with the type II chains [13,23,24]. In addition, a population of BMP-receptors exists prior to ligand binding in preformed complexes composed of BR-II and BR-IA or BR-IB [25]. Binding of BMP-2 to either preformed receptor complexes or to the high-affinity BR-I receptors results in induction of different signaling pathways [26].

BMP-2–BR-IA_{ec} Complex

Comprehensive mutational studies on BMP-2 mapped two symmetric pairs of spatially separated and functionally independent epitopes on the surface of the dimer for interactions with the two receptor classes [13]. Based on their locations on the BMP-2 hand, the type I receptor binding site is referred to as the *wrist epitope* and the type II receptor site the *knuckle epitope*. This epitope assignment is fully compatible with the crystal structure of the trimeric complex between BMP-2 and a pair of its high-affinity receptor chains BR-IA_{ec} [18]. In the complex, the twofold rotational symmetry of the ligand is retained, with the symmetry axis normal to the plane of the membrane (Fig. 2a). Each of the two receptor molecules, the structures of which can be compared to open left hands, binds with its concave palm side to the wrist of one of the two BMP-2 monomer hands. An extended groove on the receptor surface harbors ligand residues of the pre-helix loop and the α-helix (residues Phe49–Val63 in BMP-2). The binding site on BMP-2 extends to the inner side of the fingers of the adjacent BMP-2 subunit, and thus covers the complete *finger-helix-groove* [12]. Central to the contacts in this second part of the binding epitope is a hydrophobic pocket on the ligand surface, which is found in all currently

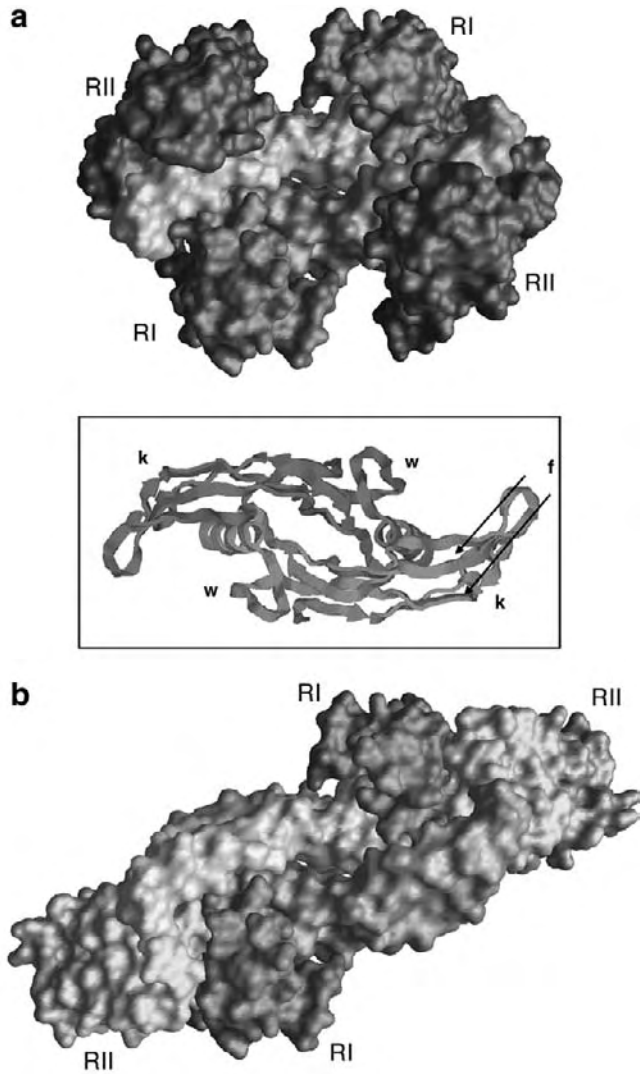


Figure 2 (a) Model of the complete pentameric complex for the BMP-2 subfamily with BMP-2 (L, orange), BR-IA_{ec} (RI, red), and ActR-II_{ec} (RII, blue). The model is based on the crystal structure of the complex between the ligand and the type I receptors. ActR-II_{ec} was fitted onto the knuckle epitope of BMP-2. The view is approximately along the two-fold symmetry axis, (i.e., onto the plane of the membrane). The receptor chains are not in contact with each other. (b) Model of the pentameric complex for the TGFβ subfamily according to Hart *et al.* [19], based on the crystal structure of TGFβ3 and TGFR-II_{ec}, with the ligand (L) and the type I receptor (RI) colored orange and red, respectively, as in part (a), and the type II receptor (RII) in green. Note that in this model, the two receptor chains are in close contact with each other. The orientation is the same as in part (a). The insert shows a ribbon diagram of the ligand depicting the fingers (f, shown in one monomer only), knuckle (k), and the wrist (w) epitopes.

known ligand structures of the superfamily [18]. The counterpart to this pocket is a large hydrophobic residue (Phe85 in BR-IA_{ec}) that is functionally conserved within the class of type I receptors. This residue is located in helix α1, a secondary structure element not present in the type II receptors ActR-II and TGFβR-II. Based on the high degree of conservation of this knob-into-hole motif, it was deduced that this is a key interaction between the ligand and the type I receptors. Two observations may explain why little cooperation is observed between binding of the two receptor types: (1) the knuckle epitope on BMP-2, which so far is defined only by functional analyses and which provides the putative type II receptor binding site (experimentally shown for BR-II_{ec} and ActR-II_{ec}), does not overlap with the binding epitope for type I receptors [13]; and (2) the structure of BMP-2 in the complex differs only by a slight opening of the finger-helix-cavity from that of the free ligand [12,18].

Alanine scanning mutagenesis of ActR-II_{ec} [27] identified a cluster of hydrophobic residues that are essential for binding to activin A. This cluster colocalizes with the central part of the palm epitope on BR-IA_{ec} (i.e., the part of the molecule that forms the contacts to the ligand), suggesting a mode of binding of this type II receptor to its ligands that is very similar to that described for the BMP-2–BR-IA_{ec} complex.

Complex Formation with TGFβ Is Different than for BMP-2

Our understanding of the receptor–ligand complex, with well-separated type I and type II binding epitopes and assumed to be valid for the entire TGFβ superfamily, had to be revised with the publication of the crystal structure of TGFβ3 in complex with TGFβR-II_{ec} [19]. Surprisingly, the receptor does not bind to a region corresponding to the knuckle epitope on BMP-2. Instead, it interacts with a hydrophobic cleft in the fingertips of the ligand and is in close proximity to the expected type I receptor binding site. Although TGFβR-II_{ec} shares the overall fold topology of ActR-II_{ec} and BR-IA_{ec} (Fig. 1), a few structural modifications in loop regions result in a completely different binding mode to the ligand. Most prominent among these modifications is an insertion of seven residues in loop 4 of TGFβR-II which packs against the central β-sheet in a way that the residues corresponding to those crucial to binding the ligand in ActR-II_{ec} as well as in BR-IA_{ec} are no longer accessible. The ligand binding residues in TGFβR-II_{ec} are located in β-strands 1 and 4, whereas the equivalent amino acids in BR-IA_{ec} are on the back side of the receptor and do not contact the ligand. Furthermore, the orientation of the receptor relative to the cell membrane differs significantly; in BR-IA_{ec} the plane of the central β-sheet is almost perpendicular to the membrane, but in TGFβR-II_{ec} it is almost parallel to the plane of the membrane.

Based on these findings a model for the pentameric signaling complex of TGFβs was suggested that combines the evidence from the two known crystal structures of ligand–receptor complexes [19]. In this model, the type I receptor binds to TGFβ in a way described by the BMP-2–BR-IA_{ec} complex, as this is accepted to be common within the superfamily, and the type II receptor binds as found in the TGFβ3–TGFβR-II_{ec} complex (Fig. 2b). As a consequence, both receptors are in direct contact with each other, which may account for the cooperativity found for TGFβ receptor complex formation.

In conclusion, current structural knowledge shows that, despite high structural similarities among ligands as well as receptors within the TGFβ superfamily, substantial differences

exist among subfamilies upon formation of the receptor–ligand complexes. Due to the relatively low stability of the pentameric arrangement of ectodomains and ligands, it will remain challenging to obtain three-dimensional structures of the complete complexes.

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CHAPTER 52

Insulin Receptor Complex and Signaling by Insulin

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Introduction

Insulin is an important regulatory hormone that mediates energy uptake by the body. It is the major food storage hormone and is secreted in response to rising blood sugar levels following a meal. It regulates energy uptake by inhibiting glucose production by the liver and by increasing sugar uptake into muscle and fat, directing this sugar into the storage forms of glycogen in liver and muscle and fat in adipose cells. Insulin deficiency or resistance to its actions leads to the profound metabolic dysfunctions of Type 1 or Type 2 diabetes, respectively, which are major diseases of the Western world. There is, therefore, intense interest in understanding the mechanisms of action for insulin. Insulin exerts a wide variety of effects on cells, including both metabolic and mitogenic actions that are triggered by binding to its cell surface receptor. This review briefly explores the nature of this receptor, its interaction with insulin, and its signaling to a major metabolic target, glucose transport.

Insulin Receptor Domain Structure

The insulin receptor (IR) is a glycosylated, disulfide-linked homodimer, with each monomer being made up of an α-chain that is entirely extracellular and a β-chain that spans the cell membrane once. The α-chain contains the insulin-binding determinants of the receptor, while the intracellular portion of the β-chain includes a protein–tyrosine kinase domain and domains involved in binding signal transduction proteins. The αβ monomer of the IR is encoded by a gene with 22 exons; alternative splicing of the IR pre-mRNA leads to the tissue-specific expression of two isoforms differing by the presence or absence of a 12-residue segment (exon 11) near the C terminus of the α-chain. The receptor is synthesized as a single chain with a 27-residue signal sequence and is glycosylated, oxidized to the disulfide form, and proteolytically processed to the two-chain form during transport to the cell surface. The mature α-chain of the human IR has 731 amino acid residues, while the β-chain has 620. Two receptors with close sequence and structural homology to the IR are the receptor for insulin-like growth factor 1 (IGF-1R) and the orphan receptor, the insulin-receptor-related receptor (IRR) (see references [1] to [3] for reviews).

Analysis of the sequence of the IR has shown that the molecule can be divided into a number of modules or domains [4]. The ectodomain has two large homologous domains of approximately 150 residues, L1 and L2, separated by a 150-residue cysteine-rich domain; in this respect, the IR is similar to the epidermal growth factor receptor (EGFR). C-terminal to these, the IR has three fibronectin type III domains (FnIII-1, -2, and -3) of approximately 100 to 130 residues each. One of these, FnIII-2, has an insertion of 125 residues that contains the α-β cleavage site, resulting in the N-terminal region of FnIII-2 and part of the insert domain (ID) being found at the C terminus of the α-chain, and the remaining portions of these two domains being located at the N terminus of the β-chain. The domain structure of the IR ectodomain is shown schematically in Fig. 1. The discussion in this section focuses largely on the structure of the ectodomain and its interaction with the ligand, insulin. The structure of the kinase domain is discussed in Chapter 53.

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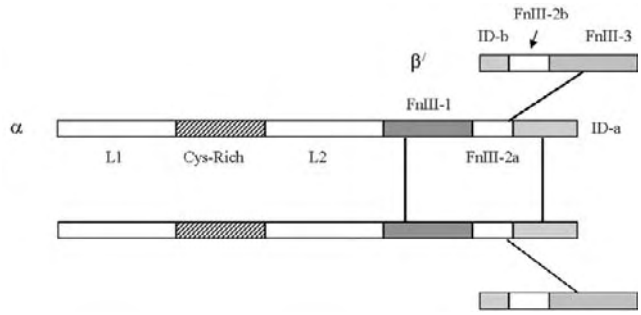


Figure 1 Domain structure of the IR. Heavy lines, chain-linking disulfide bonds; β′ refers to that portion of the β-chain that is extracellular.

The IR ectodomain has been shown to have a single disulfide bond linking the α- and β-chains, joining residues 647 and 872 (exon 11 plus form), and at least two disulfides symmetrically linking the α-chains of the dimer, one at residue 524 and the second at one of residues 682, 683, and 685. The human IR has 18 predicted sites for N-linked glycosylation: 14 on the α-chain and 4 on the β-chain. Of the 18 predicted N-linked sites, 16 are occupied by carbohydrate and one is unoccupied; O-linked glycans have been shown to occur on only 4 threonine and 2 serine residues in the 22 residues at the N terminus of the β-chain.

Although no crystal structure of the IR is yet available, a structure for a fragment of the homologous receptor, IGF-1R, has been published [5]. The structure of this fragment (Fig. 2), an expressed recombinant protein encompassing the L1–Cys-rich–L2 domains of the IGF-1R, should give a strong indication of the structure of the corresponding fragment of the IR.

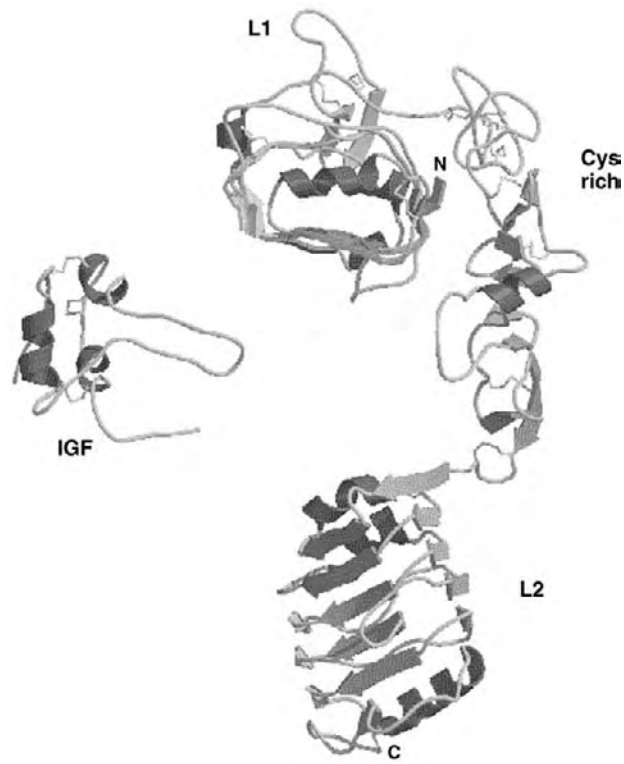


Figure 2 Structure of the fragment, L1–Cys-rich–L2, of the IGF-1R. Helices are depicted as broad ribbons and β-strands as broad arrows. Also shown, to scale, is the ligand IGF-1.

Binding Determinants of the IR

Insulin is thought to have two distinct receptor binding surfaces, with one site encompassing at least the residues G1, E4, Q5, and N21 of the insulin A-chain and the other site being made up of residues V12, Y16, F24, F25, and Y26 of the B-chain; other residues apart from those listed above are almost certainly involved in this interaction [6]. Binding of insulin to the native, dimeric IR is characterized by curvilinear Scatchard plots and the phenomenon of negative cooperativity, both interpreted as showing the presence of two states of the receptor, one of high-affinity insulin binding and the other of low affinity. Half receptors, i.e., αβ-monomers, formed by mild reduction of the dimeric $(\alpha\beta)_2$ receptor, do not show these phenomena and bind insulin with only low affinity [7], as does the expressed recombinant IR ectodomain. High-affinity binding is restored when the IR is truncated below the transmembrane domain or when a dimerization moiety such as the IgG-γ domain or a leucine-zipper segment is fused to the C terminus of the expressed ectodomain [2]. Clearly, not only is the dimeric state of the IR essential for high-affinity binding, but also the relative disposition of the two monomers in the dimer is critical; it seems that the C termini of the two α-chains must be in close proximity for effective high-affinity binding to insulin.

The isolated α-chain of the IR binds insulin, although with an affinity lower than that of the wild-type receptor, and thus appears to have all the insulin-binding determinants of the receptor. By alanine-scanning mutagenesis, studies of chimeric receptors, and direct cross-linking of insulin to the IR, binding determinants on the receptor have been located in the L1 domain, in the Cys-rich region, and near the N terminus of the L2 domain. Additionally, important determinants are also found close to the C terminus of the α-chain [2]. For both the IR and the IGF-1R, the L1–Cys-rich–L2 fragment does not bind the cognate ligand, despite the presence of many of the binding determinants and despite the fact that the horseshoe-like structure reveals a cavity of sufficient dimensions to partly encircle the ligand. Thus, the X-ray structure of this fragment does not yield details of the complete ligand-binding site. However, ligand binding can be restored by adding to the C terminus of this fragment a 16-residue peptide that includes the binding determinants identified at the C terminus of the α-chain. The peptide can be attached directly to the L1–Cys-rich–L2 fragment or by using linkers of varying length. This suggests that the mode of attachment is perhaps not critical, a view supported by the observation that the addition of the free peptide to the fragment also restores ligand binding [8]. The 16-residue peptide itself probably does not directly bind insulin, as

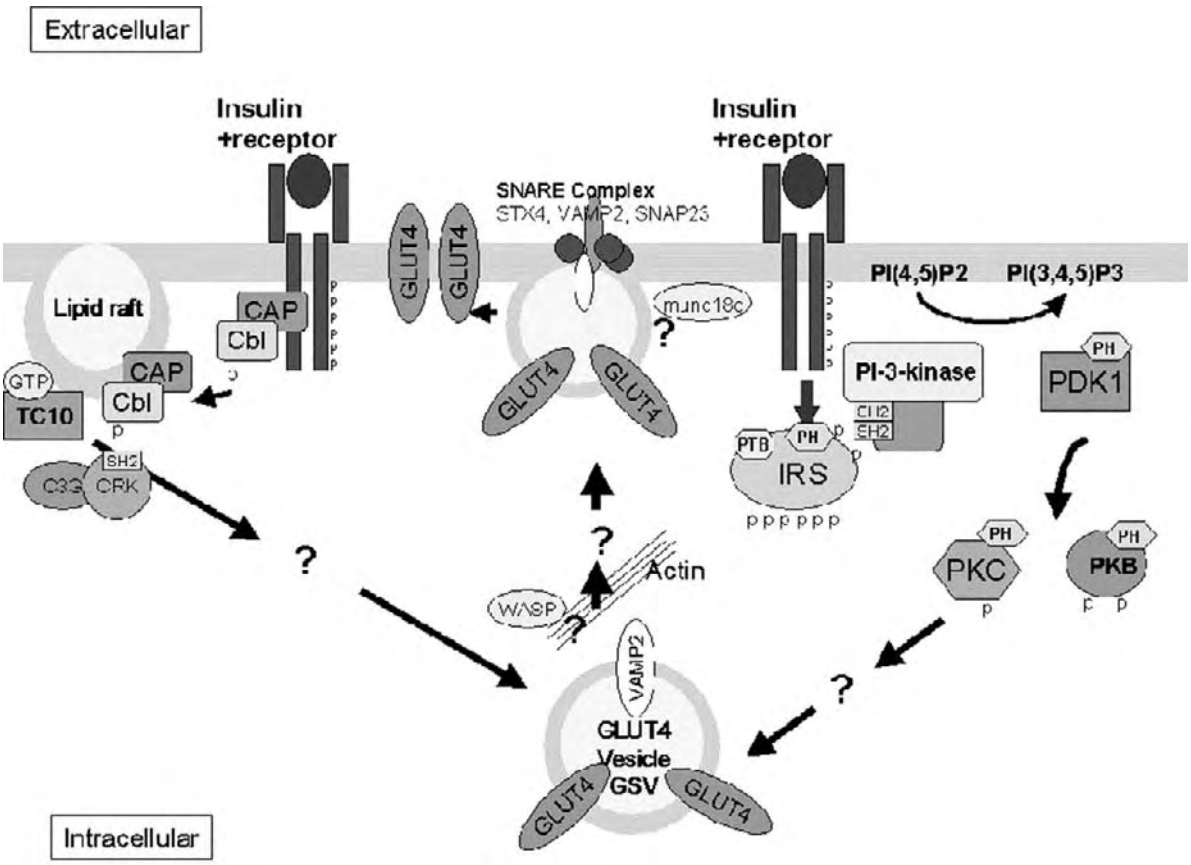


Figure 3 Putative signaling pathways involved in insulin-stimulated GLUT4 trafficking.

C-terminal fragments of the IR comprising two or three FnIII domains and including this 16-residue segment of the α-chain do not bind insulin [9].

Although each α-chain of the IR has all the determinants necessary for binding of insulin, high-affinity binding requires two α-chains held in appropriate juxtaposition by at least two disulfide bonds between these chains and by a single disulfide bond linking each α-chain to a β-chain that is itself anchored to the cell membrane. This suggests that two αβ monomers are involved in binding a single insulin molecule, thus providing a cross-link between the chains in addition to the disulfide bonds. A significant observation is that the IR undergoes an obvious conformation change (Stokes radius from 9.1 to 7.5 nm) on binding insulin [7]. The nature of this change is unclear, but it may in some way bring together the two kinase domains of the homodimer so that the activation loop of one is accessible to the active site of the other. Such a movement may be equivalent to that observed for other receptor molecules, such as the EGFR, that undergo dimerization following ligand binding, resulting in activation of the receptor tyrosine kinase. Full details of the binding of insulin to its receptor and the subsequent conformation change will probably not be known until crystal structures of the receptor with and without bound ligand are available. Activation of the IR kinase is accompanied by autophosphorylation at up to six tyrosines which both further activates the kinase and creates binding sites for signaling proteins, which in turn become phosphorylated and bind their downstream targets.

Insulin Signaling to Glucose Transport

A major, if not *the* major, endpoint of insulin signaling is the stimulation of glucose uptake (transport) in muscle and fat, the focus of the remainder of this chapter. Currently, at least two distinct pathways (Fig. 3) have been implicated in this process. Both pathways may be required for translocation of the insulin-regulated glucose transporter, GLUT4, a 12-transmembrane-spanning protein, from a vesicular storage compartment (GSV) within the cell, to the plasma membrane. Its insertion makes it competent to transport glucose into the cell (for reviews, see references [10] to [12]). The most extensively characterized of these pathways begins with the IRS family of proteins (see Chapter 71). Tyrosine phosphorylation of these proteins by the insulin receptor provides recognition sites for Src homology domain 2 (SH2)-containing proteins. Of significance for several pathways in insulin action, including those leading to glucose transport and glycogen synthesis, is the binding and activation of the type 1A phosphatidylinositol 3 (PI3) kinase (for reviews, see references [10], [12], and [13]). This enzyme phosphorylates inositol phospholipids in the plasma membrane, increasing PI3,4,5-trisphosphate (PI345P3)

levels and enabling the recruitment and activation of a serine/threonine kinase, phosphoinositide-dependent protein kinase (PDK1), via association of its pleckstrin homology (PH) domain with this phospholipid. PDK1 can then phosphorylate and activate two families of proteins implicated in stimulating glucose transport, the atypical protein kinases C ζ/λ, and protein kinase B (PKB) isoforms (for reviews, see references [12] and [13]). Unfortunately, downstream targets of these kinases relevant for stimulation of glucose transport have remained elusive despite intensive investigation.

Another putative pathway implicated in insulin stimulation of glucose transport is PI3-kinase independent and involves tyrosine phosphorylation of the protooncogene Cbl by the insulin receptor tyrosine kinase, possibly via association with the Cbl-associated protein (CAP) to the receptor within caveolin-rich lipid rafts (for reviews, see references [10] and [11]; Fig. 3). This enables the recruitment of the adaptor protein CrkII and its associated guanine exchange factor, C3G, resulting in activation of the Rho family GTPase, TC10. How this pathway intersects the PI3-kinase-dependent pathway to stimulate GLUT4 translocation is unclear. At least one of the pathways must act on the vesicular compartments storing GLUT4 to move them to the cell surface.

Recent data suggest involvement of cytoskeleton proteins such as vimentin, α-tubulin, dynein, and/or cortical actin in moving GLUT4 to the cell surface [14–16]. Indeed, one recent study provided a possible link between the TC10 pathway and mobilization of cortical F-actin, demonstrating in adipocytes that insulin causes cortical localization of the regulatory Wiscott–Aldrich syndrome protein (WASP) and actin-related protein 3 (Arp3), as well as actin polymerization [15]. Thus, it seems likely that the cytoskeleton is involved in trafficking GLUT4, although the detail remains to be determined.

Following translocation of GLUT4 vesicles to regions below the cell surface, the final stages of trafficking and fusion of the GLUT4 vesicles have been shown to involve soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (for reviews, see references [11] and [16]). These were first described as proteins mediating the fusion of neurotransmitter vesicles at synaptic terminals, and much of the molecular detail of SNARE protein interactions has been elucidated for this system [18]. In the case of plasma membrane trafficking of GLUT4, the functional **v**esicle-**a**ssociated **m**embrane **p**rotein (VAMP; also known as synaptobrevin) isoform is VAMP2. This protein binds two plasma membrane SNARE proteins, syntaxin 4 (STX4) and SNAP23, to form a so-called SNARE complex involving interaction between coiled-coil regions of the three proteins. Although the formation of this complex is possibly sufficient to enable fusion of the GLUT4 vesicle with the plasma membrane, thus placing GLUT4 proteins on the cell surface to transport glucose into the cell, effective fusion also requires N-ethylmaleimide-sensitive factor (NSF) and soluble NSF attachment protein (α-SNAP)-driven hydrolysis of adenosine triphosphate (ATP). The precise role of NSF and α-SNAP in fusion, however, is unclear. Additional accessory proteins have been demonstrated to regulate this fusion process, although the roles of these accessory factors are likewise unclear (for reviews, see references [11] and [16]). Munc18c is a STX4-binding protein that may have a role as a molecular chaperone for STX4 and in fusion. Other proteins implicated in regulation of fusion events include synip (STX4-interacting protein), synaptotagmin, VAP-33, and Rab4. How these proteins interact with the fusion proteins to regulate this important process remains to be established. Further regulation is provided by the endosomal trafficking of the transporters via clathrin-coated pits for recycling via constitutive or insulin-regulated compartments (for reviews, see references [12] and [17]). Although there is general agreement that the majority of GLUT4 is segregated into a unique insulin-sensitive endosomal compartment (GSV) in the basal state, the nature of this compartment and its regulation remain unclear despite the identification of targeting motifs within the C- and N-terminal regions of GLUT4. These areas offer fruitful avenues for investigation over the coming years.

Acknowledgment

The authors regret that due to space restrictions there was not space to cite the significant contributions of many investigators to the field; we have instead cited representative reviews that cover the respective areas in greater detail. The authors thank Dr. Tom Garrett for permission to use Fig. 2.

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CHAPTER 53

Structure and Mechanism of the Insulin Receptor Tyrosine Kinase

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Introduction

The hormone insulin activates signaling pathways that regulate cellular metabolism and growth [1]. The actions of this essential hormone are mediated by the insulin receptor [2,3], a member of a large family of transmembrane receptors known as receptor tyrosine kinases (RTKs), which contain intrinsic tyrosine kinase activity in the cytoplasmic domain. These receptors catalyze the transfer of the γ-phosphate of adenosine triphosphate (ATP) to tyrosine residues in protein substrates. The RTK family also includes, among others, the receptors for insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF). There is also a large family of non-receptor tyrosine kinases that includes Src, Abl, Jaks, Syk/Zap70, and Fak. Receptor and nonreceptor tyrosine kinases are critical components of signaling pathways that control cellular proliferation, differentiation, migration, and metabolism [4].

In contrast to most other members of the RTK family, which are monomeric and activated by ligand-induced oligomerization, the insulin receptor is an $\alpha_2\beta_2$ hetero-tetramer (Fig. 1), with disulfide linkages between the two extracellular α-chains and between the α-chains and the transmembrane β-chains. Insulin binding to the α-chains induces a structural rearrangement within the receptor, resulting in autophosphorylation of specific tyrosines in the β-chain: at least one in the juxtamembrane region (Tyr972), three in the tyrosine kinase domain (Tyr1158/1162/1163), and two in the C-terminal tail (Tyr1328/1334) (Fig. 1). The phosphorylated tyrosines either stimulate catalytic activity (pTyr1158/1162/1163) [5] or serve as recruitment sites for downstream signaling proteins (pTyr972) [6]. Both phosphotyrosine functions—stimulation of catalytic activity and recruitment—are critical to the activation process. Following insulin stimulation, two distinct downstream pathways are activated to facilitate fusion of Glut4-containing vesicles with the plasma membrane for glucose uptake [7].

Structural/Mechanistic Studies

Overview

Structural studies of the insulin receptor have been limited largely to the cytoplasmic portion of the β-subunit. Within the cytoplasmic portion are 36 residues in the juxtamembrane region (membrane-proximal), 295 residues in the tyrosine kinase domain, and 72 residues in the C-terminal tail (Fig. 1). Crystal structures of the insulin receptor kinase domain (IRK) have been determined in different states of phosphorylation [8,9] and for several mutants [10] (S. R. Hubbard, unpublished data). IRK shares a similar overall architecture with protein serine/threonine kinases [11], with an N-terminal lobe composed of a five-stranded antiparallel β-sheet and one α-helix, and a larger C-terminal lobe that is mainly α-helical (Fig. 2). ATP binds in the cleft between the two lobes, and the tyrosine-containing segment of a protein substrate interacts with residues in the C-terminal lobe. Several residues are highly conserved in all functional tyrosine kinases, including several glycines in the nucleotide binding loop; a lysine in β-strand 3 (β3); a glutamic acid

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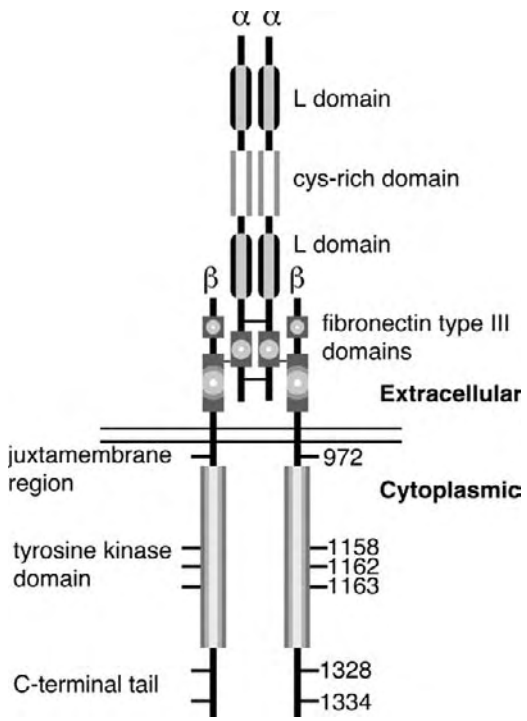


Figure 1 Overall architecture of the insulin receptor. The α-chains are extracellular and the β-chains pass through the plasma membrane. The major sites of tyrosine autophosphorylation are numbered according to Ebina *et al.* [2]. There are two fibronectin type III domains in the extracellular portion of the receptor. The N-terminal portion of the first domain is on the α-chain, and the C-terminal portion of this domain resides in the beginning of the β-chain, which is followed by a second, intact domain. Solid lines between the α-chains and between the α- and β-chains represent disulfide linkages.



Figure 2 Ribbon diagram of the tyrosine kinase domain of the insulin receptor [9]. The α-helices are lettered, and the β-strands are numbered. The ATP analog (AMP-PNP) and the side chain of the substrate tyrosine are shown in ball-and-stick representation. The N terminus is denoted by N; the C terminus is not visible but follows shortly after αJ.

in α-helix C (αC); an aspartic acid, arginine, and asparagine in the catalytic loop; and, in the activation loop, a DFG motif in the beginning and a proline at the end (Figs. 2 and 4).

Although no crystal structure has been reported for the extracellular region of the insulin receptor, a crystal structure of the first three domains of the highly related IGF-1 receptor has been published [12]. This structure shows the probable site of interaction between the ligand (IGF-1 or insulin) and the L domains and cysteine-rich domain of the receptor. Recently, a low-resolution image reconstruction of the full-length insulin receptor with bound insulin has been determined using electron microscopy, providing a putative model for the spatial organization of the various domains in the intact receptor [13].

Receptor Activation Mechanism

The binding of insulin to its receptor induces a conformational change in the receptor, measured (among other techniques) as a reduction in the Stokes radius of the insulin-occupied versus -unoccupied receptor [14]. The physical mechanism by which binding of insulin to the extracellular portion of the receptor is transduced to the cytoplasmic portion is not well understood. From the image reconstruction of the insulin-occupied receptor, a model for this mechanism has been proposed in which insulin binding produces a movement of the transmembrane helices toward one another, facilitating autophosphorylation between the kinase domains [15].

Once the insulin-triggered conformational change has been transmitted to the cytoplasmic domains, most biochemical data are consistent with tyrosine autophosphorylation proceeding via a *trans* mechanism, in which the tyrosine kinase domain of one β-chain phosphorylates tyrosines on the other β-chain within the same heterotetramer [16]. Simple modeling studies based on IRK crystal structures [8,9] indicate that the activation loop is too short to be autophosphorylated in *cis*, and solution studies support a *trans* mechanism for the activation loop [17]. Autophosphorylation of Tyr972 in the juxtamembrane region is also likely to occur in *trans*, for steric reasons, whereas autophosphorylation of the C-terminal tyrosine sites could potentially occur in *cis* after *trans*-autophosphorylation of the activation loop.

Activation Loop Autoinhibition

A key mechanism by which the insulin receptor and other tyrosine and serine/threonine kinases regulate catalytic activity is through positioning of the activation loop [18]. The activation loop of the insulin receptor begins with a protein kinase-conserved ^{1150}DFG motif and ends with tyrosine-kinase-conserved Pro1172. Between these conserved residues are three sites of tyrosine autophosphorylation: Tyr1158, Tyr1162, and Tyr1163. The activation loop in the crystal structure of the unphosphorylated, low-activity form of

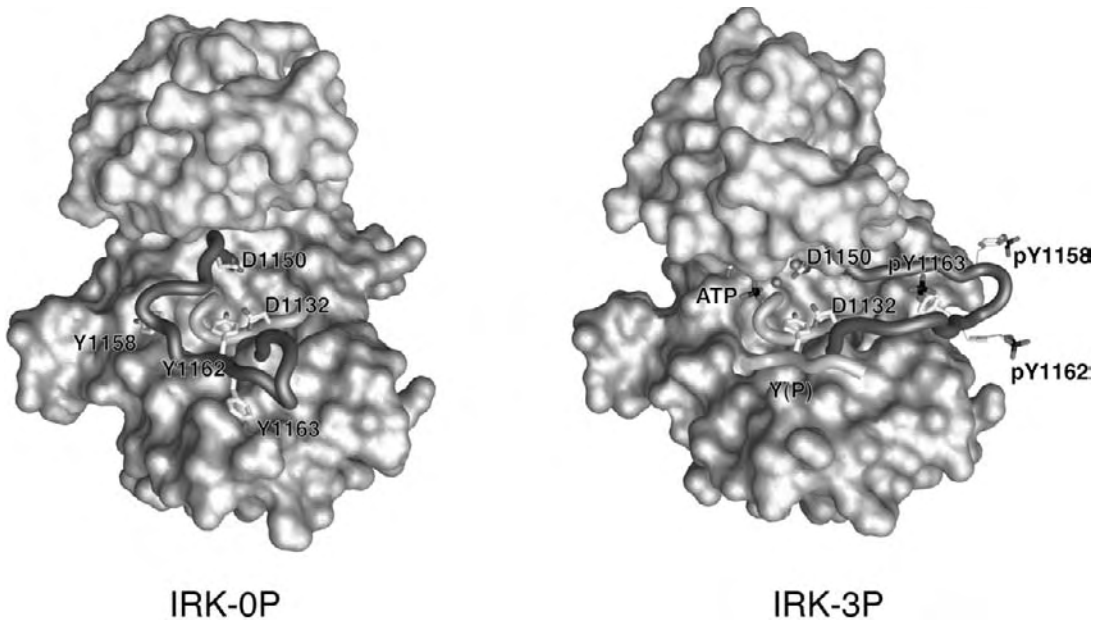


Figure 3 Comparison of the activation loop conformations in unphosphorylated IRK (IRK-0P) [8] and tris-phosphorylated IRK (IRK-3P) [9]. The activation loop contains Asp1150, Tyr1158, Tyr1162, and Tyr1163. The catalytic loop contains Asp1132. The remainder of the protein in each case is represented by a molecular surface. For IRK-3P (night), the substrate peptide (containing Y(p)) and the ATP analog are shown, the latter of which is partially masked by the N-Terminal lobe of IRK-3P.

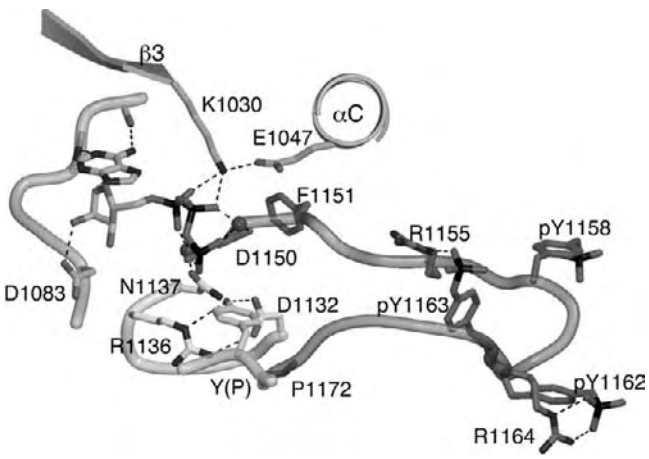


Figure 4 Properly configured active site in IRK for phosphoryl transfer derived from the ternary IRK-3P structure [9] with the following modifications: the Lys1030 side-chain rotamer and the ATP γ-phosphate dihedral angle have been changed to coincide with those in the structure of ternary protein kinase A (PKA) [28]. Two active site Mg^{2+} ions are shown as spheres. Hydrogen bonds are represented as black dashed lines.

IRK (IRK-0P) traverses the cleft between the two kinase lobes, with Tyr1162 bound in the active site, hydrogen-bonded to conserved residues Asp1132 and Arg1136 of the catalytic loop (Figs. 3 and 4) [8]. This conformation of the activation loop obstructs the substrate and nucleotide binding sites via Tyr1162 (acting as a pseudosubstrate) and residues of the DFG motif, respectively. Solution studies of IRK indicate that in the presence of millimolar quantities of ATP (comparable to cellular levels), the activation loop is actually in equilibrium between inhibiting, gate-closed conformations, as represented by the IRK-0P crystal structure, and gate-open conformations in which the activation loop is displaced from the active site cleft [19].

The crystal structure of the tris-phosphorylated, activated form of IRK (IRK-3P), co-crystallized with a Mg-ATP analog and substrate peptide, reveals how autophosphorylation of the three activation loop tyrosines stabilizes a particular gate-open conformation that is optimal for catalysis (Figs. 3 and 4) [9,20]. More specifically, the autophosphorylation-induced rearrangement of the activation loop accomplishes four main mechanistic objectives: (1) release of steric constraints to Mg-ATP and substrate binding, (2) proper positioning of Asp1150 (DFG motif) for coordination of an active site Mg^{2+} ion, (3) proper positioning of Phe1151 (DFG motif) to facilitate lobe rotation for productive ATP binding, and (4) proper positioning of the end of the activation loop, which acts as a platform for substrate binding. The positions of residues in the catalytic loop remain virtually unchanged in the basal (unphosphorylated) and activated (tris-phosphorylated) states of IRK.

The activation loop conformation in the IRK-3P structure is stabilized by direct interactions between the phosphate groups of pTyr1162 and pTyr1163 and basic residues in the activation loop (Arg1164 and Arg1155, respectively) and by short, anti-parallel β-strand interactions between segments of the activation loop and the C-terminal lobe. These interactions are also observed in the structure of the related IGF-1 receptor kinase [21]. Interestingly, the phosphate group of pTyr1158 makes no contacts with other kinase residues and

has been reported to bind a subset of Src homology 2(SH2) domain-containing proteins including SH2-Bγ [22] and APS [23].

It has not been possible to crystallize IRK-0P with bound Mg-ATP analog and substrate peptide, presumably due to the high K_m values for ATP and substrate peptide in the unphosphorylated state (≈1 and 2 m*M*, respectively) [24]. An approximate view of the initial, basal-state autophosphorylation event was provided by the crystal structure of an IRK activation loop mutant, Asp1161 → Ala, with bound Mg-ATP analog [10]. This structure, in combination with biochemical studies [24,25], demonstrates that a single residue change can result in loss of pseudosubstrate autoinhibition by the activation loop. In the Asp1161 → Ala mutant, K_m for ATP is decreased and k_{cat} is increased to levels comparable to the tris-phosphorylated form [24], although K_m for substrate peptide remains high.

The structure of this mutant with bound Mg-ATP analog shows that the activation loop is disengaged from the active site and mostly disordered. In the beginning of the activation loop, the residues of the DFG motif, particularly Phe1151, are not properly positioned for catalysis. Positioning of the Phe1151 side chain in a hydrophobic pocket below αC is critical for free rotation of the N-terminal lobe with respect to the C-terminal lobe, required for productive ATP binding, and for the independent (with respect to the N-terminal β-sheet) rotation of αC, which positions Glu1047 (αC) for salt-bridge formation with Lys1030 (β3) (Fig. 4) [10]. This structure indicates that despite release of activation loop autoinhibition (in this case through mutation), autophosphorylation of the activation loop tyrosines is still necessary to stabilize the loop in a configuration optimized for catalysis.

Prospects

An understanding of the signal transduction mechanisms intrinsic to the insulin receptor is not only of academic interest. The prevalence of non-insulin-dependent diabetes mellitus (NIDDM) in the populations of developed countries is increasing at an alarming rate. One therapeutic strategy for NIDDM is to activate (in the absence of insulin) or potentiate (in the presence of insulin) the insulin receptor. Indeed, small-molecule activators/potentiators of the insulin receptor which act cytoplasmically have been discovered through screening procedures [26,27]. Knowledge of the structural transitions that occur in the insulin receptor kinase during the activation process should aid in the design of more potent and specific small-molecule activators of the insulin receptor.

Acknowledgment

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CHAPTER 54

What Does the Structure of Apo2L/TRAIL Bound to DR5 Tell Us About Death Receptors?

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Introduction

The cell-surface receptors of the tumor necrosis factor receptor (TNF-R) superfamily mediate many cellular functions, including cell death, proliferation, and inflammatory responses. Within this superfamily, the death receptors, including TNF-R1, FAS, DR3, DR4, DR5, and DR6, activate the apoptotic machinery [1]. Ligand binding and clustering of the extracellular domains (ECDs) of these receptors are thought to trigger the oligomerization of their cytoplasmic death domains and assembly of the death-inducing signaling complex (DISC), leading to activation of apoptotic pathways. While the downstream effects of signals from the death receptors are very different from those of other TNFR family members, the extracellular clustering process is thought to be conserved throughout the superfamily.

The ECDs of the members of the TNFR superfamily are composed of cysteine-rich pseudo-repeat domains termed CRDs, which typically contain six cysteine residues. The crystal structures of TNF-R1 both unliganded and bound to lymphotoxin (LT) reveal that the structure of these CRDs consists of a ladder of disulfide bridges connected by intervening loops [2,3]. The ligands all have the prototypical fold first seen in the structure of TNF [4]. This fold is comprised of a trimer of jelly-roll domain protomers, with each protomer consisting of two flat β-sheets composed of strands A′AHCF and B′BGDE (Fig. 1). The B′BGDE sheet is more solvent accessible than the A′AHCF sheet. In the complex of LT with TNF-R1, the thin and elongated receptors bind at the interfaces between ligand monomers (Fig. 2) [3].

Novel Features in the Structure of Apo2L/TRAIL

Two independent crystal structures of unbound Apo2L/TRAIL show that it resembles the TNF trimer in its basic architecture [5,6]. Apo2L/TRAIL has two unusual features with respect to other TNF family members. First, it has a distinct, long loop between strands A and A′ (residues 130 to 150) that traverses its surface but is poorly ordered or even disordered between residues 131 and 143 (Fig. 1). Second, a novel zinc-binding site buried in the trimer interface is formed by the single cysteine residue from each monomer [6,7]. This binding site appears to be unique to Apo2L/TRAIL among TNF family members. Its importance for the structural integrity and biological activity of the protein was revealed when the site was removed using chelating agents or mutation of the cysteine residue [6,8,9]. The resulting protein showed changes in its circular dichroism and tryptophan fluorescence spectra, indicating a more open trimer, and had reduced thermal stability, receptor-binding affinity, and bioactivity. Therefore, the role of the zinc site is to stabilize the trimer and, in particular, the conformation of the adjacent loops that interact with receptor.

Apo2L/TRAIL:DR5 structures

Overview

The crystal structure of Apo2L/TRAIL bound to the ECD of one of its signaling receptors, DR5, has recently been

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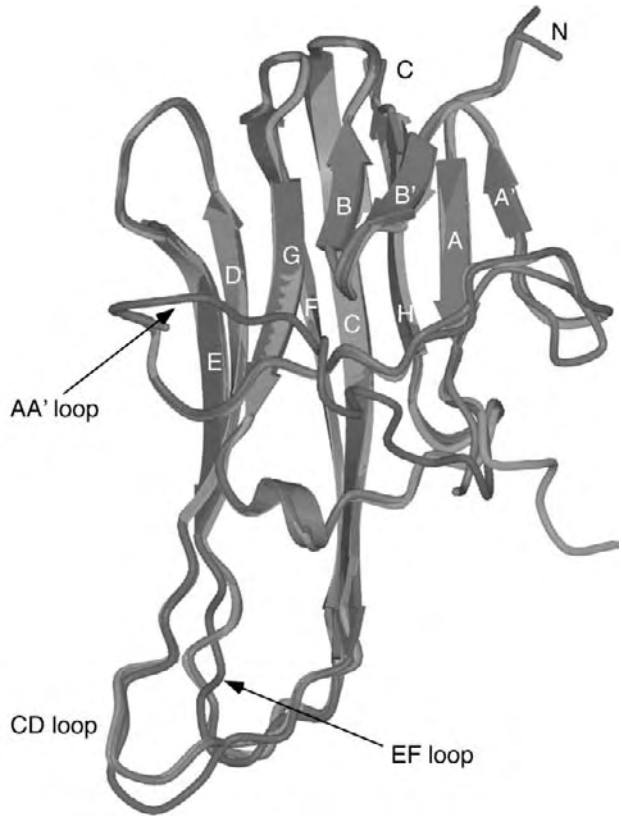


Figure 1 Superposition of monomers from independent crystal structure determinations of Apo2L/TRAIL taken from complex structures with DR5, showing the variations in loop conformation. Monomer A (Hymowitz *et al.* [7]) is rendered in light gray; monomer B (Mongkolsapaya *et al.* [10]), in medium gray; and monomer D (Cha *et al.* [11]), in dark gray.

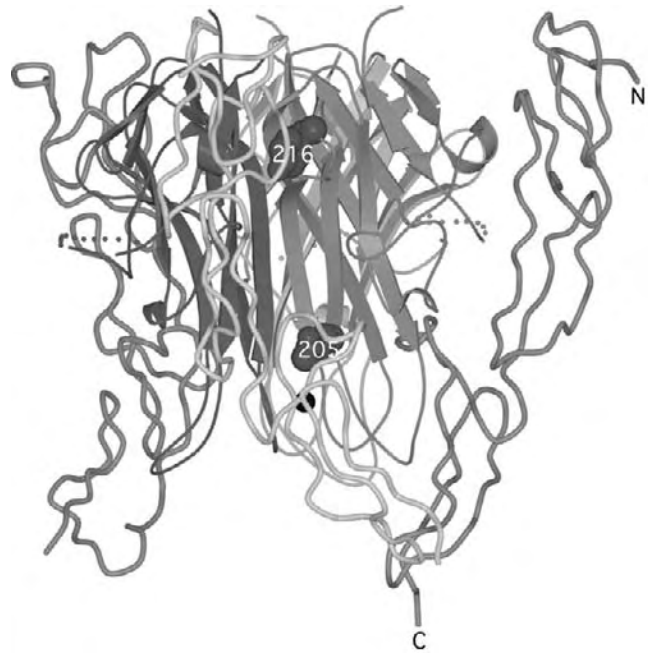


Figure 2 Crystal structure of Apo2L/TRAIL (light gray) bound to the ECD of DR5 (dark gray and black) (Hymowitz *et al.* [7]). Residues 132 to 143 of Apo2L/TRAIL are disordered and shown as small balls. The internal zinc ion is shown as a small dark sphere at the tip of the Apo2L/TRAIL trimer. The side chains of Apo2L/TRAIL residues Tyr 216 and Gln 205 are shown in close-packed rendering for one protomer (in gray).

determined by three different groups [7,10,11]. These three independent structures are in good overall agreement, especially around the receptor binding site. As in the LT–TNF-R1 complex, the three symmetrical binding sites on the ligand are nestled between the monomer–monomer interfaces; each site forms two distinct patches, one centered around Tyr 216 and the other around Gln 205 (Fig. 2). Both of these residues have been shown to be critically important for Apo2L/TRAIL activity [6]. Two loops from DR5, the 50s loop in CRD2 and the 90s loop in CRD3, make almost all the contacts with the ligand. The conformation of the 90s loop is very different between DR5 and TNF-R1, and its sequence in other TNF-Rs is highly variable. This loop was proposed to be important for defining the specificity and cross-reactivity among TNF-R family members [11]. In contrast, the 50s loop has a backbone conformation very similar to the corresponding loop in TNF-R1 (the Cα atoms of DR5 residues 50 to 62 superimpose to within 0.4 Å on the Cα atoms of TNF-R1 residues 60 to 72). Moreover, the sequence of this loop is well conserved among TNF-R family members, suggesting that its conformation and binding characteristics may be conserved as well (Fig. 3). Most of the differences in the independent structures of Apo2L/TRAIL–DR5 occur at loops on the ligand away from the receptor binding site and involve poorly ordered parts of the molecule. In exception to this, the most dramatic and functionally relevant difference occurs in the EF loop of Apo2L/TRAIL in the structure by Mongkolsapaya *et al.* [10], for which the lack of zinc resulted in a different, less ordered conformation than that seen in the other two structures. As indicated above, zinc is required for maintaining the structural integrity and activity of Apo2L/TRAIL, and the conformation observed in the structures by Hymowitz *et al.* [6,7] and Cha *et al.* [11] likely represents the native conformation.

Variation in the Orientation of CRD3 of DR5

Overall, the conformation of the receptors, and particularly of the loops that contact Apo2L/TRAIL, is well conserved among the three independent structures. One exception is revealed by a comparison of all independent copies of DR5 (Fig. 3). This comparison shows that the orientation of CRD3 with respect to the other domains is variable even among receptors within the same complex, resulting in a displacement of structurally equivalent residues by up to 5 Å. Similar variation is seen when individual DR5 structures are compared to TNF-R1 as well as in comparisons between different structures of TNF-R1 alone or bound to LT (Fig. 3). Therefore, this variability is likely to be an intrinsic property of the structure of TNF-Rs with multiple CRDs rather than a source for specificity among family members as was suggested on the basis of a single unique copy of DR5 in the structure of Mongkolsapaya *et al.* [10]. A structure of Apo2L/TRAIL bound to the extracellular

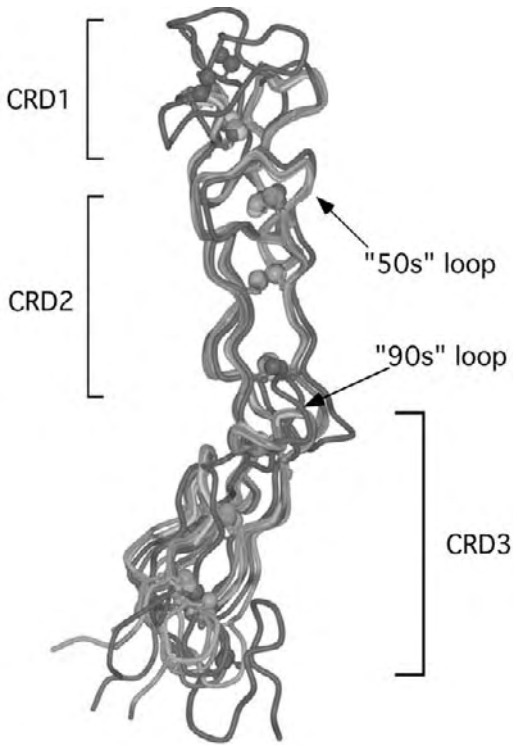


Figure 3 Superposition of the Cα traces of ten independent DR5 structures. The three chains from Hymowitz *et al.* [7] are shown in light gray; one chain from Mongkolsapaya *et al.* [10], in medium gray; six chains from Cha *et al.* [11], in dark gray; and TNF-R1 from Banner *et al.* [3], in black. The DR5 chains were superimposed using the Cα atoms of residues 22 to 101 (the ordered portion of CRD1, all of CRD2, and the first loop of CRD3). TNF-R1 was superimposed on DR5 using the Cα atoms of the structurally equivalent residues in CRD2.

domain (ECD) of DR4 or to one of its decoy receptors would reveal if variation in CRD3 orientation is a common feature of other Apo2L/TRAIL receptors.

The AA′ Loop of Apo2L/TRAIL

Much interest has centered on a possible role for the unusually long AA′ loop of Apo2L/TRAIL (residues 130 to 150). In the structures by Hymowitz *et al.* [7] and Mongkolsapaya *et al.* [10], this loop turns away from the receptor at residue 131 and becomes poorly ordered. In contrast, in the structure by Cha *et al.* [11] residues 130 to 135, while still poorly ordered, are in a different conformation, allowing for additional contacts to DR5. However, in this model there appears to be insufficient space between DR5 and Apo2L/TRAIL for the disordered part of the chain (residues 136 to 145) to fold back across the protein, suggesting that the conformation of the last few marginally ordered residues in this model may not be biologically relevant.

In all three structures, the rest of the AA′ loop uses residues 130 and 145 to 149 to make minor contacts with the receptor; however, none of these contacts involves burying significant accessible surface area. These observations are consistent with the lack of effect on binding (to DR4, DR5, or DcR2) or on the activity of single alanine substitutions at residues 130, 134, 136, 138, 140–143, and 149 within this loop. The reduction or elimination of receptor binding activity observed upon deletion (Δ137–152 [5]), shortening (Δ132–135 [11]), or multiple mutation [10] of this loop may therefore be an indirect effect on the conformation of other binding determinants. In light of the point mutation results and the high degree of conformational heterogeneity seen in this loop among the three crystal structures, we conclude that these peripheral interactions are likely to vary in other death receptor complexes and that, even when present, they are unlikely to contribute as much binding energy as contacts made by residues analogous to Apo2L/TRAIL Tyr 216 and Gln 205.

Ligand-Independent Receptor Assembly

In addition to the role of CRDs 2 and 3 in ligand binding, CRD1 of receptors DR4, FAS, CD40, TNF-R1, and TNF-R2 mediates pre-ligand-binding receptor association into inactive receptor oligomers, which are thought to be altered upon ligand binding to allow intracellular DISC assembly [12–14]. In all DR5 structures, CRD1 is only partially ordered (Fig. 3). Its sequence and structure are very different from the corresponding domain in TNF-R1; furthermore, the sequences of DR4 and DR5 vary most in this CRD, and CRD1 contains no obvious motifs conserved with other TNF-R family members. For these reasons, it is unclear if ligand-independent preassociation is a universal feature of the death receptor family.

Intracellular Consequences of Ligand Binding

Ligand binding causes the cytoplasmic domain of the death receptors to interact with downstream adaptor proteins and other members of the apoptotic cascade. Some signaling components are shared; in particular, Fas, DR4, and DR5 recruit FADD and caspase 8 after binding ligand [15–17]. The geometry of the signaling complex appears to be conserved for all ligand–TNF-R complexes. Ligand binding by DR5 and TNF-R1 results in a defined geometry in which the membrane proximal residue of the three symmetrical receptor monomers forms a triangle with sides of approximately 50 Å. This geometry is likely to be biologically relevant, as a cytoplasmic triangle of these same dimensions was observed in the structure of the trimeric adaptor protein TNF-R-associated factor 2 (TRAF2) bound to peptides from intracellular portions of TNF-R2 or CD40 [18,19]. These shared features suggest that the components and geometry of the signaling complex for other death receptors are likely to be similar to those of DR5.

Conclusion

The three independent structures of death receptor 5 interacting with Apo2L/TRAIL agree well with each other

and with the general features of TNF-R1 bound to LT, indicating that the diverse receptors of the TNF-R superfamily interact with their trimeric ligands in a conserved manner. These receptors bind their ligands through modular, flexible CRDs with conserved cysteine locations presenting intervening loops of varying size and sequence. Despite the great diversity of sequence among both the receptors and the ligands, this basic mode of interaction appears to be conserved. In both the DR5 and the TNF-R1 complexes, CRD2 and the first loop of CRD3 provide almost all receptor contacts. Therefore, a structural motif barely larger than a single CRD would appear to be the minimal ligand-binding unit. Interestingly, a recently discovered, distantly related TNF-R-like molecule, B-cell maturation activity, contains only a single CRD [20]. Further insights into this important family of signaling molecules await determination of either multicomponent interactions or the structures of more distantly related death receptor–ligand pairs.

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SECTION D

Membrane Proximal Events

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CHAPTER 55

TNF Receptor Associated Factors

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Unlike receptors with intrinsic kinase activity in their intracellular domains or in direct association with intracellular enzymes, members of the tumor necrosis factor (TNF) receptor superfamily and the interleukin-1 (IL-1) receptor/Toll-like receptor (IL-1R/TLR) superfamily use adapter proteins to couple to enzymatic activation and signal amplification. The TNF-receptor-associated factors (TRAFs), which currently consist of six members (TRAF1–6) in mammals [1–8], have emerged to be the major adapter proteins for these receptors. Through versatile protein–protein interactions, TRAFs link receptor activation to downstream kinase activation and eventually the stimulation of nuclear factor κB (NF-κB) and AP-1 transcriptional activity. Collectively, a wide range of cellular effects including cell survival, proliferation, and differentiation may be elicited by TRAF signaling.

The TRAFs participate in receptor signal transduction by either direct association with receptors or indirect interaction through additional adapter proteins, in at least three distinct pathways (Fig. 1). Members of the TNF receptor superfamily that do not contain intracellular death domains, such as TNF-R2 and CD40, recruit TRAFs directly via short sequences in their intracellular tails [1,2,5,7]. The Epstein–Barr virus transforming protein LMP1 has also been shown to directly recruit TRAFs for viral survival and cell transformation [3]. Receptors that contain an intracellular death domain, such as TNF-R1, first recruit an adapter protein TRADD via a death domain–death domain interaction [9]. TRADD then serves as a central platform of the TNF-R1 signaling complex, which assembles TRAF2 [10] and RIP [11,12] for survival signaling and Fas-associated death domain (FADD) and caspase-8 for inducing apoptosis [10,13]. Members of the IL-1R/TLR superfamily contain a protein interaction module known as the TIR domain, which recruits sequentially MyD88 [14], a TIR-domain- and death-domain-containing protein, and IRAK [15], an adapter Ser/Thr kinase with a death domain. Oligomerization of IRAK appears to result in its association with intracellular TRAF6 to elicit signaling [8].

Members of the TRAF family are characterized by the presence of a novel TRAF domain at the C terminus which in turn consists of a coiled-coil domain followed by a conserved TRAF-C domain 1 (Fig. 2). The TRAF domain plays an important role in TRAF function by mediating self-association, receptor interaction, and interactions with other signaling proteins such as TRADD and IRAK [8,10,16]. The N-terminal portion of most of the TRAF proteins contains a RING finger and several (five to seven) zinc finger motifs, which is important for downstream signaling events [16,17]. The presence of TRAFs is conserved genetically in other multicellular organisms such as *Drosophila* [18], *Caenorhabditis elegans* [19], and *Dictyostelium discoideum* [4].

Crystal structures and biochemical characterizations have revealed a conserved trimeric association of TRAFs that is mediated by both the coiled-coil domain and the TRAF-C domain (Fig. 3A) [20–22]. This trimeric stoichiometry of TRAFs provides a structural basis for the signal transduction across the cellular membrane after receptor trimerization by trimeric extracellular ligands in the tumor necrosis factor (TNF) superfamily [23]. Interestingly, recent studies suggest that specific ligand-induced receptor trimerization may be primed by nonsignaling receptor preassociation prior to ligand binding [24]. Thermodynamic characterization reveals the low-affinity nature of monomeric TRAF2–receptor interactions, thereby confirming the importance of oligomerization-based affinity enhancement or avidity in receptor-mediated TRAF recruitment [25].

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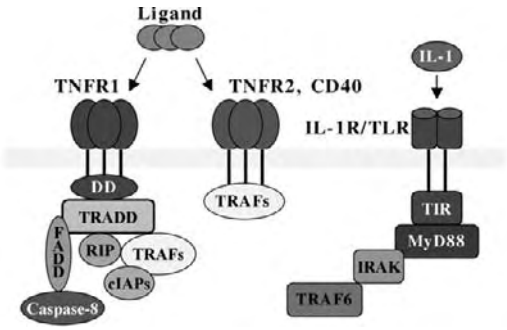


Figure 1 Membrane-proximal events in TRAF signaling, showing direct and indirect TRAF recruitment to post-receptor signaling complexes.

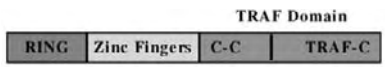


Figure 2 Domain organization of TRAFs.

Receptor sequences bind symmetrically to the surface groove on the TRAF-C domain of TRAF2 in an extended conformation (Fig. 3A) [20,22]. It makes main-chain hydrogen bonding interactions with the edge of the β-sandwich structure of the TRAF-C domain. Specific side-chain interactions observed in multiple TRAF2–receptor complexes have led to the establishment of short TRAF2-binding motifs [26]. Residues on the TRAF2 surface used for receptor interaction is generally conserved in TRAFs 1, 3, and 5, suggesting that these TRAFs interact with similar receptor sequences. However, a somewhat different binding mode has been observed for the interaction of TRAF3 with the same sequence from CD40, which forms a hairpin on the TRAF3 surface [27].

The mode of TRAF2 recruitment by TRADD has been revealed by the crystal structure of the TRAF2–TRADD complex (Fig. 3B) [28]. The more extensive TRAF2–TRADD interface overlaps spatially and therefore potentially competes with TRAF2–receptor–peptide interactions. Biochemical characterization of the interaction has shown that TRAF2 has a significantly higher affinity for TRADD than for the peptide motifs in direct receptor interactions, which leads to more efficient initiation of TRAF2 signaling by TRADD. In addition, TRADD interacts with only TRAF1 and TRAF2, but not other members of the TRAF family. It appears that TRAF1 and TRAF2 work in conjunction with associated caspase inhibitors, cIAP-1 and cIAP-2, to fully suppress TNF-induced apoptosis in the TNF-R1 signaling complex [28,29]. This leads to a dominance of survival signaling for TNF-R1 under most circumstances.

The TRAFs appears to undergo specific intracellular trafficking upon receptor stimulation, from either a diffuse or punctate cytoplasmic distribution to a cell-surface relocalization [3,30]. Accumulating evidence suggests that the recruitment of TRAFs to membrane microdomains or rafts is a crucial step for the initiation of TRAF signaling events [31–33]. The formation of TRAF-containing rafts may stabilize the receptor–signaling complex and bring TRAFs to the proximity of kinases and other signaling proteins. Because many different receptors recruit the same TRAFs, the availability of intracellular TRAFs can be a limiting factor in TRAF-mediated signal transduction. It has been shown that receptor activation can lead to depletion of the cytoplasmic pool of TRAF2 via relocalization to insoluble fractions [34] and/or TRAF degradation [35,36]. This suggests a competitive nature of TRAF signaling by different receptors and adds to the potential complexity of TRAF-mediated signal regulation.

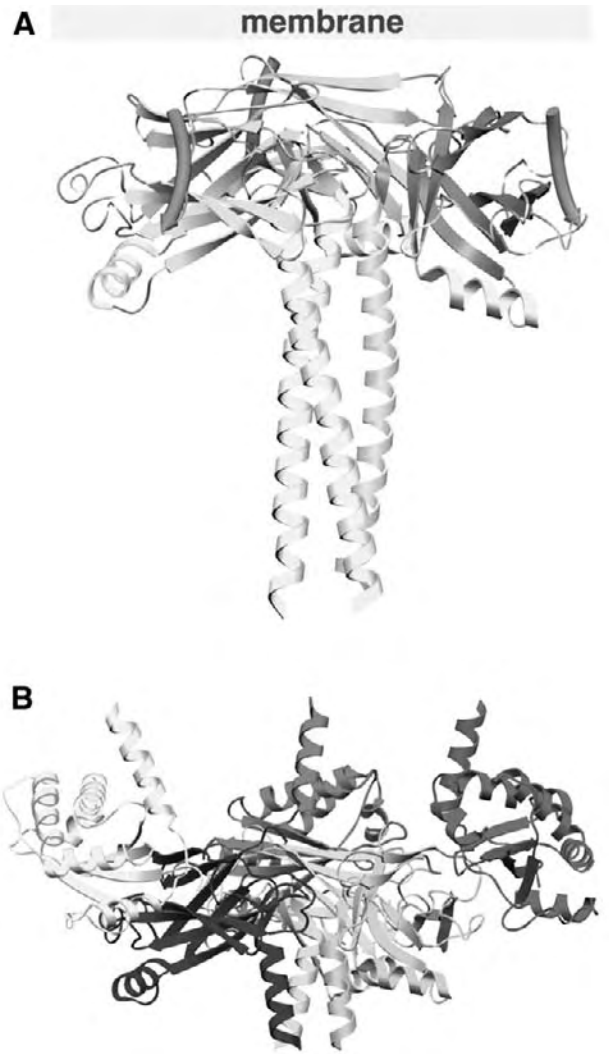


Figure 3 TRAF structures. (A) Mushroom-shaped trimeric structure of the TRAF domain of TRAF2 in complex with TNF-R2 [20], shown with the three-fold axis vertical. The coiled-coil region (stalk) is in yellow. The β-strands of the three TRAF-C domains are shown, respectively, in blue, green, and purple. Bound peptides from TNF-R2 are shown as orange arrows indicating the direction of the peptide chains. (B) Ribbon diagram of the complex between TRADD and TRAF2 [28], shown with the three-fold axis vertical. TRAF2: blue, green, and purple; TRADD: magenta, red, and yellow.

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CHAPTER 56

Assembly of Signaling Complexes for TNF Receptor Family Molecules

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Introduction

The tumor necrosis factor receptor family (TNFR-F) of molecules is a large, diverse group of molecules that participate in the regulation of cellular activation, development, and programmed cell death [1]. This brief overview summarizes and integrates currently available information on how this interesting family of receptors assembles signaling complexes at the cell membrane and how this may regulate cellular signaling pathways.

Receptor Aggregation

A common property of TNFR-F molecules is their trimeric ligands, which can be found in both membrane-bound and soluble forms [1]. Many other cell surface receptors can be fully activated by dimerization, so it has been asked whether trimerization of TNFR-F receptors is integral to their proper signaling. Numerous studies of TNFR-F molecules have used agonistic receptor-specific antibodies to successfully mimic ligand-initiated signaling. Additionally, fusions of the cytoplasmic domains of CD120a (TNF-R1), CD120b (TNF-R2), and CD40 to extracellular domains that can be dimerized but not trimerized results in chimeric molecules that clearly retain many signaling functions *in vitro* [2–4]. It has also been shown that TNF receptors and CD95, when overexpressed in transformed epithelial cells, can self-aggregate and signal in the absence of ligand binding [5,6]. However, as a greater variety of signaling events have been examined, it has become clear that dimerization of TNFR-F receptors does not reproduce all the effects of trimerization, nor is it likely that endogenous levels of receptors are normally sufficient for self-aggregation. Sensitivity of T cells to apoptosis mediated by CD95/Fas is substantially altered depending upon whether agonistic anti-CD95 Ab or various forms of CD95L are used to ligate CD95 [7]. Similarly, CD40-mediated interleukin-6 (IL-6) production by B cells cannot be effectively induced by anti-CD40 or even soluble CD154 (CD40L) but requires membrane-bound trimeric ligand [8].

Raft Recruitment

While aggregation of TNFR-F members in the plasma membrane is required to initiate signaling (Fig. 1), additional organization of the receptors and their associated signaling molecules may also be necessary. Recent work indicates that the signaling complexes of several members of the TNFR-F are assembled in specialized regions of the plasma membrane known as membrane microdomains, or *rafts*. These regions of the plasma membrane, enriched in sphingolipids and cholesterol, are known to be sites of organization for various transmembrane signaling complexes [9]. Members of the TNFR-F associated with rafts include CD40 [10,11], CD95 [12], and TNFR1 [13]. The oncogenic Epstein–Barr virus protein LMP-1, which mimics CD40 signaling in many respects, also localizes to rafts [14,15].

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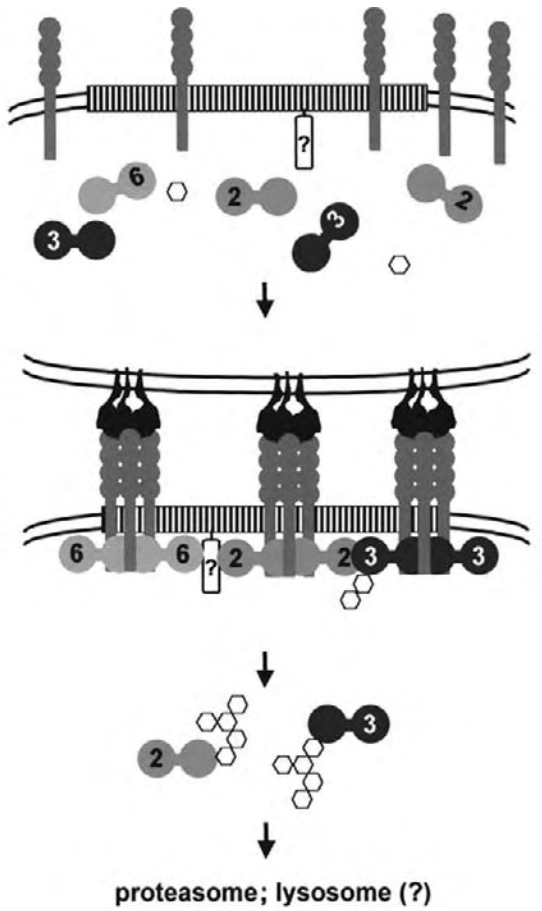


Figure 1 Working model of assembly of TNFR-F molecule signaling complexes in the plasma membrane. Aggregation of receptors (gray) by their trimeric ligands can lead to their movement into cholesterol rich lipid rafts (striped bars), to which they can recruit the TRAF molecules. TRAFs can interact with one another, and with incompletely characterized raft-resident proteins (box with question mark). It is likely that there is diversity of complexes of the same receptor, due to binding of different TRAFs to individual trimers; also, distinct members of the TRAF family may show different temporal association with particular receptors. Following receptor binding, some of the TRAF molecules can induce their own ubiquitin-dependent degradation (hexagons), in addition to facilitating the degradation of neighboring TRAFs. This may be an important mechanism for regulation of the signal cascade.

Assembly of TNFR-F signaling complexes in microdomains may serve several purposes. First, concentrating receptors in rafts may promote interactions between intracellular molecules that do not otherwise interact in unstimulated cells. For example, CD40 is known to bind several members of the TNF-receptor-associated factor (TRAF) family, including TRAF2, TRAF3, and TRAF6. Based on the crystal structures of TRAF2 or TRAF3 complexed with CD40 peptides [16–18] and the tendency of TRAF2 and 3 to form homotrimers [19], it is unlikely that a single CD40 trimer will contain both TRAFs 2 and 3. However, the aggregation of CD40 trimers in microdomains may allow TRAF2 molecules associated with one CD40 trimer to interact with TRAF3 (or other signaling molecules) bound by a neighboring CD40 trimer. Assembly of TNFR-F signaling complexes in microdomains may also foster interactions of the TRAFs (acting as adaptor molecules) with as yet uncharacterized signaling molecules residing in microdomains [10]. Finally, membrane rafts may be integral to the timely termination of signaling. The CD40 signaling complex is internalized following its aggregation at the plasma membrane [10,20], and while it is not yet known if this internalization is raft mediated, membrane microdomains do appear to participate in a number of endocytic processes [9].

Ubiquitination

Potentially related to the downregulation of TNFR-F signaling is the activation-induced degradation of receptor-associated molecules. The activation of cells through CD30 or CD40 results in the degradation of TRAF2 and, in the case of CD40, TRAF3 as well [10,21,22]. Similarly, degradation of TRAF6 has also been observed following stimulation of cells through the TNFR-F member RANK [23]. Degradation appears to be preceded by ubiquitination of the TRAFs through a mechanism that involves their Zn-RING domains [24]. Interestingly, the oncogenic Epstein–Barr virus protein LMP-1, whose signaling also involves TRAF proteins, is deficient in mediating the activation-induced degradation of TRAFs 2 and 3 [22]. It has been suggested that the failure to engage this potentially important regulatory mechanism contributes to the oncogenic activity of LMP-1. While perhaps important for the negative regulation of signaling, ubiquitination events may also contribute to the activation of certain signaling pathways by TNFR-F members. The Zn-RING domain of TRAF6 appears to have the ability to activate inhibitory factor κB (I-κB) kinase and nuclear factor κB (NF-κB) through a novel ubiquitin-dependent mechanism [25].

Receptor Interactions

The majority of studies seeking to understand assembly of signaling complexes focus upon individual receptors. However, receptors deliver their signals *in vivo* to cells that receive a variety of other stimuli preceding, simultaneous with, or subsequent to the ligation of any one particular receptor, and it is often demonstrated that signals from one receptor can enhance or inhibit signals from another. The most frequently studied points of interaction between distinct receptors, including those of the TNFR-F, include production of pro- or anti-apoptotic proteins, including members of the Bcl family and various transcription factors. However, key points of receptor cross-talk may occur much earlier following receptor ligation and involve molecules participating in the assembly of receptor signaling complexes at the plasma membrane. This may be particularly important for effective inhibition of cell death mediated by pro-apoptotic members of the TNFR-F, such as CD95. Irreversible DNA damage mediated by such receptors can occur too quickly for an intervention mechanism that relies exclusively upon *de novo* transcription and translation, and

it has been shown that CD95L-triggered apoptosis in lymphocytes and hepatocytes cannot be blocked by Bcl family proteins [26], suggesting that signals influencing CD95 effects may do so at early points in the signal cascade. The finding that proteins found in the signaling complexes for CD95 (Fas-associated death domain, or FADD), CD120a (TNF-receptor-associated death domain, or TRADD), or both (receptor interacting protein, or RIP) can associate with each other via their death domain motifs [27] supports this possibility. A number of subsequent studies have demonstrated that CD95-mediated apoptosis can be altered by modifying early stages of the signaling pathway. Induction of activation of protein kinase C [28,29] or phosphatidylinositol 3′-kinase [30] by signals through B- or T-cell antigen receptors can block CD95-mediated apoptosis and inhibit the aggregation and lateral diffusion of CD95 in the plasma membrane. As mentioned earlier, it has recently been reported that CD95 is constitutively located in membrane rafts [12,31], and both the BCR and TCR show ligation-induced raft localization [32], which could facilitate receptor interactions. Interestingly, the first protein to associate with ligated CD95 in its signaling complex, FADD, is not localized to rafts until CD95 binds its ligand [12], and it has been shown that BCR signals can prevent the CD95–FADD association [33]. Additional B-cell activation signals through major histocompability complex (MHC) class II and CD40 molecules also inhibit CD95-induced apoptosis independently of *de novo* RNA or protein synthesis [34,35]; both of these receptors are also recruited to rafts following ligation [10,36,37]. However, neither of these signals inhibits CD95–FADD binding, but instead they act at the subsequent step of the pathway to decrease the cleavage and activation of caspase-8 [34,35]. TCR-enhanced membrane recruitment of the protein FLIP is a powerful mechanism for inhibition of CD95-activated caspase-8 [38]; FLIP is structurally similar to caspase-8 but lacks residues necessary for catalytic activity and may thus act as a decoy to block signaling in the receptor complex [39]. It is thus clear that a variety of events that occur during formation of receptor signaling complexes by TNFR-F molecules can be regulated by signaling cascades initiated by other TNFR-F receptors, as well as members of additional receptor families.

Conclusions

Although this chapter has focused upon mechanisms by which members of the TNFR family of receptors assemble membrane signaling complexes, knowledge gained through study of these receptors will undoubtedly apply to signaling complexes formed by a variety of receptors. The degree and intensity of receptor clustering, initiated either by ligand binding or self-aggregation, affects the nature of the signal delivered by a wide variety of receptors. Similarly, it is now appreciated that membrane microdomains contribute to the regulation of signaling through a number of receptor families, serving to influence interactions between receptors and cytoplasmic signaling proteins and between receptors themselves, as well as regulating receptor movement and internalization. The way in which receptor complexes are organized in membrane microdomains can also provide a major mechanism by which degradation of components of the complex is regulated, by affecting trafficking of the complex, and/or associations leading to regulatory posttranslational modifications. Thus, these features of TNFR-F complex assembly and signal regulation illustrate principles common to signal transduction by many different types of receptors.

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CHAPTER 57

Mechanisms of CD40 Signaling in the Immune System

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Introduction

CD40 is a 48-kDa type I membrane glycoprotein belonging to the tumor necrosis factor receptor (TNFR) superfamily. It is expressed on a wide variety of cells including B cells, dendritic cells (DCs), macrophages, endothelial cells and a number of human carcinomas [1]. The ligand for CD40 is CD154, a 39-kDa type II membrane glycoprotein belonging to the TNF superfamily which exists as a self-associating homotrimer mainly on activated CD4+ T cells. However, CD154 is also present on a small population of activated CD8+ T cells, natural killer (NK) cells, monocytes, basophils, mast cells, eosinophils, and activated platelets [1,2].

The CD40–CD154 interaction plays a critical role in both humoral and cell-mediated immune responses [2,3]. The importance of CD40 signaling during humoral immunity is highlighted in patients with hyper IgM syndrome, an immunodeficiency characterized by mutations in CD154 that render it nonfunctional, and in mice deficient in either CD40 or CD154. Under these circumstances, B cells still develop but, due to the absence of CD40 signaling, they fail to undergo antibody class switching in response to T-dependent antigens. Patients with this syndrome produce only IgM antibodies [2].

In cell-mediated inflammatory responses, CD40 is critical in controling the maturation of antigen-presenting cells (APCs), such as DCs, macrophages, and B cells. CD40 signaling appears particularly important in triggering the release of proinflammatory cytokines, such as interleukin-12 (IL-12), and the upregulation of a number of adhesion and costimulatory ligands, such as CD80, CD86, CD134 ligand, and CD137 ligand on APC [3]. In this way, helper T cells, by upregulating CD154 and other costimulatory ligands, can initiate the maturation of DCs, which in turn allows them to drive CD8+ cytotoxic T cell (CTL) responses [3]. Hence, through intermediary DCs, helper T cells can use accessory molecules such as CD154 to control CTL responses without necessarily making direct cell-to-cell contact with the responding CD8 cells [4–6].

Finally, it has recently been found that certain neoplastic cells, such as high-grade non-Hodgkin's lymphoma, express both CD40 and CD154 on their plasma membranes in signaling structures called *signalosomes* that form within the cholesterol- and sphingolipid-rich plasma membrane microdomains known as *membrane rafts*. This configuration seems to allow the malignant cells to continuously trigger CD40 without a need for CD154-expressing accessory cells [7]. Because of its profound effects on all aspects of the immune system, strategies to inhibit or stimulate signaling via CD40 are currently being pursued as a means to alleviate a range of autoimmune and malignant diseases [8,9]. Work in a number of laboratories has demonstrated that extraneous stimulation via CD40, with monoclonal antibody (mAb) or soluble CD154, is particularly effective in boosting CD8 responses against weak tumor antigens, resulting in eradication of existing tumors and long-term immunity [8].

Signaling Pathways Triggered by CD40 Engagement

The CD40 cytoplasmic tail lacks intrinsic enzymatic activity, and, as such, signaling via CD40 is mediated through

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its interaction with a family of genetically conserved adaptor proteins known as TNF-receptor-associated factors (TRAFs). These are the major signal transducers for the TNFR and IL-1 receptor/Toll-like receptor superfamilies (see below) [10]. Although TRAFs themselves have no intrinsic enzymatic activity, they are thought to be capable of activating Ser and Thr kinases, which in turn activate the downstream inhibitory factor κB (I-κB) kinase and mitogen-activated protein kinase cascades (JNK, p38, ERK2 MAPKs) [11].

Engagement of CD40 also results in the activation of protein tyrosine kinases, including the Src-family kinase Lyn and the Syk kinase [12–15]. Interestingly, the binding of CD154 to CD40 can result in recruitment of CD40 to membrane rafts and thus may facilitate the association with raft-located Src-family kinases, such as Lyn [15]. To date, however, no direct interaction has been demonstrated between CD40 or TRAF proteins and Lyn. Inhibition of Lyn and other Src-family kinases following engagement of CD40 on DCs, using the selective inhibitor PP1, resulted in diminished activity of the ERK2 MAPK but had only minor inhibitory effects on the activity of p38 MAPK [15]. These data suggest that CD40-mediated activation of ERK2 is dependent on the activation of upstream Src-family kinases, most probably Lyn. The role of ERK2 and p38 MAPK in CD40-triggered regulation of proliferation and gene expression has recently been established. In DCs, CD40-mediated ERK2 activation regulates IL-1α, IL-β, and IL-1Ra and to a lesser extent IL-12 p40 mRNA levels, whereas CD40-triggered p38 MAPK activation appears to be a potent regulator of IL-12 p40 mRNA [15]. CD40-mediated activation of p38 MAPK has also been shown to play an important role in B-cell activation, for which it is required for CD40-induced B-cell proliferation, NF-κB activation, and the expression of CD54 [16,17].

CD40 Signaling Is Mediated by TRAF-Dependent and TRAF-Independent Pathways

The TRAF family consists of six members (TRAFs 1 to 6), of which TRAFs 1, 2, 3, and 6 can bind directly to the cytoplasmic tail of CD40 through their C-terminal TRAF domain [18–20]. TRAF1 and TRAF5 also interact with CD40 indirectly as a result of forming hetero-oligomers, with TRAF2 and TRAF3, respectively. With the exception of TRAF1, the N-terminal portion of TRAF proteins contains a RING finger and several zinc finger motifs that are involved in mediating downstream signaling events. Indeed, deletion of the RING domain results in the generation of dominant-negative TRAF mutants.

TRAF2 and TRAF3 bind to an overlapping region of the CD40 cytoplasmic tail, whereas TRAF6 binds to a remote proximal region of the CD40 tail [18,20]. Engagement of CD40 by CD154 results in the recruitment of trimeric TRAF proteins to the receptor complex [21,22]. The crystal structure of the complex between the receptor-binding fragment of TRAF2 and its corresponding recognition site within the CD40 cytoplasmic tail showed that one trimeric TRAF protein is capable of binding to three cytoplasmic tail regions [23]. This implies that the binding of trimeric CD154 to CD40 organizes the cytoplasmic tails of the receptor in an orientation that allows the docking of a single TRAF2 trimer; however, recent studies have shown that receptor trimerization may not be sufficient to trigger all CD40 signaling pathways effectively and that higher order oligomers are required for optimal signaling [19,24]. We have recently described a method for producing a soluble dodecameric form of CD154 by fusing the extracellular domain of CD154 to a truncated form of the tetrameric lung surfactant protein-D. This highly defined multimeric form of CD154, which is more active than soluble trimeric CD154, could have potential for *in vivo* use [24]. It is possible that highly oligomeric forms of CD40 are more effective at sustaining the interaction with TRAF proteins, particularly those that bind to the trimeric cytoplasmic tail of CD40 with low affinity, such as TRAF6 [19]. Alternatively, it is conceivable that highly multimerized forms of CD40 are recruited into kinase-rich membrane rafts more effectively than trimeric CD40. Interestingly, CD95 (Fas), another member of the TNFR superfamily, is recruited into rafts only after engagement by soluble trimeric CD95 ligand that is additionally cross-linked with antibodies to induce further aggregation [25].

CD40 signaling has recently been shown to trigger the translocation of acid sphingomyelinase (ASM) to the plasma membrane, where it initiates the release of extracellularly orientated ceramide, which in turn mediates the clustering of CD40 into membrane rafts [26]. Interestingly, ASM-deficient cells or cultures where surface ceramide had been neutralized were resistant to antibody-triggered CD40 clustering and CD40-mediated cell signaling [26] in a manner similar to that reported for CD95 [27]. Moreover, CD40 clustering into membrane rafts is also triggered by the more physiologically relevant, membrane-bound CD154 [28]. Importantly, Grassme *et al.* also inferred that clustering of CD154 occurs during the CD40–CD154 interaction through an ASM/ceramide-dependent process and that this clustering is in fact critical for efficient aggregation and signaling through CD40. However, it should be noted that ASM may not be required for CD40 signaling in all circumstances; at high concentrations of anti-CD40 antibody, CD40 clustering into rafts occurs in the absence of ASM [26].

Following engagement of CD40 by soluble or membrane-bound CD154, TRAF2 and TRAF3, but surprisingly not TRAF6, are translocated to membrane rafts [22]. Subsequently, stimulation of CD40 signaling leads to proteasomal degradation of TRAF2 and TRAF3, and this probably serves as an elegant mechanism to control signaling through CD40 [29]. It is interesting to note that this degradation of TRAF proteins is not promoted by the Epstein–Barr virus transforming protein LMP-1, which mimics many structural and signaling aspects of CD40 (including recruitment of TRAFs to membrane rafts) independent of CD154 ligation [29]. Recently it has been shown that CD40-mediated TRAF2 degradation requires the ring domain of TRAF2 and is

preceded by TRAF2 ubiquitination. This was confirmed by blocking 26 S proteasomal degradation, which maintained TRAF levels and resulted in sustained CD40-mediated JNK activity, similar to the enhanced activity seen from LMP-1 [29]. It has also been shown that signaling by TNF-R2 leads to TRAF2 degradation, again mediated by ubiquitination. In this case, cIAP1(cellular inhibitor of apoptosis 1) associates with TRAF2 in the TNF-R2 signaling complex [30]. Therefore, it is possible that cIAP1 could play a similar role in CD40-triggered degradation of TRAF2.

While the mechanisms by which CD40 mediates signaling have been intensely studied and important mediators have been identified, it is clear that a number of factors remain to be revealed. For example, recent data suggest that CD40 mediates signaling independently of TRAF proteins. The importance of these TRAF-independent signaling pathways was studied using CD40 mutants that lack either the TRAF2/3 site or the TRAF6 site, or both [31,32]. In B cells, a CD40 mutant lacking the TRAF2/3 site was defective in the phosphorylation of I-κBα, JNK, and p38 MAPK, although I-κBα phosphorylation was not completely abolished. In contrast, a mutant without the TRAF6 site was capable of phosphorylating I-κBα, JNK, and p38 MAPK to an extent similar to wild-type CD40. Furthermore, even in the absence of both TRAF2/3 and TRAF6 sites, some I-κBα phosphorylation was apparent, confirming that at least some CD40-induced signaling is independent of TRAF binding to the CD40 cytoplasmic tail [32]. These results are in contrast to previous studies conducted using epithelial 293 cells, where both TRAF2/3 and TRAF6 sites were required for optimal NF-κB and JNK activation, and the TRAF6 site was primarily required for p38 MAPK activation [19]. These differences demonstrate the disparate requirements for activation of these signaling pathways in these two cell types.

Recently, the generation of transgenic mice expressing mutated CD40 protein lacking the ability to bind to TRAFs has provided for the first time a critical evaluation of the role of TRAF adapters in CD40 signaling *in vivo* [32]. Although CD40-induced B cell growth, upregulation of cell surface molecules, and early antibody production are independent of the interaction with TRAF proteins, later events such as germinal center formation are dependent on the binding of either TRAF2/3 or TRAF6. Furthermore, affinity maturation of antibodies and the generation of long-lived bone marrow plasma cells specifically require recruitment of TRAF6 to the CD40 receptor complex. Thus, TRAF proteins appear to regulate only the terminal phases of CD40-mediated B-cell differentiation [32]. CD40 still raises many questions, particularly with regard to how it mediates signaling within membrane rafts and the importance of non-TRAF signaling pathways. Furthermore, CD40 is expressed on many different cell types and exerts a variety of effects ranging from the induction of proliferation and differentiation in B cells [33] to the initiation of apoptosis in a number of carcinomas [34]. The immediate challenge is to completely define the signaling pathways that mediate the many functions of CD40 in order to exploit its full potential in future therapeutics.

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CHAPTER 58

Role of Lipid Domains in EGF Receptor Signaling

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Introduction

Epidermal growth factor (EGF) is a 53-amino-acid polypeptide that promotes growth and differentiation of a wide variety of cells. Its existence was first recognized over 40 years ago when it was identified as a factor in mouse submaxillary glands that induces precocious eyelid opening when injected into newborn mice [1].

Epidermal growth factor elicits its cellular effects through binding to the cell-surface EGF receptor. The EGF receptor is a transmembrane tyrosine kinase composed of an ≈620-amino-acid extracellular domain, a 24- to 26-amino-acid transmembrane domain, and an ≈450-amino-acid cytoplasmic portion that contains the kinase domain [2]. The EGF receptor is a member of the ErbB receptor family, which has four members: EGF receptor (ErbB1), HER2/neu (ErbB2), ErbB3, and ErbB4. The receptors are structurally similar; however, in ErbB3 the kinase domain is inactive. The ErbB receptors bind a family of ligands, with each receptor having a slightly different ligand selectivity. ErbB2 is the exception in that there is no known ligand for this receptor (for review, see Olayioye *et al.* [3]).

Upon ligand binding, the EGF receptor dimerizes and undergoes interchain autophosphorylation, primarily in the C-terminal tail of the receptor. This promotes complex formation with proteins containing Src homology domain 2 (SH2) and the phosphotyrosine binding (PTB) domain and initiates intracellular signaling cascades. The EGF receptor can also form heterodimers with ErbB2, yielding a complex with slightly different signaling and regulatory properties than EGF receptor homodimers. Both the EGF receptor and ErbB2 are frequently found over-expressed in tumors, particularly in breast cancer. A recently introduced therapy for breast cancer is based on the targeted downregulation of ErbB2 through the use of an antibody directed against this receptor (Herceptin). In addition, small-molecule tyrosine kinase inhibitors specific for the EGF receptor are also in clinical trials [4].

A variety of studies have demonstrated that the EGF receptor is enriched in low-density, cholesterol-enriched regions of the membrane known as *lipid rafts* [5–8] and that this localization is important in receptor function. This review describes what is known regarding the mechanism through which the EGF receptor is targeted to lipid rafts and discusses the role of localization to lipid rafts in EGF-receptor-mediated signaling.

Localization of the EGF Receptor to Lipid Rafts

Data from early studies suggested that the EGF receptor resided in a low-density membrane fraction that also contained the marker protein caveolin-1 [5–8]. As a result, the EGF receptor was initially thought to reside in the caveolin-1-coated membrane invaginations known as *caveolae*. However, recent studies suggest that the EGF receptor is present in flattened, low-density lipid rafts that lack caveolin-1, rather than in caveolae proper [9,10]. The initial confusion was due to the fact that caveolae are isolated along with EGF-containing lipid rafts in most subcellular fractionation protocols. Although no specific data are available, it appears from these studies that, in most cells, the majority of EGF receptors are present in the low-density lipid raft fraction.

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The EGF receptor localizes to lipid rafts under basal conditions; however, upon stimulation with EGF, the receptor rapidly exits lipid rafts and is eventually internalized by coated pits [11]. Thus, the association of the EGF receptor with caveolae/lipid rafts is readily reversible. Experiments have demonstrated that movement of the EGF receptor out of rafts requires an active kinase domain as well as at least one available autophosphorylation site; therefore, EGF receptor localization to rafts appears to be a highly regulated phenomenon [11].

Several structural features of proteins have been identified that serve to target the proteins to lipid rafts. These include palmitoylation, myristoylation, and prenylation of the protein as well as the addition of a GPI-anchor [12–14]; however, none of these mechanisms appears to function in the case of the EGF receptor, which has never been shown to acquire any such posttranslational lipid modifications. Mutational analysis of the EGF receptor demonstrated that a receptor in which the entire cytoplasmic domain has been deleted still localizes to lipid rafts, indicating that the intracellular domain is not required for localization of the receptor to these low-density domains [11]. Similarly, a receptor lacking the first 270 amino acids of the extracellular domain continues to localize to rafts, indicating that this portion of the receptor is not required for localization [11]. Recent work has demonstrated that a 60-amino-acid segment in the most membrane-proximal region of the extracellular domain of the EGF receptor is responsible for targeting the receptor to lipid rafts [15]. This suggests that the EGF receptor may localize to lipid rafts as a result of the interaction of its extracellular domain with a resident raft protein or lipid. It also suggests that the ability of ligand to promote release of the EGF receptor from lipid rafts may be due to an EGF-induced conformational change in the extracellular domain that reduces its affinity for its raft-localized binding partner.

Rafts and EGF-Receptor-Mediated Signaling

Because lipid rafts harbor a variety of proteins that are involved in signal transduction, it has been hypothesized that these low-density domains serve to organize cell signaling, enhancing the specificity or efficiency of the processes. While some experiments have demonstrated that the integrity of caveolae/lipid rafts is necessary for signal transduction via some pathways, other data seem to indicate that EGF-receptor-mediated signaling can take place both inside and outside lipid rafts.

Both EGF- and bradykinin-stimulated phosphatidylinositol turnover appear to occur exclusively in lipid rafts [16]. Disruption of lipid rafts by depletion of cholesterol completely ablated the ability of both hormones to stimulate phosphatidylinositol turnover, implying that intact rafts are absolutely required for this response [7]. By contrast, while EGF receptor autophosphorylation in intact cells appears to occur largely in low-density, noncaveolar membranes [8], depletion of cholesterol is associated with an increase in both receptor binding and autophosphorylation [9,17]. These findings suggest that both EGF receptor binding and kinase activity are suppressed when the receptor is present in lipid rafts. Similarly, EGF stimulation of the initial steps in activation of mitogen-activated protein (MAP) kinase (namely, the recruitment of ras and raf to plasma membranes) appears to take place within the lipid raft compartment [6]; however, lipid rafts are not essential for the EGF-induced activation of MAP kinase. Depletion of cholesterol from cells leads to hyperactivation of MAP kinase by EGF [17,18]. As cholesterol depletion results in disruption of lipid rafts, these data suggest that activation of MAP kinase can occur in the absence of intact rafts. This is consistent with recent studies that indicate that GTP binding causes H-ras to move out of lipid rafts and that activation of Raf by H-ras is less efficient when it occurs in rafts as compared to bulk plasma membrane [19]. Thus, the events leading to the activation of the kinases upstream of MAP kinase can apparently occur outside of lipid rafts.

Because of the differential effects of raft disruption on EGF-receptor-mediated signaling pathways as well as the EGF-dependent movement of the receptor out of lipid rafts, it is possible that some pathways are stimulated only through EGF receptors residing in lipid rafts, while other pathways are stimulated only when the EGF receptors exit caveolae/lipid rafts. Additional studies with nonlocalizing EGF receptors will be necessary to test this hypothesis.

The EGF Receptor and Caveolin

Caveolin is a 21-kDa membrane protein that was first identified as a substrate for $pp60^{src}$ (20) and appears to be responsible for stabilizing the invaginated structure of caveolae [21,22]. Cells lacking caveolin-1 do not exhibit plasma membrane caveolae, yet these cells still have low-density, cholesterol-enriched lipid rafts that contain high levels of signaling proteins. The relationship between caveolae proper and lipid rafts is not clear, but they appear to be distinct entities based on the ability to separate caveolin-containing low-density membranes from other similar membranes that contain raft markers such as GPI-linked proteins [23]. The EGF receptor appears to be present in lipid rafts rather than caveolae [9,10].

Despite the fact that the two proteins appear to reside in different compartments, numerous studies have reported evidence for a physical or functional relationship between caveolin-1 and the EGF receptor. Upregulation of caveolin-1 expression was shown to inhibit EGF-stimulated MAP kinase activation in human diploid fibroblasts [24]. This has been attributed to the ability of caveolin to interact directly with the EGF receptor and inhibit its activity. Using GST-caveolin-1 and synthetic peptides corresponding to the scaffolding domain of caveolin-1 (residues 81 to 101), Couet *et al.* [25] reported a physical association of the EGF receptor with caveolin-1 as well as an inhibition of EGF receptor kinase activity by caveolin-1 *in vitro*. Similar findings were

reported regarding the effect of caveolin-1 expression on the activity of the EGF receptor homolog, ErbB2 [26]. However, EGF-mediated MAP kinase activation appeared to be normal in caveolin-1 knockout mice [27], suggesting that in the *in vivo* situation, caveolin-1 does not suppress EGF receptor signaling. The discrepancy may be due to the use of recombinant proteins and synthetic peptides in the *in vitro* studies that apparently do not accurately reflect the relationship of caveolin-1 and the EGF receptor *in vivo*.

Studies from other investigators have provided data consistent with the results from the caveolin-1 knockout mice. Immunodepletion of low-density membrane fractions with an antibody to caveolin results in the physical separation of EGF receptors from caveolin-containing membranes [8]. In addition, Waugh *et al.* [8] reported that coimmunoprecipitation of the EGF receptor and caveolin was "irreproducible," consistent with the absence of a stable association between the two proteins. Thus, direct interaction between the EGF receptor and caveolin-1 *in vivo* seems unlikely.

Epidermal growth factor does affect caveolin-1 phosphorylation and trafficking. Over-expression of the wild-type EGF receptor or a C-terminally truncated form of the EGF receptor results in an EGF-dependent tyrosine phosphorylation of caveolin-1 that appears to be mediated by $pp60^{src}$ [28]. Furthermore, EGF induces the redistribution of caveolin-1 from the plasma membrane to an early endocytic compartment [29]. The recent observation that EGF also stimulates the phosphorylation and endocytosis of phospholipid scramblase 1, a resident lipid raft protein [30], is consistent with the notion that EGF may regulate the trafficking of a variety of proteins present in low-density membrane domains. An interesting question is whether the EGF receptor itself can be internalized via lipid rafts.

Summary

The EGF receptor localizes to low-density, cholesterol-enriched plasma membrane domains known as lipid rafts via an interaction that is mediated by its extracellular domain. Raft integrity appears to be important for the appropriate function and regulation of EGF-receptor-mediated signaling. Other receptor tyrosine kinases, including ErbB2, the PDGF receptor, the NGF receptor, and the insulin receptor, have been found to reside in or be recruited to lipid rafts [11,31–35]. Disruption of rafts by depletion of cholesterol has been shown to modulate signaling mediated by many of these growth factors [36,37]. Thus, lipid rafts appear to play a general role in the signaling function of receptor tyrosine kinases.

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CHAPTER 59

Structure and Function of B-Cell Antigen Receptor Complexes

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Introduction

Upon activation, B lymphocytes produce antibodies or immunoglobulins (Igs), thus they are an essential part of the humoral immune system. The activation of a B lymphocyte and its differentiation into the antibody-producing plasma cell is controlled by the B-cell antigen receptor (BCR) (*antigen* being the term immunologists use to describe a substance that elicits an immune response). In most cases, B-cell antigens are substances foreign to the body and include a heterogeneous group of molecules, such as proteins, polymeric sugars, polyglycans, DNA, or other polymeric molecules. The ability of the BCR to become activated upon binding to such a structurally diverse library of antigens indicates that the activation mechanism of the BCR must be different from that of other receptors that have only one or a limited set of ligands [1]. The latter receptors are often brought into a precise signaling-active conformation by the bound ligand, whereas signaling of the BCR is largely independent of the precise structure of the antigen.

The Structure of the B Cell Antigen Receptor

The BCR is composed of two protein modules, the membrane-bound immunoglobulin (mIg) molecule and the Ig-α/Ig-β heterodimer, which mediate antigen binding and signaling, respectively (Fig. 1A) [2,3]. The proper assembly between the mIg molecule and the Ig-α/Ig-β heterodimer in the membrane of the endoplasmic reticulum is a prerequisite for the transport of the BCR to the cell surface. All five major classes of mIg (mIgM, mIgD, mIgG, mIgA, and mIgE) are associated with the Ig-α/Ig-β heterodimer, presumably in a 1:1 stoichiometry. In the case of the IgM- and IgD-BCR, evidence suggests an oligomeric organization of the BCR [4]. These data support the model that conformational changes within oligomeric antigen receptors lead to initiation and amplification of the BCR signal (see below).

The mIg molecule is a tetramer consisting of two identical heavy (H) chains and two identical light (L) chains. The H chain and L chain each contain an N-terminal, variable (V) Ig domain, and an antigen-binding site is formed by the combination of one V_H and one V_L domain. Thus, a given mIg molecule has two identical antigen-binding sites. During B-cell development, the sequence coding for a V domain is assembled from a library of variable gene segments. The highly variable assembly process generates a huge set of different V domains and, thus, antigen-binding sites. However, expression of these genes is clonally and allelically regulated so that each B cell expresses only one set of H and L chains [5]. The mIgM molecule does not contain a cytosolic part that can interact with intracellular proteins; thus, the signaling function of the BCR relies mostly on the Ig-α/Ig-β heterodimer. Ig-α and Ig-β share many structural features. Both proteins carry a glycosylated extracellular Ig domain, a linker region with the heterodimer-forming cysteine, one transmembrane part, and a cytoplasmic tail sequence of either 61 (Ig-α) or 48 (Ig-β) amino acids. These cytoplasmic sequences are the most conserved parts of these transmembrane proteins, indicating that they have an important cellular function. The cytoplasmic tails of Ig-α and Ig-β contain the consensus sequence immunoreceptor tyrosine-based

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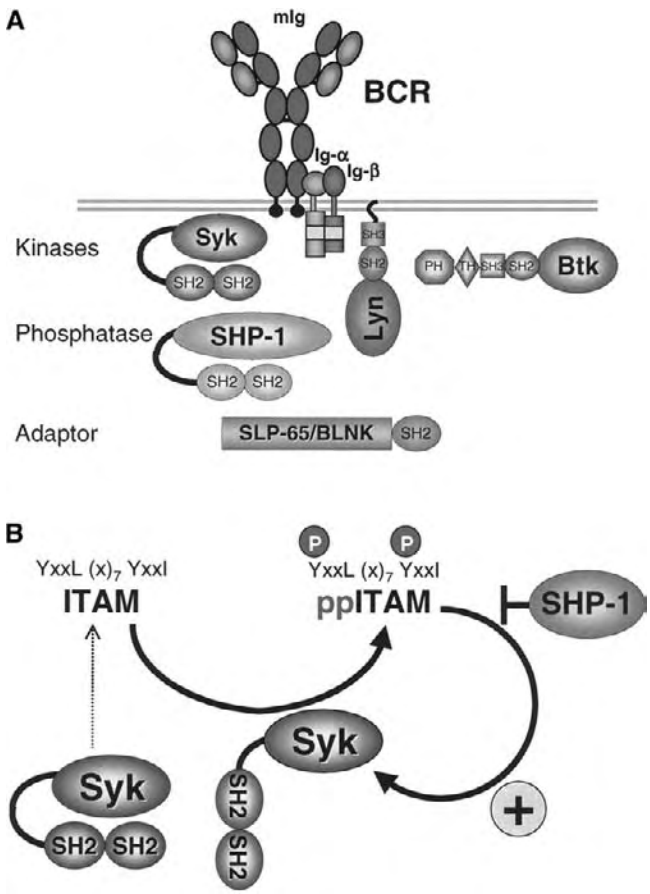


Figure 1 The BCR, its early signaling elements, and an ITAM-mediated positive feedback mechanism. (A) The BCR is comprised of the membrane-bound immunoglobulin molecule (mIg) and the Ig-α/Ig-β heterodimer, which are responsive for antigen binding and signaling, respectively. The early signaling elements of the BCR signal transduction machinery are the tyrosine kinases Syk, Lyn, and Btk, as well as the tyrosine phosphatase SHP-1 and the adaptor protein SLP-65. (B) Syk in its closed conformation is able to slowly phosphorylate the ITAM tyrosines. Once dual-phosphorylated, the ITAM can be bound by Syk via the two Syk SH2 domains, resulting in stabilization of Syk in its open, active conformation. Active Syk can now amplify the initial signal by phosphorylating further ITAMs within the oligomeric BCR complex, thus establishing a positive feedback. SHP-1 can stop the positive feedback of Syk and the ITAM by dephosphorylating the ITAM phosphotyrosines.

activation motif (ITAM), which is also found in other receptors of the multi-subunit immune recognition receptor (MIRR) family and has the following consensus sequence: D/ExxYxxL(x)$_7$YxxL/I. The two tyrosines in the ITAM sequence are phosphorylated during activation of the BCR and become a binding-target for interactions with signal-transducing elements.

Initiation of BCR Signaling Is Controlled by Redox Regulation

How signals are initiated upon antigen binding to the BCR is not completely understood and is still a matter of controversy. It has been found that the three protein tyrosine kinases (PTKs) Syk, Lyn, and Btk, as well as the adaptor protein SLP-65/BLNK and PLC-γ2, are required for an optimal calcium response following engagement of the BCR (Fig. 1A) [6]. In the absence of Syk and Btk, no calcium response is observed, but in the absence of the Src-family kinase Lyn the amplitude of the calcium response is only reduced [7]. A synergy between Src- and Zap-70/Syk family kinases in the tyrosine phosphorylation response was also observed in cotransfection studies with COS cells [8]. These data support the model of sequential interaction between the two PTKs and the antigen receptor. According to this model, it is thought that Lyn is preassociated with the receptor and that, upon cross-linking of the BCR by antigens, Lyn becomes active and phosphorylates the two ITAM tyrosines. This modification then leads to the binding of the two N-terminal Src homology 2 (SH2) domains of Syk to the double-phosphorylated ITAM, cross-wise Syk phosphorylation and activation, phosphorylation of several Syk substrate proteins, and progression of the signal.

Recent data, however, contradict this BCR cross-linking and sequential Lyn/Syk ITAM interaction model. For example, exposure of B cells to reactive oxygen species (ROS), such as H_2O_2 or pervanadate (VaO_4^{3-}/H_2O_2), results in substrate tyrosine phosphorylation and calcium flux in the absence of any engagement of the BCR by antigen [9]. These data suggest that BCR cross-linking is not a mandatory step for signaling; rather, it seems that signal initiation is under the direct control of protein tyrosine phosphatases (PTPs) such as SHP-1, which is efficiently inhibited in contact with H_2O_2 or pervanadate [10–12].

In Lyn-deficient DT40 B cells, Syk becomes activated via the BCR (albeit less efficiently than in Lyn-containing cells), suggesting that Lyn is not located upstream of Syk in the BCR signaling cascade [7]. In a new experimental system allowing the inducible coexpression of the BCR and its signaling elements in *Drosophila* S2 cells, it was found that only Syk but not Lyn can phosphorylate both ITAM tyrosines [13]. Furthermore, in the presence of at least one ITAM sequence, that of either Ig-α or Ig-β, the kinase activity of Syk is strongly increased. These data suggest that initiation of signaling at the BCR involves a positive feedback between Syk and the ITAM sequences resulting in rapid amplification of the BCR signal (Fig. 1B).

A key to understanding this signal amplification process is the regulation of the kinase activity of Syk. A detailed mutational analysis showed that Syk is an allosteric enzyme, whose activity is regulated by its tandem SH2 domains. Apparently, in the absence of an ITAM sequence, Syk resides primarily in a closed conformation in which the two SH2 domains block the kinase domain. Therefore, Syk in solution has only low kinase activity. However, once Syk encounters an ITAM sequence, it phosphorylates the two ITAM tyrosines and binds to this sequence via its tandem SH2 domains. This binding fixes Syk in an open, active conformation, which results in a strongly increased kinase activity. At the same time, the increased activity of Syk is focused at the inner leaflet of the plasma membrane and can rapidly phosphorylate

neighboring ITAM sequences, resulting in more Syk activation and rapid amplification of the signal (Fig. 1B). This amplification process occurs even more efficiently inside an oligomeric BCR complex where ITAM-containing sequences are located close to each other. This Syk activation model is supported by the phenotype of a Syk mutant carrying a C-terminal SH2 domain deficient in ITAM binding. This mutant is nearly as deficient in tyrosine phosphorylation as a kinase-dead mutant of Syk.

Another important protein controlling initiation of the BCR signal is SHP-1 [14]. This PTP was previously thought to play a role only in the termination phase of signaling by dephosphorylating PTK substrates. However, we found that the Syk/ITAM signal amplification process can occur only at the BCR under conditions where PTPs are absent or inhibited. In the S2 cell system, the Syk-mediated signal amplification at the BCR is completely prevented by coexpression of SHP-1 [13]. In this system, the inhibitory function of SHP-1 seems not to be due to a dephosphorylation of Syk but rather to the dephosphorylation of the dual-phosphorylated ITAM, which removes the sequence required for the allosteric activation of Syk. Thus, signal initiation at the BCR not only involves activation of a kinase but also inactivation of a phosphatase. Newer studies on other receptor systems also have found that signal transduction from these receptors requires both kinase activation as well as phosphatase inhibition [12].

Given that kinases are often activated by conformational changes, how then are phosphatases inhibited? All phosphatases carry an invariant, reactive cysteine (Cys–SH) in their catalytic center that takes part in the removal of phosphate groups from phosphorylated substrate proteins. In the presence of H_2O_2, this cysteine is reversibly oxidized (Cys–SOH), thus the phosphatase activity is transiently inhibited [10]. Via the inhibition of phosphatases, H_2O_2 can function as a second messenger in signal transduction [15]. H_2O_2 is indeed produced in stimulated B and T cells via activation of the membrane-bound NADPH–oxidase complex producing singlet oxygen molecules ($^1O_2$) which react with water to yield H_2O_2. Interestingly, recently it was found that the BCR and TCR, apart from their binding and signaling function, are enzymes that efficiently catalyze this reaction and thus contribute to H_2O_2 production [16,17].

In summary, the following scenario of BCR activation seems feasible. Upon antigen binding, the BCR is rapidly translocated to a membrane region where Lyn and the NADPH–oxidase reside. A transient activation of Lyn and Syk by the BCR give a synergistic signal that activates the NADPH–oxidase which results in increased H_2O_2 production and subsequent PTP inhibition in the vicinity of the BCR. This inhibition of the PTP by Lyn and Syk establishes a double-negative feedback at the BCR that may explain the observed synergy between the two kinases in signal transduction [18]. In the absence of PTP activity, the Syk/ITAM signal is rapidly amplified, resulting in full BCR activation. Thus, at least two feedback loops are involved in BCR signal initiation: a positive feedback between Syk and the ITAM sequences and a double-negative feedback between the ITAM, SHP-1, and the H_2O_2-producing enzymes (Fig. 2).

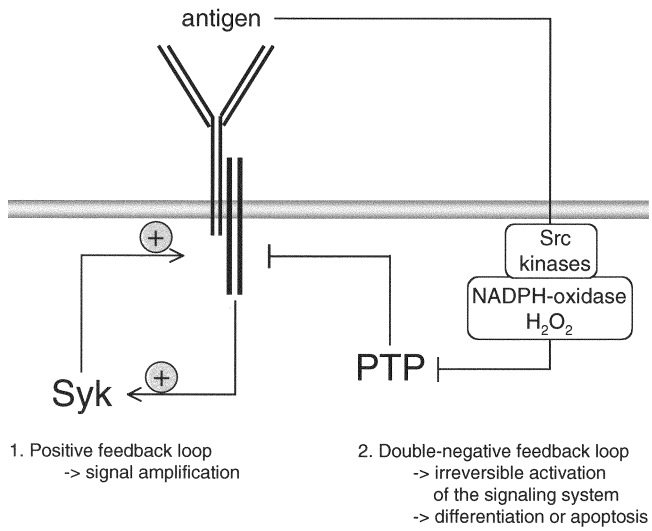


Figure 2 The BCR and its early signaling elements establish a positive feedback as well as a double-negative feedback loop. The positive feedback involving Syk and the ITAMs and the double-negative feedback involving the ITAMs, SHP-1, and the H_2O_2-producing enzymes are described in the text. In principle, double-negative feedback systems have the potential to convert graded inputs into irreversible responses such as differentiation or apoptosis.

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CHAPTER 60

Lipid-Mediated Localization of Signaling Proteins

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Introduction

Extracellular signals elicit responses by binding to receptors at the cell surface and activating signal transducers localized at the inner leaflet of the plasma membrane. Signals propagate to intracellular effectors, resulting in changes in enzyme activity, ion channel gating, or protein–protein interactions. The components of a signaling pathway must be oriented spatially and temporally to execute the appropriate response to an extracellular cue. Many effectors are constitutively present at the membrane, but others must be recruited from the cytoplasm in response to signals. Lipids participate in the localization or recruitment of signaling proteins to cellular membranes through several mechanisms. First, lipids covalently modify proteins, thereby increasing their hydrophobicity and affinity for membranes. Second, lipids anchor proteins to membranes by serving as ligands for protein domains. Third, lipids form domains rich in sphingolipids and cholesterol that may organize signal transduction complexes at the plasma membrane.

Covalent Attachment of Lipids

Some signal transducers do not harbor transmembrane-spanning segments that confer membrane association. Instead, heterotrimeric G proteins, Src family kinases, and monomeric GTPases associate with cellular membranes through covalent lipid modifications [1]. Src family kinases and G-protein α subunits are fatty acylated, whereas G-protein γ subunits and most monomeric GTPases are prenylated. Inhibiting protein lipidation leads to mislocalization of the signal transducer, either to the cytoplasm or intracellular membranes, and an inability to convey the signal from receptor to effector. Thus, covalent lipid modifications are essential for function and are discussed in greater detail in this chapter.

Lipid-Binding Domains

A large number of proteins involved in signal transduction and membrane trafficking contain protein modules that bind to specific lipids embedded in cell membranes [2,3]. The phosphatidylinositol phospholipids (PIs) represent the largest class of lipid ligands. The inositol headgroup can be phosphorylated at the D3, D4, and D5 positions, singly or in combination, thereby generating at least seven unique PI species. Regulation of the production and degradation of these versatile lipids spatially and temporally provides a mechanism for reversible recruitment of proteins to specific cellular membranes. PI-binding protein modules include PH (pleckstrin homology), FYVE (Fab1p/YOTP/Vac1p/EEA1), and PX (Phox homology) domains. Phosphoinositides and their effectors are covered in Volume II, Section E.

Lipid Rafts

Our view of the lateral organization of plasma membrane constituents has evolved from the conventional picture of membrane proteins diffusing freely in a sea of lipid to one where there is selective confinement of lipids and proteins in discrete regions of the membrane [4]. These domains, lipid rafts, are rich in sphingolipids and cholesterol and have been implicated in a variety of cellular processes, including signaling. It has been proposed that the spatial concentration of specific sets of proteins increases the efficiency and

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specificity of signal transduction by facilitating interactions between proteins and by preventing inappropriate crosstalk between pathways. This chapter focuses on how covalent lipid modifications target proteins to rafts.

Protein Lipidation

N-Myristoylation

Protein N-myristoylation is the addition of myristic acid to a protein through an amide linkage to an N-terminal glycine residue. Modification of the protein occurs cotranslationally upon removal of the initiator methionine by methionylpeptidase [5], or posttranslationally following proteolytic cleavage that exposes an N-terminal glycine [6]. N-myristoylation is a stable modification. Examples of signaling proteins that are N-myristoylated can be found in Table 1.

Prenylation

Prenylated proteins are recognized by their characteristic C-terminal motifs (Table 1) [7]. Ras, other monomeric GTPases, and G-protein γ subunits undergo posttranslational processing at a CaaX motif (C, cys; aa, small aliphatic amino acid; X, uncharged amino acid). When the protein terminates in serine, methionine, or glutamine, the protein is modified with a C15 farnesyl isoprenoid. When the protein terminates in leucine, the protein is modified with a C20 geranylgeranyl isoprenoid. The prenyl group is added through a thioether linkage. This is just the first of a series of posttranslational processing steps. Following prenylation in the cytoplasm, the protein associates with the endoplasmic reticulum where the –aaX peptide is proteolytically cleaved, followed by methylation of the carboxyl group on the prenylated cysteine. Although prenylation is a stable modification, methylation of the prenylated cysteine residue is reversible and may be regulated. Members of the Rab family of GTPases are modified with two geranylgeranyl groups at C-terminal motifs –CC or –CXC. Methylation of the prenylated cysteine occurs only on proteins ending with –CXC.

S-Palmitoylation

In addition to modification with amide-linked myristate, proteins are also fatty acylated at cysteine residues through reversible thioester linkages [8]. This is commonly referred to as palmitoylation, although other long-chain fatty acids besides palmitate are incorporated into proteins through this mechanism. Palmitoylation is a posttranslational event that occurs both on intracellular membranes and at the plasma membrane. Palmitate modifies cysteine residues in a variety of sequence motifs (see Table 1). In signal transducers, palmitoylated

Table I Lipid Modifications of Selected Signaling Proteins[a]

Protein	Modified sequence	N-Myr	S-Palm	C15	C20	–aaX	CM
G-protein coupled receptors							
Rhodopsin	-----^{319}TTL**C**C**C**GKN^{326}---	–	+	–	–	–	–
β_2-adrenergic Receptor	-----^{337}QELL**C**LRR^{344}---	–	+	–	–	–	–
Src family kinases							
Src	^{1}M**G**SNKSKPK---	+	–	–	–	–	–
Fyn	^{1}M**G**CVQCKDK---	+	+	–	–	–	–
Lck	^{1}M**G**CGCSSHP---	+	+	–	–	–	–
G-protein α Subunits							
αt1	^{1}M**G**AGASAE---	+	–	–	–	–	–
αi1, αi2, αi3, αo, αz	^{1}MGC---	+	+	–	–	–	–
αs[b]	^{1}MGCLGNSKT---	–	+	–	–	–	–
αq	^{1}MTLESIMACCLS---	–	+	–	–	–	–
α12	^{1}MSGVVRTLSRCLL---	–	+	–	–	–	–
α13	^{1}MADFLPSRSVLSVCFPGCLL---	–	+	–	–	–	–
G-protein γ subunits							
γ1	---^{65}KELKGG**C**VIS-$_{COOH}$	–	–	+	–	+	+
γ2	---^{63}EKKFF**C**AIL-$_{COOH}$	–	–	–	+	+	+
Monomeric GTPases							
K-Ras 4B	---^{175}KKKKKKSKTK**C**VIM-$_{COOH}$	–	–	+	–	+	+
H-Ras	---^{181}CMSCK**C**VLS-$_{COOH}$	–	+	+	–	+	+
Rho A	---^{181}ARRGKKKSG**C**LVL-$_{COOH}$	–	–	–	+	+	+
Rab3a	---^{216}GD**C**A**C**-$_{COOH}$	–	–	–	+	–	+
Rab1a	---^{201}GGG**CC**-$_{COOH}$	–	–	–	+	–	–

[a]Sequences shown are human except for rhodopsin (bovine). N-myristoylated (N-myr) glycines are boldface; S-palmitoylated (S-palm) cysteines are shown in outline; prenylated cysteines are boldface and underlined. C15, farnesyl isoprenoid; C20, geranylgeranyl isoprenoid; –aaX, proteolytic removal of –aaX motif; CM, carboxyl methylation.

[b]Gαs has an unidentified lipid modification on the amino terminus in addition to thioester-linked palmitate at Cys 3.

Source: Kleuss and Gilman, *Proc. Natl. Acad. Sci. USA*, **94**, 6116–6120, 2001.

cysteines are frequently located near N-myristoylated glycine residues or immediately upstream of prenylated C-terminal cysteines. However, some G-protein α subunits are modified exclusively with palmitate. Integral membrane proteins can also be palmitoylated. Many G-protein-coupled receptors are modified at cysteine residues in the C-terminal cytoplasmic domain. Insertion of the fatty acid into the membrane creates a fourth cytoplasmic loop in these serpentine receptors. Palmitate turnover is regulated in response to agonist stimulation on heterotrimeric G proteins and receptors. This topic is discussed in Volume II, Section D.

Other Lipid Modifications

Fatty acylation and prenylation are modifications of intracellular proteins. On the extracellular face of the plasma membrane, proteins are anchored by glycosylphosphatidylinositols (GPI) through insertion of the acyl chains of the phosphatidylinositol moiety into the bilayer [9]. The GPI anchor is added *en bloc* by transamidation to the C-terminal carboxyl group of a protein that has been cleaved previously near the C-terminus. This process occurs in the lumen of the endoplasmic reticulum. GPI-modified proteins then travel through the secretory pathway to the cell surface.

Hedgehog proteins, which are important for growth and patterning in animal development, undergo unusual lipid modifications [10]. Hedgehog is secreted from cells following a complex series of posttranslational processing steps. Following synthesis and translocation into the lumen of the ER, the signal sequence of Hedgehog is cleaved, leaving an N-terminal cysteine residue. Hedgehog then undergoes an autocatalytic cleavage reaction yielding an N-terminal signaling domain and a C-terminal fragment. The signaling domain is modified at its C terminus with cholesterol and with palmitate at its N terminus through a stable amide bond. Lipidation of Hedgehog influences the range and potency of the signals generated. How the lipids exert these effects remains to be determined.

Membrane Interactions

Covalent lipid modifications promote membrane association by direct insertion of the fatty acid or isoprenoid into the lipid bilayer [5]. However, N-myristoylated and prenylated proteins are found in the cytoplasm as well as on cellular membranes, whereas S-palmitoylated proteins appear to be exclusively associated with membranes. Measurements of the apparent affinities of lipid-modified peptides for liposomes demonstrate that a farnesyl or myristoyl group is not sufficient to confer stable membrane association. Thus, proteins modified singly by myristate or a farnesyl isoprenoid are predicted to be exquisitely sensitive to other properties of the protein that promote or interfere with membrane association.

Two well-characterized properties that act cooperatively with N-myristoylation or farnesylation to promote membrane binding are electrostatic interactions or additional lipid modifications [5]. Examples of both mechanisms are found in the Src family kinases. All members of this family are N-myristoylated. Positively charged residues near the N terminus of Src cooperate with the myristoyl moiety to provide a stable membrane anchor by binding to the negatively charged phospholipid head groups of the lipid bilayer. Lck and Fyn are palmitoylated at cysteine residues adjacent to or nearby the myristoylated N terminus. The additional hydrophobicity provided by the thioester-linked fatty acids promotes long-lived interactions with membranes [11]. Similar mechanisms operate in prenylated proteins. Electrostatic interactions promote membrane interactions of the G-protein βγ complex [12] and K-Ras proteins [13]. H- and N-Ras are modified with palmitate subsequent to prenylation and C-terminal processing [13]. In addition, membrane affinity of prenylated proteins is increased approximately 10-fold by carboxyl methylation [11].

How do lipidation motifs target proteins to specific membranes? A short peptide sequence that directs tandem N-myristoylation and palmitoylation is sufficient for plasma membrane localization, suggesting that the lipid modifications themselves can provide specificity [14]. The kinetic membrane-trapping model proposes that an N-myristoylated protein will undergo transient associations with membranes until it encounters a membrane with an appropriate "membrane-targeting receptor" [12]. This interaction leads to the addition of the second lipid modification, palmitate, and the dually lipidated protein is now "trapped" on the membrane. A protein acyltransferase (PAT) at the plasma membrane would fulfill such a role, and PAT activity for N-myristoylated substrates is enriched in the plasma membrane [8]. However, it is clear that protein interactions and lipidation work in concert to localize signaling proteins appropriately. For example, interaction of G-protein α subunits with Gβγ is important for Gα palmitoylation and localization at the plasma membrane [15,16]. Furthermore, not all signaling proteins are palmitoylated at the plasma membrane. Palmitoylation of myristoylated Gα and Fyn appears to be coincident with their arrival at the plasma membrane [17], but other palmitoylated signal transducers such as Lck [18] and H-Ras [19,20] are palmitoylated early in the secretory pathway and then traffic to the plasma membrane. This is consistent with the membrane-trapping model in that palmitoylated H-Ras or Lck move to their final destination as membrane-bound cargo.

Protein Lipidation and Lipid Rafts

Raft lipids have been proposed to exist in a separate phase from the rest of the bilayer, in a state similar to the liquid-ordered (l_o) phase described in model membranes [21]. Lipid raft formation in the exoplasmic leaflet of the plasma membrane is driven by the tight packing of the long saturated acyl chains of sphingolipids with cholesterol. The structure of raft lipids in the cytoplasmic leaflet is less clear, but these domains are also believed to exist in a state where acyl chains of lipids are tightly packed, highly ordered, and extended. Proteins with a high affinity for an ordered

lipid environment are selectively recruited to rafts. These are proteins with GPI anchors (which contain predominantly saturated fatty acids) or proteins such as Src family kinases that are modified with dual fatty acylation motifs. Interestingly, the signaling domain of the Hedgehog protein is also associated with lipid rafts [10]. How the cholesterol and palmitate modifications of Hedgehog contribute to its raft association is unknown. Other lipid modifications exclude proteins from rafts. Proteins modified with unsaturated fatty acids or prenyl groups do not associate with lipid rafts in cells or in model membranes [22–24]. Unsaturated acyl chains or bulky prenyl groups prefer regions of the bilayer that are disordered. Thus, lipid modifications are sufficient for directing proteins to specific subdomains of the plasma membrane.

Summary

In the study of signal transduction, much of the effort in the past has focused on identifying the protein players and how they interact with one another. However, the last decade has led to a greater appreciation of the role that lipids play as second messengers, in protein localization, and in the organization of cellular membranes. These areas will continue to be active areas of investigation as we try to extend our understanding of how cells respond to extracellular signals.

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CHAPTER 61

G-Protein Organization and Signaling

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Introduction

A remarkably large number of extracellular stimuli, including peptides, polypeptide, and glycoprotein hormones, monoamine neurotransmitters, autacoids, photons, odorants, and even ions, such as Ca^{2+}, signal to their target cells through binding and activation of heptahelical membrane receptors that belong to the superfamily of G-protein-coupled receptors (GPCRs). On the inner membrane surface of cells, heterotrimeric G proteins (composed of α, β, and γ subunits) transduce receptor-activated signals into intracellular responses that underlie physiological responses of cells and tissues [1].

In the inactive heterotrimeric state (Gαβγ), GDP is bound to the Gα subunit. Upon activation by a GPCR, guanosine diphosphate (GDP) is released and, in intact cells, guanosine triphosphate (GTP) binds immediately to the empty guanine-nucleotide-binding pocket. An activated GPCR goes through multiple rounds of G-protein activation, leading to signal amplification (Fig. 1). The Gα subunit in the GTP-bound form dissociates from Gβγ and the receptor. Both free Gα–GTP and Gβγ are able to activate downstream effectors. The timing of the activity of the effector is controlled both by the turnoff of GPCRs and by GTP hydrolysis by the intrinsic GTPase activity of Gα, which terminates the actions of both the G protein and its effectors. The turn-off reaction of Gα can be accelerated either by the effector itself or by a family of proteins termed regulators of G protein signaling (RGSs), which act as GTPase-activating proteins (GAPs) to reduce the lifetime of the Gα–GTP form. Gα–GDP reassociates with Gβγ, reconstituting the inactive heterotrimer that is ready for a new activation cycle (Fig. 1). This chapter reviews and summarizes current knowledge on heterotrimeric G-protein structural and functional organization and signaling.

G-Protein Molecular Organization

The crystal structures of inactive (GDP-bound) [2], active (GTP-bound) [3–5], and transition-state ($GDP–AlF_4^-$) [4, 6] $G\alpha_t$, $G\alpha_i$, and $G\alpha_s$, as well as structures for inactive heterotrimeric complexes [7,8], have provided the framework for understanding the biochemical basis of G proteins as molecular switches. In addition, comparison of Gα structures delineates the molecular basis for heterotrimeric G-protein specificity. A discussion of the specific intramolecular contacts within heterotrimeric G proteins can be found in a detailed review [9] and in another chapter of this handbook.

We currently know of 20 Gα, 5 Gβ, and 12 Gγ subunits. On the basis of Gα subunit similarities, G proteins are grouped into four families: $G_{i/o}$, G_s, $G_{q/11}$, and $G_{12/13}$ (Table 1). Classically, members of the $G\alpha_s$ group stimulate isoforms of adenylate cyclase (AC); however, $G\alpha_s$ proteins also modulate the activity of other effector molecules (Table 1). The $G\alpha_{i/o}$ group can be subdivided into $G\alpha_{i/o/z}$ and $G\alpha_{t/g}$ subunits. The former subunits inhibit some isoforms of AC but also interact with and activate other proteins (Table 1). The latter subunits stimulate the retinal cGMP–phosphodiesterase ($G\alpha_t$) and presumably a related gustatory effector ($G\alpha_g$). The $G\alpha_{q/11}$ subunits activate the β-isoforms of phospholipase C (PLC)

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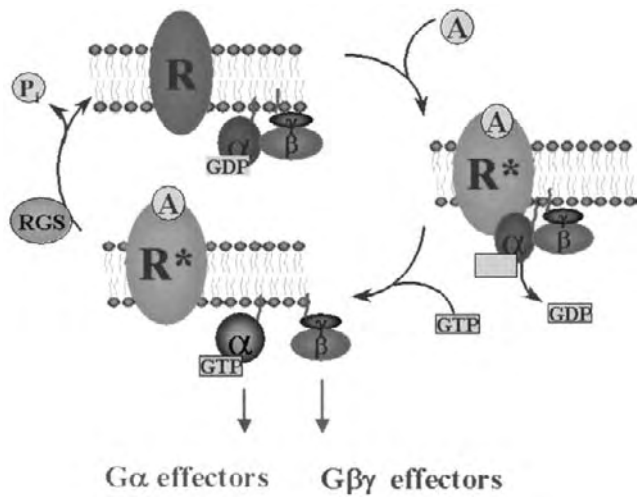


Figure 1 Schematic of the GTP-binding-protein regulatory cycle. Receptor (R) interacts with a specific agonist to induce a conformational change leading to activation (R*). R* is able to interact with the heterotrimer, Gα–GDPβγ and catalyze the release of GDP from the Gα subunit. Upon the binding of GTP, Gβγ and R* are released from Gα–GTP. Gα–GTP and free Gβγ regulate the appropriate effectors. The intrinsic hydrolysis of GTP to GDP, with the assistance of GTPase-activating proteins (RGS proteins) returns the system to the resting, basal state.

Table I Classification of Gα Subtypes and Their Effectors

Family	Subtype	Effector
G_s	$G\alpha_{s(S)}$	↑ Adenylyl cyclase
	$G\alpha_{s(L)}$	↑ GTPase of tubulin[a]
	$G\alpha_{olf}$	↑ src[a]
		↑ Adenylyl cyclase
G_i	$G\alpha_{i1}$	↓ Adenylyl cyclase
	$G\alpha_{i2}$	Rap1 GAP
	$G\alpha_{i3}$	GRIN 1 and 2
	$G\alpha_{oA}$	↑ GTPase of tubulin[a]
	$G\alpha_{oB}$	↑ src[a]
	$G\alpha_z$	Ca^{2+} and K^+ channels[a]
	$G\alpha_{t1}$	↑ cGMP–PDE
	$G\alpha_{t2}$	
	$G\alpha_g$	?
G_q	$G\alpha_q$	
	$G\alpha_{11}$	↑ PLCβs
	$G\alpha_{14}$	↑ Bruton's tyrosine
	$G\alpha_{15}$	kinase ($G\alpha_q$)
	$G\alpha_{16}$	
G_{12}	$G\alpha_{12}$	↑ NH-1[a]
	$G\alpha_{13}$	↑ PLD[a]
		↑ p115RhoGEF
		↑ iNOS[a]

[a]See references [89] to [95].

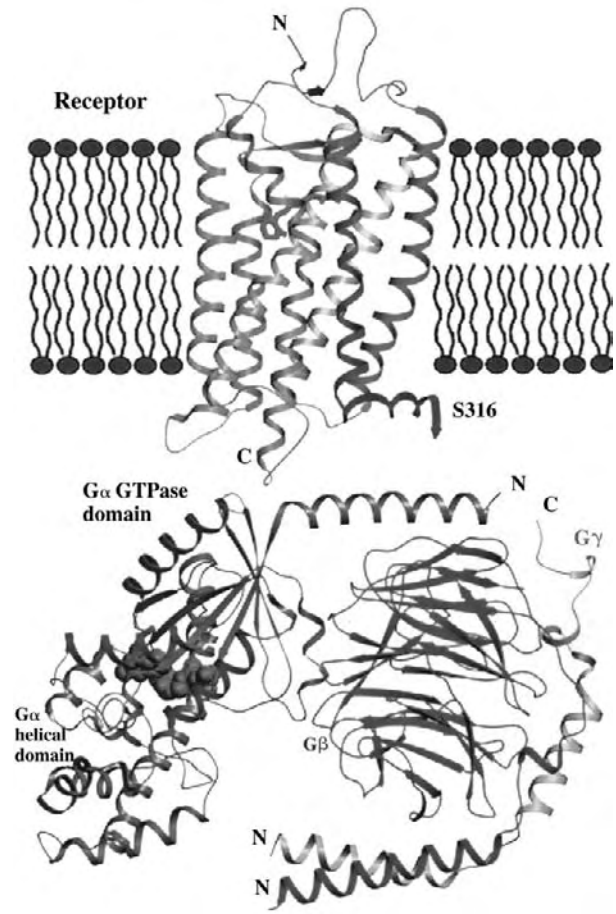


Figure 2 Hypothetical model showing the orientation of rhodopsin, the photoreceptor G protein G_t, and the membrane. The refined rhodopsin structure is from reference [98], and G_t is from reference [7]. The individual ribbon drawings are based on the crystal structures and are shown to scale. The C-terminal residues after S316 are not shown, as there is no evidence that these residues interact with G_t, and they appear to occlude the G_t binding site. The orientation of G_t with respect to rhodopsin and the membrane is modeled on the basis of the charge and hydrophobicity of the surface, the known rhodopsin-binding sites on G_t, and the sites of lipidation of Gα and Gγ [7].

and nonreceptor protein kinases of the *Btk*-family (Bruton's tyrosine kinase). The $G\alpha_{12/13}$ subunits regulate the activity of low-molecular-weight G proteins of the Rho family, the Na^+/H^+-exchanger, phospholipase D (PLD) and the inducible isoform of NO synthase (iNOS).

All Gα subunits are formed by two domains: a GTPase domain that is involved in binding and hydrolysis of GTP and a helical domain that buries the guanine nucleotide within the core of the protein (Fig. 2). The helical domain is the most divergent domain among Gα families and may have a role in directing specificity of interaction with partner proteins. In the GTPase domain, three nonconsecutive flexible regions undergo structural change between the GDP- and GTP-bound conformations [2,3]. These regions, designated Switch I, II and III, become more rigid and well ordered in the GTP-bound conformation. The structures of the extreme amino (N)- and carboxyl (C)-terminal domains are not solved in the isolated Gα crystals, as the N- and C-termini were either removed from the protein or disordered [2–4].

However, in two separate crystal structures of heterotrimeric complexes, the N-terminal helix is ordered by its interaction with Gβ [7,8].

Crystallographic studies of Gβγ heterodimer alone [10] and in the heterotrimeric complex [7,8] revealed that the Gβ subunit has a β-propeller structure containing 7 WD-40 repeats (Fig. 2). The Gγ subunit interacts with the Gβ subunit through an N-terminal coiled-coil and makes extensive contacts along the base of the Gβ subunit. Gβ, but not Gγ, contacts Gα in two regions, the N-terminal helix and a hydrophobic pocket present in Gα-GDP that includes the switch II region [7,8].

Structural Features of G Protein Activation

The rate-limiting step in G protein activation is the release of GDP from the nucleotide-binding pocket. GDP is spontaneously released from the heterotrimeric G protein at a rate that varies depending on the type of Gα subunit. However, in the basal state, the rate of GDP release is much lower than the rate of GTP hydrolysis, and Gβγ binding controls this state. GDP release is greatly facilitated by receptor activation of the G protein [11]. Mutations in the TCAT sequence of Gα in the β6–α5 loop of the GTPase domain enhance receptor-independent spontaneous GDP release [12–14]. In addition to the TCAT motif, residues within the helical domain as well as the N- and C-terminal domains of Gα subunits are mediators of spontaneous GDP release. For example, in $G\alpha_t$, mutations of three residues located on the inward facing surface of the α5 helix dramatically increase the basal nucleotide exchange rate and enhance the receptor-catalyzed nucleotide exchange rate [15]. Mutations in the switch IV helical domain of $G\alpha_s$ decrease the rate of GDP release, GTPγS binding, and GTP hydrolysis [16]; disruption of the contacts between the helical and GTPase domains also influences basal dissociation rates [17,18]. The C-terminal domain of a $G\alpha_t/G\alpha_i$ chimera moves into a more hydrophobic environment upon AlF_4^- activation as determined by the fluorescence increase of a covalently bound probe at Cys^{347} [19]. Substitution of 31 N-terminal residues of a $G\alpha_{t/i}$ chimera (low intrinsic exchange rate) with the corresponding 42 residues of $G\alpha_s$ (high intrinsic exchange rate) significantly enhanced the nucleotide exchange rate [20]. Thus, structural interactions between the N- and C-termini of $G\alpha_t$ appear to be important to the maintenance of a slow GDP release rate for $G\alpha_t$.

Receptor-mediated GDP release is depends on the ability of the GPCR to interact with the G protein and trigger conformational changes in the Gα subunit that cause release of GDP. Comparing the crystal structures with biochemical data, we can deduce that the receptor contacts Gα at a site that is more than 20 Å away from the guanine nucleotide binding site [1,20]; thus, GPCRs work at a distance to release GDP. Interaction of intracellular loops of an activated GPCR with the Gα C-terminus leads to conformational changes that are propagated to the GDP binding site of Gα [1]. However, the requirement of Gβγ for receptor–G-protein coupling and G-protein activation suggests that the heterodimer may actively participate in the GDP release by opening an exit route for the guanine nucleotide [21]. The heterotrimer structure also suggests that the activated receptor could use Gβγ as a lever to release GDP [22]. Thus, the Gβγ dimer may not simply be a passive binding partner stabilizing inactive conformation of Gα, but it may actively participate in receptor-mediated G-protein activation.

Structural Determinants of Receptor–G-Protein Specificity

The last five C-terminal residues of Gα play an import role in receptor–G-protein interaction [21,23,24]. Much experimental evidence supports such a role: (1) ADP-ribsosylation of a Cys residue at position −4 by *Pertussis toxin* uncouples $G_{i/o}$ proteins from receptors [25]; (2) mutations in this region alter receptor–G-protein specificity [26–28]; (3) antibodies targeting Gα C-terminal domains block receptor–G-protein signaling [29]; (4) sequence-specific C-terminal synthetic peptides can stabilize the active agonist-bound form of the receptor by mimicking the G protein [30–32] and serve as competitive inhibitors of receptor–G protein interactions [31–33].

The C-terminus is not the only region that controls the specificity of receptor–G-protein interactions. Several Gα subunits possess identical or nearly identical residues within the extreme C-terminus but exhibit differential coupling to receptors. Two residues within the α4 helix of $G\alpha_{i1}$ are critical for specific coupling between G_{i1} and $5\text{-}HT_{1B}$ receptors [34,35]. Key residues for coupling specificity have also been identified within the N-terminus [27,30,36], the α2 helix and α2–β4 loop regions [37,38], and the α4 helix and α4–β6 loop domain [34,38,39]. Segments of the β and γ subunits also seem to participate in forming the heterotrimer interaction surface for receptors [36,40–43]. Receptor–G-protein specificity is clearly not mediated by only one structural feature, but appears to result from a network of specific contacts between the receptor and heterotrimeric G protein that differ for each G protein and receptor, resulting in a large number of possible combinations on top of a basic general framework.

Gα Interactions with Effector Molecules

The specific effectors activated by Gα–GTP are dependent on the Gα subtype and are summarized in Table 1. Some properties of Gα-mediated effector activation deserve mention: (1) Each Gα family activates a distinct profile of effectors. Co-crystallization of $G\alpha_s$ with the catalytic domains of adenylyl cyclase (AC) has allowed the identification of specific contact sites within the subunit at the α2 helix and α3–β5 loop [44]. In addition to the Gα–GTP bound form the inactive form, Gα–GDP, can also stimulate AC but with

a lower potency [45]. These data suggest that reassociation of Gα with Gβγ is required for complete termination of $G\alpha_s$ signaling. (2) Within a Gα family, each Gα subunit shows a differential profile of effector activation. For example, $G\alpha_{i2}$ was shown to be required to inhibit forskolin-mediated AC activity, while $G\alpha_{i3}$ inhibits $G\alpha_s$-activated AC [46]. (3) Some Gα subunits interact with only one effector, such as $G\alpha_t$ with cGMP-phosphodiesterase, while others couple to several different effectors. For example, $G\alpha_{i2}$ and $G\alpha_{o1}$ can inhibit AC, modulate Ca^{2+} and K^+ channels and other activities [47]. (4) Effectors for some Gα subunits have not yet been definitively identified, while a number of proteins have been recognized to directly interact with Gα subunits. For example, Hart *et al.* [48] recently identified p115RhoGEF as a direct effector for $G\alpha_{13}$. The RGS protein, p115RhoGEF, serves as the GAP for both $G\alpha_{12}$ and $G\alpha_{13}$ but only activated $G\alpha_{13}$ stimulates p115RhoGEF to trigger GDP/GTP exchange on the low-molecular-weight G protein Rho.

Other proteins have been identified via their interactions with certain types of Gα subunits and are considered as possible effector molecules. For example, direct interactions between $G\alpha_o$ and Rap1 GTPase-activating protein (Rap1 GAP), Gz GAP, and RGS17 have been identified using a yeast/two-hybrid screen [49]. These interactions seem to be specific for the G_i protein family. So far, receptor-mediated activation of these proteins has not been demonstrated. Activated $G\alpha_i$, $G\alpha_o$ and $G\alpha_z$ subunits bind to two proteins identified in neuronal tissue, named GRIN1 and GRIN2 for G-protein-regulated inducer of neurite outgrowth [50]. The calcium-binding protein calnuc is considered a potential effector for $G\alpha_{i3}$ and $G\alpha_s$ [51, 52]. Bruton's tyrosine kinase (*Btk*) is a novel effector for G_q proteins, as Gα activates *Btk* both *in vivo* and *in vitro* [53]. This activation is required for receptor-mediated stimulation of p38 mitogen-activated protein kinase (MAPK).

Gβγ Interactions with Effector Molecules

The Gβγ subunit was originally thought to passively facilitate intracellular information transfer by binding to Gα, thus facilitating the return of the heterotrimer to the plasma membrane. Overwhelming evidence now indicates that the Gβγ heterodimer plays a more active role. As mentioned before, Gβγ actively participates in the process of receptor-mediated G-protein activation by directly interacting with the receptor and facilitating GDP release from the Gα binding pocket. Moreover, Gβγ is able to interact with and activate several effectors, including PLCβ2 and β3 [54,55], ACs [56], β-adrenergic receptor kinase [57], phosphoinositide 3-kinase (PI_3 kinase) [58,59], components of the MAPK cascade [60], and K^+ and Ca^{2+} channels (Table 2) [61–64]. Currently, 5 different Gβ and 12 different Gγ subunits have been identified [65–69], so many combinations are possible. Do different dimers have different or similar effector specificity? What is the physiological significance of the formation of different Gβγ dimers?

Table II Effectors Regulated by βγ Dimers

Effector	Regulation
PLCβs	Stimulation
AC I	Inhibition
AC II, IV, and VII	Stimulation
K^+ channel (GIRK 1, 2, 4)	Stimulation
Ca^{2+} channels	Inhibition
G protein receptor kinase	Recruitment to membrane
PI_3K	Stimulation
Bruton's tyrosine kinase	Stimulation
Tsk tyrosine kinase	Stimulation
Protein kinase D	Stimulation
Calmodulin	Inhibition of CaM kinase
Tubulin	Increased GTPase activity
Dynamin I	Increased GTPase activity
Shc phosphorylation[a]	Indirect activation of MAPK (?)
Raf-1 protein kinase	Sequestration of βγ
Ras exchange factor[a]	Indirect activation of MAPK (?)
KSR-1	Sequestration of βγ

[a]See references [96] and [97].

Previously, it was thought that Gβγ dimers were for the most part interchangeable, but current research indicates that the dimer composition determines the quality and efficiency of effector activation and may mediate receptor–G-protein coupling specificity similar to Gα subunits. For example, when nine unique dimers of $G\beta_1$ or $G\beta_2$ with $G\gamma_{(1, 2, 3, 5 \text{ or } 7)}$ were tested for the ability to activate various PLCβ isoforms, all dimers could activate PLCβ isoforms, except the retinal-specific $G\beta_1\gamma_1$ [70,71]. Likewise, $G\beta_1\gamma_1$ is less effective at stimulating ACII and inhibiting ACI than other Gβγ combinations [70,71]. The $G\beta_5\gamma_2$ dimer is a much weaker inhibitor of ACI, ACV, and ACVI than $G\beta_1\gamma_2$. In addition, $G\beta_1\gamma_2$ stimulates ACII activity, while $G\beta_5\gamma_2$ inhibits the activity of this enzyme [72]. However, both dimers activate $PLC\beta_2$ with similar potency and efficacy [73]. The various Gβ subtypes also inhibit the N-type voltage-dependent Ca^{2+} channels with different potency, such as $G\beta_1 = G\beta_2 > G\beta_5 >> G\beta_3 = G\beta_4$ [74]. These data demonstrate that the primary sequence of the Gβ subunit is a major determinant for effector coupling and efficiency.

Other proteins have recently been found to interact with Gβγ subunits. In many cases, the Gβγ and Gα subunits interact with a number of common effectors, such as PLCβ, Bruton's tyrosine kinase, and certain AC isoforms. These effector interactions can be independent, synergistic, or antagonistic. In addition, Gβγ dimers interact with a number of novel effectors that are not regulated by Gα subunits. Putative Gβγ effectors recently identified include protein kinase D (PKD) [75], PI_3 kinase [58,59], tubulin [76], KSR-1 [77], dynamin I [78], calmodulin (CaM) [79], Raf-1 protein kinase [80], and Tsk protein kinases [81] (Table 2).

Recently, our laboratory has found that the receptor for activated C kinase 1 (RACK1) and the dynein intermediate chain interact with the $G\beta_1\gamma_1$ dimer [82]. Gβγ can inhibit neurotransmitter release independently of second messenger formation and ion channel modulation, perhaps by direct interaction with the exocytotic fusion machinery, as both syntaxin 1B and SNAP25B are Gβγ binding partners [83].

Unlike Gα, the conformation of Gβγ dimers does not change significantly between the inactive heterotrimeric complex and the free, active state. The only known exception is that phosducin binding to Gβγ induces a conformational change mainly in blades 1 and 7, thus preventing Gβγ association with additional effectors [84]. Several site-directed mutagenesis studies [85–87] have indicated that each effector contacts a unique but overlapping set of residues on Gβ and some of these sites also represent Gα interacting sites. Indeed, these studies are consistent with the idea that interaction with Gα precludes Gβγ binding to effector molecules.

Conclusions

Structural and functional aspects of heterotrimeric G proteins, their binding partners, and the signaling networks they participate in are the subjects of intense investigation. Dramatic progress has been made in recent years. The next frontier is to understand how signaling pathways interact with each other to form signaling networks [88]. Cells are bombarded by a multiplicity of ligands, and the cellular response is somehow integrated based on all its responses. The experimental approaches to this problem are beginning to be available but are in their infancy. Certainly, many new approaches to these issues of complexity in cellular signaling must be pioneered and will surely lead to new insights.

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CHAPTER 62

JAK–STAT Signaling

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Introduction

Initially brushed aside as "just another kinase," JAKs are now known to be central to signal transduction pathways involved in hematopoiesis and immune function. The JAK tyrosine kinases are associated with cytokine receptors and, along with the STAT transcription factors, are central components of pathways that result in the activation of gene expression upon cytokine stimulation [1]. These pathways involve a series of tyrosine phosphorylation steps resulting in the dimerization of cytoplasmic STATs and their translocation to the nucleus, where they activate gene expression. Both JAKs and STATs are large, multidomain proteins, and we are only beginning to understand the organization of these components at the biochemical and structural level. This article summarizes our current understanding of the initiation, organization, and downregulation of the JAK–STAT signaling pathway.

Cytokine Signaling Proteins

Cytokines are a loosely defined set of secreted factors that control a variety of important biological responses related to hematopoiesis and immune function. There are over 40 members of this family of small (≈15–30 kDa) glycoproteins, and many of them display functional redundancy [2]. Cytokines bind receptors that are characterized by sequence and structural similarities in their extracellular regions and the lack of catalytic domains in their intracellular portions [3]. The intracellular regions of these receptors are associated with the Janus tyrosine kinases (JAKs), which are activated upon cytokine binding to the receptor. The JAKs then phosphorylate and activate a family of intracellular proteins known as STATs (signal transducers and activators of transcription), which translocate to the nucleus and activate gene expression. This signaling pathway, referred to as the JAK–STAT pathway, is notable for the direct and rapid transmission of the signal from the cell surface to the nucleus. The essential steps in the JAK–STAT pathway are outlined in Fig. 1.

Cytokine receptors signal as oligomers, ranging from dimers to tetramers [4]. These receptors have been broadly classified into two subgroups (type I and type II cytokine receptors), based on patterns of conserved amino acid residues within their extracellular domains [5,6]. Type I cytokine receptors include receptors for interleukins, colony-stimulating factors, and hormones; type II receptors include the receptors for interferons and interleukin-10 [7]. Cytokine receptors have also been classified into subgroups based on the use of shared subunits [8]. There are three subfamilies of cytokine receptors that share common signal transducing receptor subunits within the family: (1) interleukin-6 (IL-6) subfamily of receptors, which have a common gp130 subunit; (2) granulocyte–macrophage colony-stimulating factor (GM-CSF) subfamily, which have a common β subunit (β_c); and (3) the interleukin-2 (IL-2) subfamily of receptors that share a γ subunit (γ_c). In each case, the multi-subunit receptor consists of one or more ligand-specific subunits and a common subunit that is essential for signal transduction. Thus, different cytokines can bind to distinct receptors that share a signal transducer.

The four mammalian JAKs are JAK1, JAK2, JAK3, and Tyk2. The JAKs have two tandem kinase-like domains, one of which is a functional catalytic tyrosine kinase domain and is located at the C-terminal end of the protein. Immediately upstream of this functional kinase domain is a non-functional pseudokinase domain [3] that possesses many of the sequence motifs of tyrosine kinases but lacks several residues that are essential for kinase activity.

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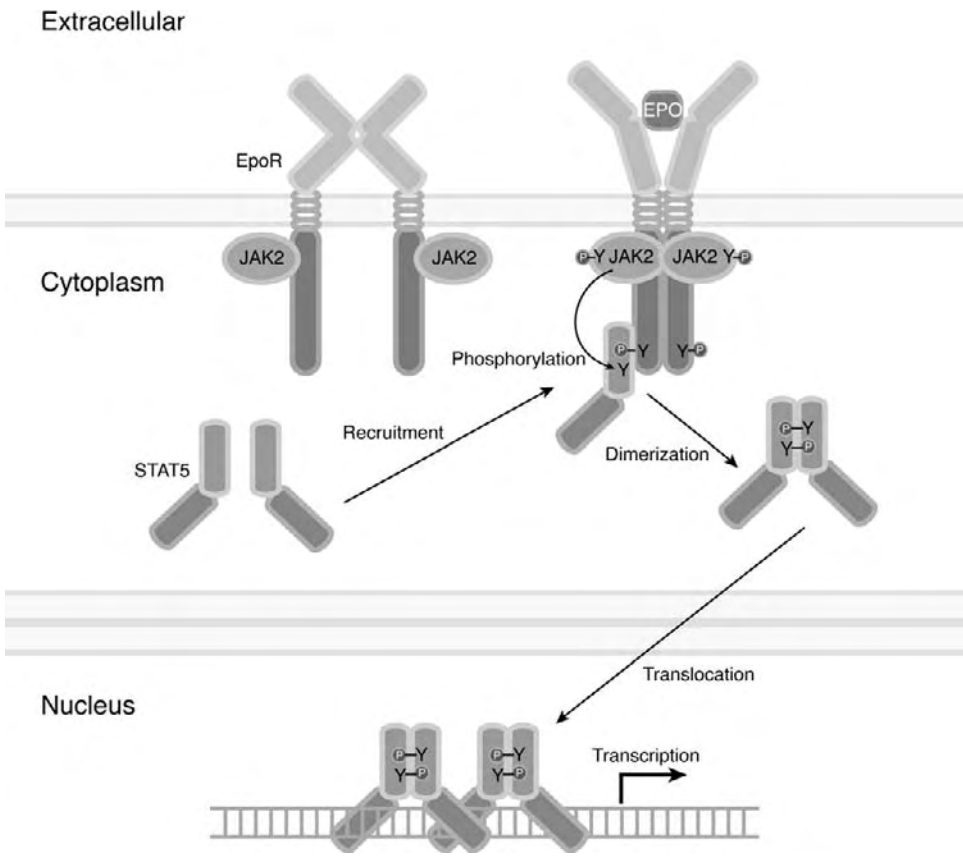


Figure 1 An overview of the JAK–STAT signalling pathway. The binding of erythropoietin (Epo) to its homodimeric cell surface receptor (EpoR) leads to a conformational change resulting in the apposition and transphosphorylation of receptor-associated JAK2 molecules. JAK2 phosphorylates EpoR on tyrosine residues, creating docking sites for the SH2 domains of STATs. The receptor-bound STAT5 molecules are phosphorylated by JAK2, leading to their dimerization via reciprocal SH2–phosphotyrosine interactions. Dimeric STAT5 molecules translocate to the nucleus, where they bind DNA and activate gene transcription.

The cytokine receptors and the JAK kinases are associated constitutively, and this requires an ≈60-amino-acid membrane proximal domain in the signaling receptor. This domain contains two sequence motifs, referred to as box 1 and box 2, that are conserved in most cytokine receptors [9]. It is believed that ligand binding to the extracellular portion of the receptor causes the two JAK molecules associated with the intracellular region of the receptors to come into apposition, such that they are now able to phosphorylate each other. This phosphorylation occurs at tyrosines in the activation loop of the functional kinase domain, in the (E/D)YY motif that is conserved in all JAKs [10]. This transphosphorylation process is essential for the activation of the tyrosine kinase.

Some examples of the various receptor types and associated JAKs are shown in Fig 2. The simplest signaling complex is that of the homodimeric hormone receptors, such as the erythropoietin receptor (EpoR) and JAK2. Structural studies on the unliganded and ligand-bound extracellular domains of the EpoR have revealed some surprises and changed the way we view ligand-mediated tyrosine kinase activation. Contrary to the idea that receptor activation is brought about by ligand-induced oligomerization, the unliganded EpoR extracellular domain crystallizes as a dimer [11]. This dimer, however, is in an open scissor-like conformation, in which the transmembrane domains are separated by ≈70 Å, suggesting that the receptor-associated JAKs would be too far from each other to allow transphosphorylation. Structures of liganded receptors have shown how a single ligand molecule binds two Epo receptors, with two distinct surfaces of the ligand making contact with equivalent binding regions on the two receptors. This causes a conformational switch in the molecule, resulting in the transmembrane regions coming closer together so that they are now separated by only ≈30 Å [12–14]. This would bring the two JAK molecules sufficiently close to each other so as to promote transphosphorylation. It is not yet certain if this mechanism is applicable to all cytokine receptors, as there are no structures of unliganded cytokine receptors other than for EpoR.

JAK Structure and Localization

The JAK kinases are relatively large proteins, with molecular weights of approximately 120 to 140 kDa. Seven regions

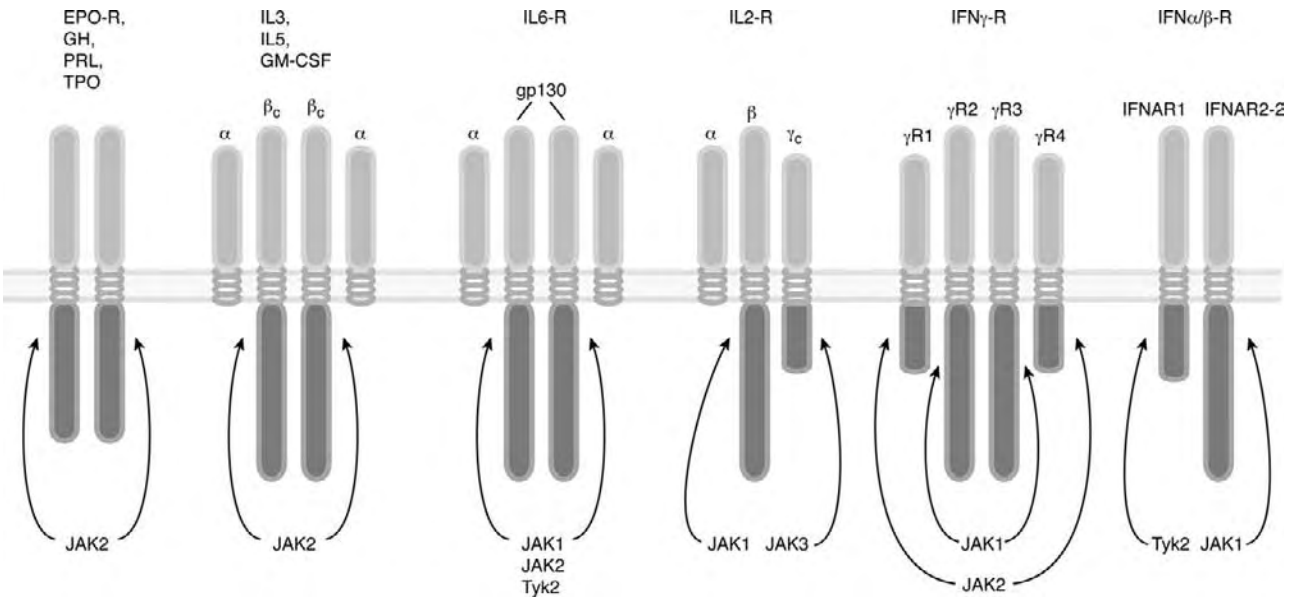


Figure 2 Cytokine receptor families and associated JAKs. From left to right, (1) the homodimeric hormone receptors, which signal via JAK2; (2) the IL-3 family of receptors, each having a unique ligand binding α chain and sharing a common beta (β_c) subunit associated with JAK2; (3) the IL-6 subfamily of receptors, which have a common gp130 subunit that can interact with different members of the JAK family; (4) the IL-2 receptor family, which shares a common gamma (γ_c) subunit and is the only cytokine receptor subunit that interacts with JAK3. The receptor-specific α or β subunit binds JAK1. The type II cytokine receptors, the interferon γ (5) and interferon α/β (6) receptors, signal via JAK1, JAK2, and Tyk2. (Adapted from Pellegrini, S. and Dusanter-Fourt, I., *Eur. J. Biochem.*, 248, 615–633, 1997.)



Figure 3 Structure of JAKs. The JAK family of kinases contains seven conserved sequence regions, JAK homology (JH) domains 1 to 7. The JH1 domain is a tyrosine kinase domain, and the JH2 domain is a pseudokinase domain without catalytic activity but is essential for normal JAK function. The region encompassing the C-terminal portion of JH4 and the JH3 domain has sequence similarity with SH2 domains. The N-terminal region of JAKs contains a FERM domain, which is critical for their association with the receptor and for kinase function.

with conserved sequence have been identified within the JAKs, and these are designated JAK homology (JH) domains 1 to 7 (Fig. 3) [4]. JH1 refers to the functional kinase domain, and JH2 is the pseudokinase domain. Sequence analysis showed that the region spanning JH7 to the N-terminal half of JH4 contains a domain known as the FERM domain (band four-point-one, ezrin, radixin, moesin homology domain) (Fig. 3) [15]. In other proteins, this domain of ≈300 amino acids is involved in binding to the cytoplasmic regions of several transmembrane proteins, thereby localizing the FERM domain containing protein to the plasma membrane [16]. The FERM domain of JAKs is now known to be responsible for anchoring JAKs to the cytoplasmic region of cytokine receptors [17,18]. Crystal structures of the FERM domains of radixin [19] and moesin [20] reveal three subdomains that form a compact, clover-shaped structure. Although there is only a low level of sequence identity between this segment of the JAKs and other FERM domains, there are several conserved blocks of sequence within this region [15], suggesting that the overall structures may be similar. The region spanning the C-terminal part of JH4 to JH3 has sequence similarity to Src homology 2 (SH2) domains, the modular phosphotyrosine binding domains [21,22]. As with the FERM domain, the level of sequence similarity of the JAK SH2-like domain with other SH2 domains is very low, and to date the function of this domain in JAK kinases is not known.

Certain mutant forms of JAK3 isolated from patients with severe combined immune deficiency (SCID) revealed point mutations in the FERM domain [18]. Interestingly, these mutations not only disrupted kinase-receptor association, but also abrogated adenosine triphosphate (ATP) binding to the kinase domain, thereby destroying kinase activity. The mutation Gly 341 to Glu that maps to the FERM domain of the *Drosophila* Hop protein (a JAK homolog) has been shown to hyperactivate the kinase [23]. Furthermore, mutations in the pseudokinase domain of JAKs have been shown to alter the activity of the kinase, with different mutations resulting in either a loss of kinase activity [24] or a hyperactive kinase [25]. These results suggest a complex interplay between the various domains of JAKs, an understanding of which will require a crystal structure of the entire JAK molecule.

STAT Structure and Function

The final signaling components in the JAK–STAT pathway are the STAT molecules. STATs are so named because they serve as both signal transducers in the cytoplasm and activators of transcription in the nucleus. Seven mammalian STAT proteins have been discovered so far [26]. Like the JAKs, STATs are also large, multidomain proteins, and the availability of three-dimensional structural information on STATs has provided us with a deeper understanding of the molecular mechanism of STAT activation [27,28]. STATs possess a DNA binding domain and a transcription activation domain. In addition, each STAT molecule also contains an SH2 domain that acts as a phosphorylation-dependent switch controlling the activation of STATs. Phosphorylation of the cytokine receptors by activated JAK kinases creates docking sites for the STAT SH2 domains, thereby recruiting STATs to the receptor–JAK complex (Fig. 1). The JAKs then phosphorylate a tyrosine residue located C-terminal to the STAT SH2 domain [29], following which the SH2 domains and phosphotyrosines in each of the two STATs interact in a reciprocal manner to form a dimer [30]. This dimer is then translocated to the nucleus, where it binds DNA and directs specific transcription initiation.

Crystal structures of tyrosine-phosphorylated STATs bound to DNA revealed that STAT dimers form C-shaped clamps around DNA that are stabilized by interactions between the SH2 domain of one monomer and the tyrosine-phosphorylated, C-terminal segment of the other (Fig. 4) [27,28]. STATs have been shown to form dimer–dimer complexes on promoters containing two neighboring STAT binding sites [31–33]. This interaction between STAT dimers is cooperative and is mediated by the amino-terminal 130 residues that form a separable functional domain (N-domain) [31,32]. STATs interact with a number of transcription factors and other proteins that form part of the transcription machinery via various domains, including the C-terminal transcription activation domain [26], the structure of which is not yet known. These interactions result in the formation of a cluster of proteins that form a transcription enchancer complex, termed an *enhanceosome* [34]. This complex is responsible for the final step in the JAK–STAT pathway—that is, the activation of gene expression.

Inhibition of Cytokine Signaling

The JAK–STAT pathway is turned off some time after signaling is activated. There are three classes of proteins that

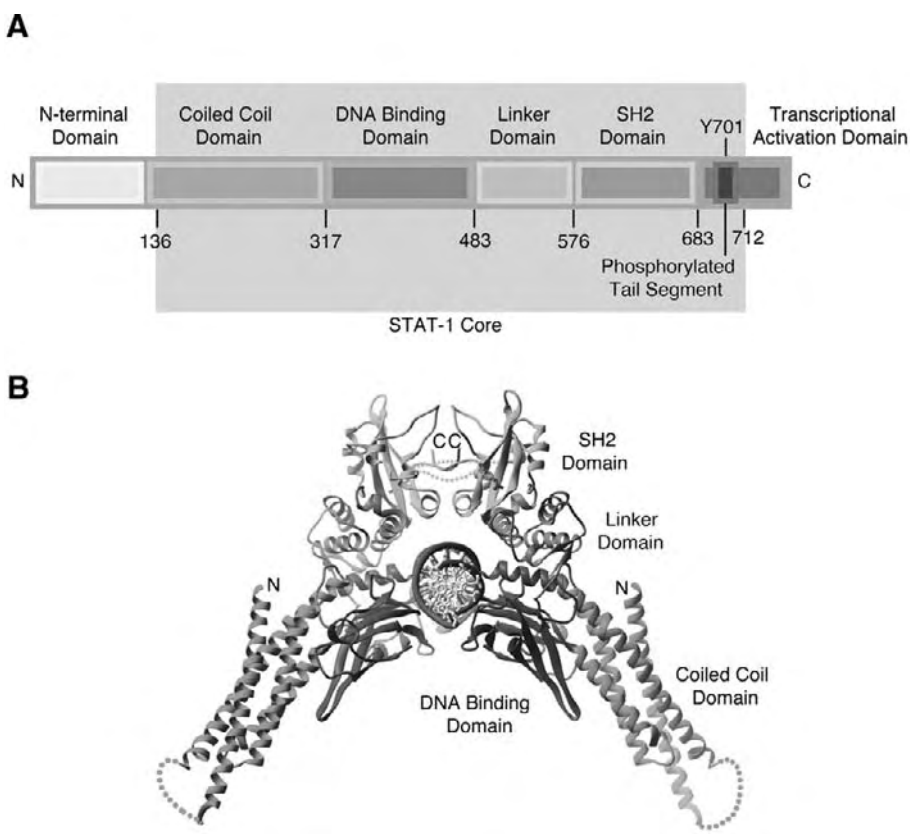


Figure 4 Structure of STATs: (A) schematic diagram showing the domains of STAT1; (B) crystal structure of the core domain of STAT1 bound to DNA. Dimeric STATs form a C-shaped clamp around DNA that is stabilized by reciprocal interactions between the SH2 domain of one monomer and a phosphorylated tyrosine of the other. The phosphotyrosine-binding site of the SH2 domain in each monomer is coupled structurally to the DNA-binding domain, suggesting a potential role for the SH2–phosphotyrosine interaction in the stabilization of DNA interacting elements. (Part B from Chen, X. *et al., Cell*, **93**, 827–839, 1998. With permission.)

deactivate cytokine signaling at a number of levels [35]. Because the entire cascade is dependent on tyrosine phosphorylation as an activation signal, dephosphorylation by tyrosine phosphatases is obviously an important regulatory step. Specifically, the SH2 domain containing protein tyrosine phosphatase, SHP-1, binds tyrosine-phosphorylated cytokine receptors, such as EpoR, via its SH2 domain and dephosphorylates JAK2 [36]. SHP-1 has also been shown to associate directly with and dephosphorylate JAK2, and this association is independent of the SH2 domain [37]. Tyrosine phosphatases are also implicated in the dephosphorylation and consequent inactivation of phosphorylated STAT molecules, although a specific phosphatase–STAT association has yet to be demonstrated [38].

Another class of proteins involved in switching off the JAK–STAT signal is the suppressor of cytokine signaling (SOCS) family of proteins [39]. SOCS proteins contain a central SH2 domain flanked by an N-terminal domain of variable length and sequence and a C-terminal region containing a conserved motif called the *SOCS box* [38]. SOCS proteins inactivate JAK–STAT signaling by different mechanisms; SOCS-1 (also known as STAT-induced STAT inhibitor [SSI-1] or JAK2 binding protein [JAB]) binds JAKs in their activation loop in a phosphorylation-dependent manner and blocks ATP binding to the kinase, thereby inhibiting any further kinase activity [40–42]. Another member of the SOCS family, CIS (cytokine-inducible, SH2-containing protein), directly binds phosphorylated tyrosine residues on the cytokine receptor, blocking STAT recruitment and phosphorylation [43]. Interestingly, as the name SSI-1 suggests, transcription of the SOCS genes is induced by cytokines, at least partially via STAT transcription factors, thereby forming a negative feedback loop. Recent studies have shown that the SOCS box interacts with components of the proteasome machinery, suggesting that binding of SOCS to the receptor–JAKs complex might target them for ubiquitination and degradation [44].

The third class of JAK–STAT negative regulators is the protein inhibitors of STAT (PIAS) family of proteins [35,45]. Unlike SOCS, PIAS proteins are expressed constitutively but associate with STATs only upon stimulation of the cell by cytokines. PIAS proteins bind activated STAT dimers and inhibit their DNA binding activity. It is thought that these proteins might buffer the concentration of active STAT dimers in the cell.

Summary

In conclusion, the JAK–STAT pathway is a rapid membrane-to-nuclear, signaling pathway that is chiefly responsible for proliferation and differentiation of cells of the immune system. Perturbation of this pathway is seen to be an underlying cause of a variety of diseases [46]. Given that the JAK–STAT pathway was only discovered about a decade ago, the pace at which we have progressed in our understanding of this signaling mechanism has been rapid. There is hope that it will not be long before a biochemical and structural picture of the JAKs and the STATs is fleshed out in detail.

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CHAPTER 63

Organization of Photoreceptor Signaling Complexes

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Phototransduction is the conversion of light into a change in the electrical potential across the cell membrane. This process involves the sequential activation of a series of signaling proteins, leading to the eventual opening or closing of ion channels in the photoreceptor cell membrane. Traditionally, it has been thought that G-protein-coupled signaling pathways, like phototransduction, transmit signals from one signaling protein to the next by diffusing freely about a cell, until randomly contacting the appropriate downstream component in the pathway. Recently, however, the phototransduction cascade in *Drosophila* has become a prime example of how signaling proteins do not roam freely about but are physically bound in macromolecular complexes, or signaling complexes [1,2]. An eye-specific scaffold protein binds to multiple signaling components, bringing them into close proximity and localizing them to a subcellular compartment specialized for phototransduction. With increasing evidence of similar strategies used in other cell types and systems, the diffusion-based model of intracellular signaling is being replaced by a model in which the formation of macromolecular complexes is essential for normal signaling [3–5].

Phototransduction is a prototypical G-protein-coupled signaling pathway. Vertebrates and invertebrates share a similar overall strategy but differ in their underlying molecular machinery [6,7]. In *Drosophila*, light stimulation of the seven-transmembrane domain receptor rhodopsin leads to the activation of a G protein, $G_{\alpha q}$, which in turn activates a phospholipase Cβ (PLCβ). Activated PLCβ catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphophate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG). These events lead to the opening of at least two types of cation channels encoded by the *transient receptor potential* (*trp*) [8–10], *trp-like* (*trpl*) [10,11], and *trp*γ [12] genes. Deactivation of the light response includes calcium-dependent activation of an eye-specific protein kinase C (eye-PKC) [13,14] and calmodulin [15].

INAD Organizes Signaling Complexes

Phototransduction in *Drosophila* is one of the fastest G-protein-coupled signaling pathways known, taking less than 20 ms from light activation to maximum response. This high speed of signaling is primarily due to the incorporation of signaling components into multi-protein complexes. Signaling complexes bring components into close proximity, promoting rapid interaction as well as ensuring the proper subcellular localization of components. The central organizer of these signaling complexes is the inactivation/no-afterpotential D protein (INAD), which functions as a scaffold for complexes. The original *inaD* mutant, $inaD^{215}$, was first isolated in a large genetic screen for mutants with a defect in light responsiveness [16]. The *inaD* gene was later identified as the affected gene [17]. The predicted protein sequence of INAD was found to contain five protein–protein interaction domains called postsynaptic-density-95/Discs-large/ZO1 (PDZ) domains [18]. In general, PDZ domains, like other protein–protein interaction domains, such as Src homology 2 (SH2), SH3, and phosphotyrosine binding (PTB) domains, constitute the glue that holds such macromolecular complexes together [2,5,3,19].

PDZ domains are conserved sequences of 80 to 100 amino acids that have been shown to most commonly bind C-terminal protein motifs (–S/T–X–V/I–COOH, or ϕ-X-ϕ-COOH, where ϕ represents a hydrophobic residue, or –X–X–C–COOH) of proteins [5,20]. Some PDZ domains,

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however, have been shown to bind internal sites of the protein [21,22] as well as other PDZ domains [23,24]. About 200 PDZ-domain-containing proteins have been identified in yeast, *C. elegans*, *Drosophila*, and humans [25]. Many scaffolding proteins have been reported to contain multiple PDZ domains [5,26–28]. PDZ domains are found more frequently in multiples within a single protein than any other protein–protein binding domain [5]. These findings suggest that multiple PDZ domains are a common feature of scaffold proteins.

As its sequence would suggest, the eye-specific INAD protein, which consists almost entirely of PDZ domains (PDZ1 to PDZ5 from N to C terminus), has been found to interact with multiple members of the phototransduction cascade. Studies in both *Drosophila* and *Calliphora erythrocephala*, using co-immunoprecipitation experiments, GST-fusion "pull-down" assays, ligand overlay assays, and the yeast-two-hybrid technique, have revealed three major proteins that interact with INAD: the effector PLCβ, the light-activated ion channel TRP, and the eye-PKC required for normal deactivation [1,2]. Each PDZ domain appears to be specific for a particular protein partner. PDZ3 binds to the TRP channel [18,21,29–32]. PDZ1 and PDZ5 bind to the extreme C terminus (–F–C–A–COOH) and an internal sequence, respectively, of PLCβ [22,33]. PDZ2 and PDZ4 have been reported to bind eye-PKC [18,34]. The interaction of PDZ4 with eye-PKC depends on the C-terminal–T–I–I–COOH of eye-PKC [34].

To understand the functional significance of INAD in photoreceptors, a null mutant of *inaD* (*inaD*^{1}) was isolated and examined [18]. In *inaD*^{1} null mutants, three key observations were made: (1) PLCβ, TRP, and eye-PKC are all mislocalized in young flies; (2) light-responsiveness in these young flies is severely impaired; and (3) with age, flies display a further degradation of PLCβ, TRP, and eye-PKC. In wild-type flies, phototransduction components, including INAD-signaling complexes, are exclusively localized to the rhabdomere (see Fig. 1); the rhabdomere is a specialized subcellular compartment of photoreceptors that contains ≈60,000 tightly packed microvilli, a structure analogous to the membranous discs of the vertebrate rod photoreceptor. In the *inaD*^{1} null mutant, PLCβ, TRP, and eye-PKC are all mislocalized from the time of eclosion (adult fly emergence from its pupal stage): PLCβ and eye-PKC are found in the cytoplasm of the cell body, and TRP localizes to the plasma membrane of the cell body outside of the rhabdomere [18]. While these core components of the INAD-signaling complex are mislocalized, other phototransduction components,

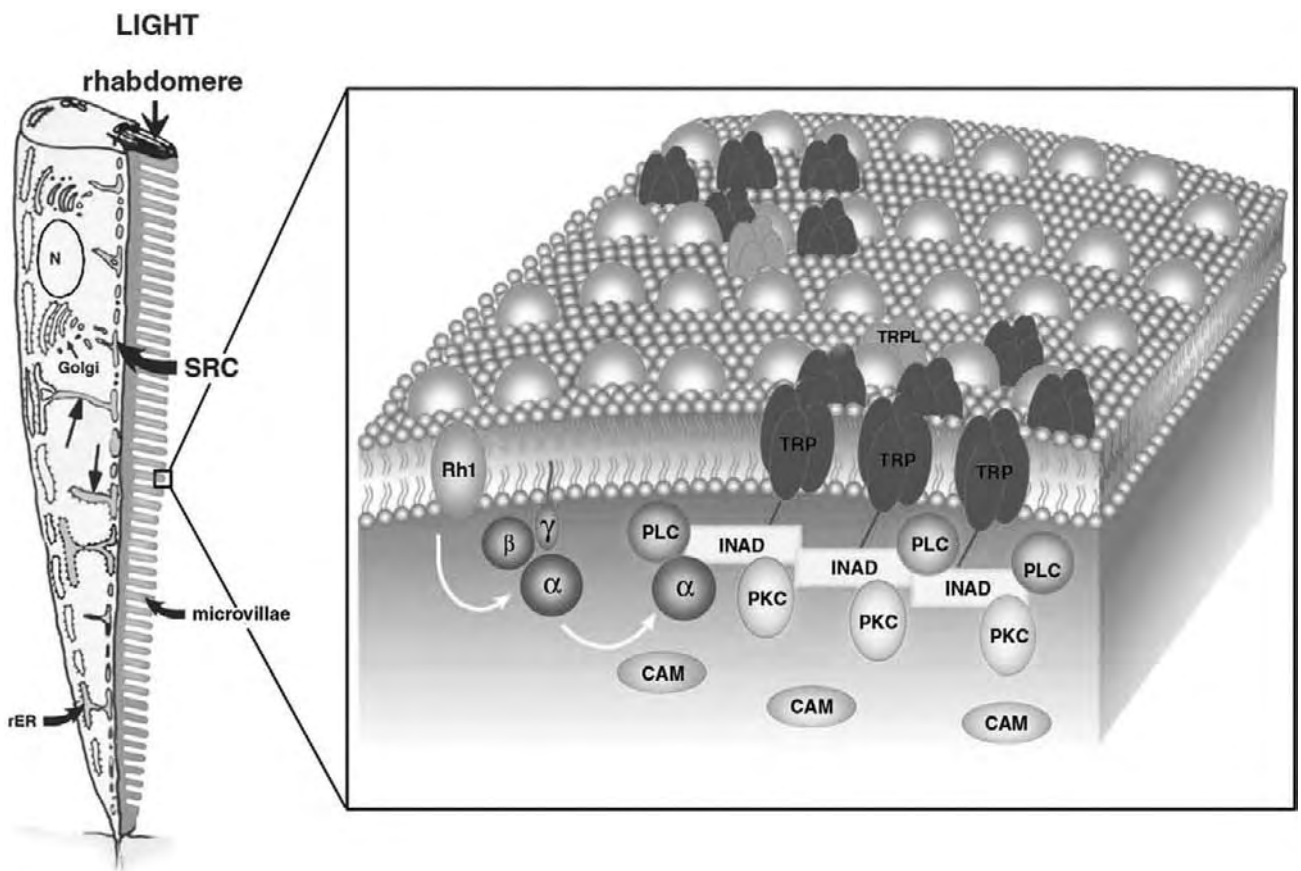


Figure 1 Illustration shows a *Drosophila* photoreceptor with the rhabdomere of the cell indicated. An expanded view of a microvillus membrane from the rhabdomere is shown. INAD-signaling complexes are clustered in the membrane, while rhodopsin molecules (Rh1, gray balls) are distributed randomly at a high density throughout the membrane. (From Tsunoda, S. and Zuker, C. S., *Cell Calcium*, **26**(5), 166, 1999. With permission.)

such as rhodopsin, $G_{\alpha q}$, and TRPL, remain normally localized in the rhabdomeres of *inaD*^{1} null photoreceptors. These results show that INAD is essential for localizing its target proteins to the rhabdomere.

Light responsiveness of photoreceptors was examined in newly eclosed flies by electroretinogram (ERG) recordings and whole-cell voltage-clamp recordings; *inaD*^{1} null flies were found to be nearly blind, displaying very small responses with slowed activation and deactivation kinetics [18,35]. Although newly eclosed *inaD*^{1} null flies have somewhat reduced levels of PLCβ, TRP, and eye-PKC, their mislocalization most likely produces the defective signaling observed. The placement of signaling complexes at the appropriate subcellular location has proven to be critical for signaling in other systems as well. The role INAD plays in assembling signaling complexes, targeting and/or anchoring components to the rhabdomere is just beginning to be explored (see below).

As *inaD*^{1} null flies age, levels of PLCβ, TRP, and eye-PKC all become progressively reduced, while rhodopsin, $G_{\alpha q}$, and TRPL protein levels are unaffected [18,36]. In addition, point mutations in single PDZ domains of INAD affect steady-state protein levels as well as the localization of the corresponding target protein, but not those of any other target proteins [18,30,36]. Thus, each target protein depends on its interaction with the scaffold protein INAD for both its subcellular localization as well as its stability.

In addition to PLCβ, TRP, and eye-PKC, there may be other protein partners that transiently or constitutively interact with INAD. INAD has been reported to bind to rhodopsin, TRPL, calmodulin, and the eye-specific unconventional myosin III, neither-inactivation-nor-afterpotential C (NINA C) [30,37,38]. These interactions are not observed under all conditions and are present in co-immunoprecipitates in much lower levels than INAD, TRP, PLCβ, and eye-PKC [39]. In addition, the localization and stability of rhodopsin, TRPL, calmodulin, and NINA C are unaffected in *inaD*^{1} null mutants [30,38]. Thus, it may be that these interactions are transient in nature. Future studies may reveal their significance.

INAD-Signaling Complexes in Phototransduction

Two of the most significant features of *Drosophila* phototransduction are a high sensitivity to light and a high speed of signaling. A single photon is thought to randomly activate one rhodopsin molecule. Thus, a high sensitivity to light is equivalent to a high probability of capturing any single photon. This is accomplished by having a high concentration of rhodopsin molecules in the photoreceptive membrane. The structure of the rhabdomere, like the discs of the outer segment of vertebrate rod photoreceptors, has evolved to maximize membrane surface area, allowing for the insertion of an enormous number ($\approx 10^8$) of rhodopsin molecules (Fig. 1). In the simplest and most likely scenario, rhodopsin would not be bound to INAD-signaling complexes but would be randomly distributed at a high density throughout the rhabdomeric membrane [1,2,39]. The role of the G protein would be to report the activation of rhodopsin molecules in a local area to the downstream INAD-signaling complexes (Fig. 1). Similarly, the G protein would also not be expected to be a part of the INAD-signaling complexes.

The organization of downstream components into signaling complexes ensures a high speed of signaling. Interaction between proteins in complexes eliminates the reliance on diffusion and coincidental collisions between components. Fast activation and deactivation gives rise to high temporal resolution, essential for a flying organism such as *Drosophila*. In fact, the temporal resolution of the visual system in flies is about five times higher than in humans. It is still unclear, however, whether the high speed of signaling in *Drosophila* photoreceptors is primarily dependent on components being tethered to signaling complexes or on components being concentrated in the rhabdomeres of photoreceptors. Signaling complexes may also prevent cross-talk between different signaling pathways, promoting specificity of signaling. For example, Ca^{2+} ions that enter the cell through TRP channels may reach higher concentrations within a restricted microdomain created by signaling complexes, enabling the specific activation of eye-PKC and calmodulin within the same complex.

Multiple INAD-signaling complexes are proposed to be bound together, making up larger transduction units, or *transducisomes* [18]. Transducisomes may promote coordinated gating of the channels that are opened in response to the activation of one rhodopsin molecule by one photon [35]. Several potential mechanisms may underlie the interaction of multiple INAD-signaling complexes. One possibility is that one INAD scaffold may interact with another INAD scaffold, perhaps via PDZ–PDZ interactions [23,24]. Another possibility is that TRP channel subunits, which form tetramers, may bring as many as four different INAD-signaling complexes together. Because INAD interacts with PLCβ at two different sites, PLCβ could also link two different INAD-signaling complexes together.

Assembly, Targeting, and Anchoring of Signaling Complexes

Because the localization of INAD-signaling complexes to the rhabdomeres of photoreceptors is critical for normal signaling, studies have begun to focus on the underlying mechanism of this subcellular localization. How and where are signaling complexes assembled? One possibility is that signaling components are targeted independently to the rhabdomere, where they are then incorporated into complexes. Another possibility is that signaling components are preassembled into complexes in the soma before being targeted together to the rhabdomere. Recent evidence suggests this latter scenario, with preassembly dependent on the scaffold INAD [32]. Preassembly of signaling complexes may serve as a regulatory mechanism for ensuring that signaling

complexes in the rhabdomere contain the correct stoichiometry of signaling components. To date, almost nothing is known about how INAD-signaling complexes are assembled, how assembly is regulated, and how signaling complexes are targeted to the rhabdomere.

Because INAD is a soluble protein that organizes signaling complexes associated with the membrane, an anchoring mechanism that retains INAD-signaling complexes in the rhabdomere must also exist. Recent studies [31,32,36] suggest that the TRP channel is one component required for anchoring INAD in the rhabdomere. INAD and TRP appear to be targeted independently to rhabdomeres but somehow require one another to remain in the rhabdomeres. Although TRP may serve as an indirect anchor to the cytoskeleton, there is likely to be another anchoring protein of INAD-signaling complexes. Little is known about what other factors and proteins function in targeting, transporting, assembling, and anchoring signaling components/complexes in the rhabdomere.

Signaling Complexes in Vertebrate Photoreceptors

The assembly of phototransduction components into signaling complexes is not limited to *Drosophila*. Vertebrate rod photoreceptor cells have been reported to share a similar organization. In vertebrate photoreceptors, rhodopsin activates the G protein transducin. Transducin activates a cGMP phosphodiesterase (PDE) that hydrolyzes cGMP to GMP. The transient reduction in cGMP levels causes the closure of cGMP-gated channels. The β-subunit of these cGMP-gated channels consists of six-transmembrane-spanning domains, which make up the characteristic structural component of the channel, and a large cytosolic N-terminal region. This large N-terminal region contains a glutamic-acid-rich region, giving this region the name GARP. Two additional splice variants give rise to soluble forms of this GARP region alone; these proteins are called GARP1 and GARP2. GARP proteins are especially intriguing because they contain four homologous repeats (R1 to R4) that have been reported to bind to different components of the vertebrate phototransduction pathway [41]. Affinity chromatography columns carrying GARP repeats R1 to R4 were found to bind to PDE, guanylate cyclase (GC), and a retina-specific ATP-binding cassette transporter (ABCR). Thus, GARP containing cGMP-gated channel subunits or soluble GARP1 or 2 may serve as a scaffold to bring PDE, GC, ABCR, and cGMP-gated channels together into macromolecular complexes. Similar to INAD-signaling complexes, vertebrate signaling complexes do not display significant interaction with rhodopsin or the G protein.

Vertebrate rod signaling complexes are also proposed to be localized to a particular subcellular region; GARPs are localized to the space between the plasma membrane and the edge or rim of the intracellular membranous discs in the outer segment [41]. It has been suggested that GARPs bridge the space between the plasma membrane and membranous discs, forming complexes consisting of cGMP-gated channels in the plasma membrane and GC and ABCR in the disc margins. The interaction of GARP with tubulin and actin may serve to tie signaling complexes to the cytoskeleton, anchoring them at the proper subcellular site.

A growing number of studies are proving that the organization of signaling proteins into macromolecular complexes is a universal cellular strategy not restricted to photoreceptors. Many PDZ-mediated complexes are found at neuronal synapses [27,42–45] and in epithelial cells [46]. Two major functions of these signaling complexes are (1) to bring components into close proximity for fast and specific interaction, and (2) to localize components to specific subcellular sites critical to their function. Understanding how signaling complexes are assembled and localized is likely to reveal much about intracellular signaling as well as how specialized subcellular structures are established and maintained.

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CHAPTER 64

Protein Localization in Negative Signaling

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Introduction

Cellular activation is regulated by the integration of both positive and negative signals. While positive signaling can be simplistically thought of as the initiation of a biochemical pathway leading to activation of a cellular process, negative signaling can have multiple meanings. From the standpoint of a cellular process such as proliferation or protein secretion, the initiation of any pathway that functions to downregulate these events can be defined as negative signaling. For instance, transforming growth factor β (TGFβ) receptor can transduce a negative signal by upregulating cyclin-dependent kinase inhibitory proteins (CKIs) leading to cell cycle arrest [1]. Negative signaling can also refer to the activation of specific pathways that inhibit cellular stimulation, such as the inhibitory function of the glycine and γ-aminobutyric acid (GABA) receptors in neurons. Upon binding to their respective ligand, these receptors undergo a conformational change that allows the entry of chloride ions into the cell, thereby generating a hyperpolarizing inhibitory signal [2]. The signals generated in the above examples have inhibitory consequences for cellular activation; however, the intricacies of these processes can be thought of as being similar to those of positive signaling. In both cases, specific signaling pathways are activated but with differing downstream effects.

This article will focus on a distinct form of negative signaling in which extrinsic cell signals serve to directly antagonize positive signals. Direct antagonism is accomplished either by recruitment of specific proteins that serve to counteract signals generated by a stimulatory receptor or by direct competition with the stimulatory receptor for ligand binding. In general, this form of negative signaling increases the ability of a cell to regulate a given activation pathway by providing a mechanism to attenuate or terminate the response. However, a potential danger in this scenario is that the negative signal could constitutively suppress the activation pathway, thereby rendering it useless. Here, we will discuss how differential protein localization of activating and inhibitory receptors regulates a critical balance between positive and negative signals. The T-cell stimulatory protein CD28 and the T-cell inhibitory protein cytotoxic T-lymphocyte antigen 4 (CTLA-4) will be used as models.

The Role of CD28 and CTLA-4 in T-Cell Activation

T-cell activation is governed by interactions between the T-cell antigen receptor (TCR) and major histocompatability complex (MHC) molecules expressed on the surface of antigen-presenting cells (APCs). When a T cell bearing a specific TCR recognizes an MHC bearing an antigenic peptide, multiple phosphorylation-mediated signaling pathways are initiated, eventually leading to cell proliferation and upregulation of specific gene products involved in T-cell function. While signaling through the TCR is required for T-cell activation, it is not sufficient and a second distinct signal is needed. CD28, expressed on the surface of the T cell, is the most potent generator of this costimulatory signal. Interactions between CD28 and B7 family member proteins expressed by APCs result in enhancement of T-cell proliferation, cytokine production, and resistance to apoptosis [3].

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CTLA-4 is homologous to CD28 and also binds to B7, although with a much higher avidity [4,5]. Both of these proteins are members of the immunoglobulin superfamily and are expressed as disulfide-linked homodimers. Importantly, while CD28 delivers an activating costimulatory signal, CTLA-4 functions to inhibit the T-cell response [6]. Perhaps the most convincing demonstration of the significance of CD28 and CTLA-4 mediated regulation of T-cell activation came from the generation of mice lacking functional gene products. CD28-deficient mice have severely compromised immune responses against a variety of pathogens [7,8], while CTLA-4-deficient mice die of a severe lymphoproliferative disorder at around three weeks of age [9–11].

The activating and inhibitory signals respectively generated by CD28 and CTLA-4 represent an interesting paradigm to study positive and negative regulation of cellular activation. The net costimulatory signal perceived by the T cell is a function of the integration of CTLA-4 and CD28 signals. The trafficking of these molecules during T-cell–APC interactions is a critical factor in regulating the balance between CD28-mediated activation and CTLA-4-mediated inhibition.

Expression and Localization of CTLA-4 and CD28: Consequences for Receptor Function

Although CD28 and CTLA-4 are homologous proteins, they have different expression and trafficking patterns within the cell. CD28 is constitutively expressed by T cells and localizes to the cell surface. In contrast, CTLA-4 is upregulated only after T-cell stimulation [12] and primarily localizes to an intracellular compartment due to a tyrosine-based internalization motif (Yxxϕ) within its intracellular tail [13]. This motif results in the binding of the medium chain of the clathrin-coated pit adaptor complex, AP2, to the intracellular tail of CTLA-4, leading to its endocytosis from the cell surface [14–17]. CTLA-4 may also bind to the adaptor protein AP1, suggesting that intracellular trafficking of CTLA-4 emanating from the Golgi apparatus may also be a highly regulated process [18].

Intracellular sequestration has two important consequences for the ability of CTLA-4 to inhibit the T-cell response. First, sequestration provides a mechanism for decreasing the amount of CTLA-4 that can interact with ligand at the cell surface and thereby deliver an inhibitory signal. Second, it provides access to lysosomal compartments which allows for greater regulation of CTLA-4 expression by increasing the protein turnover rate.

CTLA-4 Expression

CTLA-4 expression is controlled by multiple factors. In addition to transcriptional and translational regulation [19,20], several studies have demonstrated that protein levels are limited through posttranslational mechanisms. While CTLA-4 is found in several different intracellular compartments, a significant portion localizes to lysosomes [18,21,22], presumably accounting for its rapid turnover rate in activated T cells [22,23]. A short protein half-life ensures that CTLA-4 gene activity is tightly linked to expression levels within the cell. Thus, when gene expression is reduced or terminated, protein levels will rapidly reflect this change. A protein with a slower rate of turnover, such as CD28, could persist in the cell even after gene expression had been terminated [23]. Thus, the rapid rate of CTLA-4 degradation provides a high level of control over when and where CTLA-4 is capable of inhibiting the T cell response.

CTLA-4 Protein Trafficking

Because inhibitory signals generated by CTLA-4 are mediated through interactions with B7 molecules on the APC surface, a pathway must exist to specifically localize CTLA-4 to the T-cell plasma membrane. Intracellular CTLA-4 is known to rapidly polarize to a site facing antigenic stimulation upon T-cell encounters with APCs [24]. This pattern of trafficking may serve at least two purposes. First, it may ensure that CTLA-4 interacts only with B7 molecules expressed by APCs that are also displaying antigenic peptide. This would limit the origin of CTLA-4-mediated inhibitory signals to the specific APC with which the T cell is engaged. Second, it provides a regulatory point for controlling CTLA-4 expression levels on the T-cell surface. Factors controlling both exocytosis of CTLA-4 from intracellular vesicles and its subsequent stabilization at the cell surface play an important role in regulating CTLA-4-mediated inhibitory signals (Fig. 1).

During T-cell activation, CTLA-4 surface expression is regulated by at least two events that occur downstream of TCR signaling. First, an increase in intracellular calcium levels can enhance the rate of fusion between CTLA-4-containing vesicles and the plasma membrane, thereby leading to increased surface expression [24]. Second, tyrosine phosphorylation of the intracellular localization motif within the cytoplasmic tail of CTLA-4 prevents interactions with the AP2 complex, suggesting that TCR-mediated kinase activity can stabilize CTLA-4 on the cell surface [14,16]. These trafficking properties may explain the observation that CTLA-4 expression on the cell surface at the T-cell–APC interface is regulated by the strength of the TCR signal. TCR signals of higher quality (affinity of TCR for antigenic peptide–MHC) are more efficient at translocating CTLA-4 from intracellular sources to the T-cell plasma membrane [23]. This may represent a negative feedback mechanism in which a T cell receiving a strong stimulatory signal will specifically recruit CTLA-4 to the cell surface, where it can function to inhibit the response.

By coupling the recruitment of an inhibitory molecule to the stimulatory signal, the cell is able to achieve a higher degree of regulation over cellular activation. Under conditions of

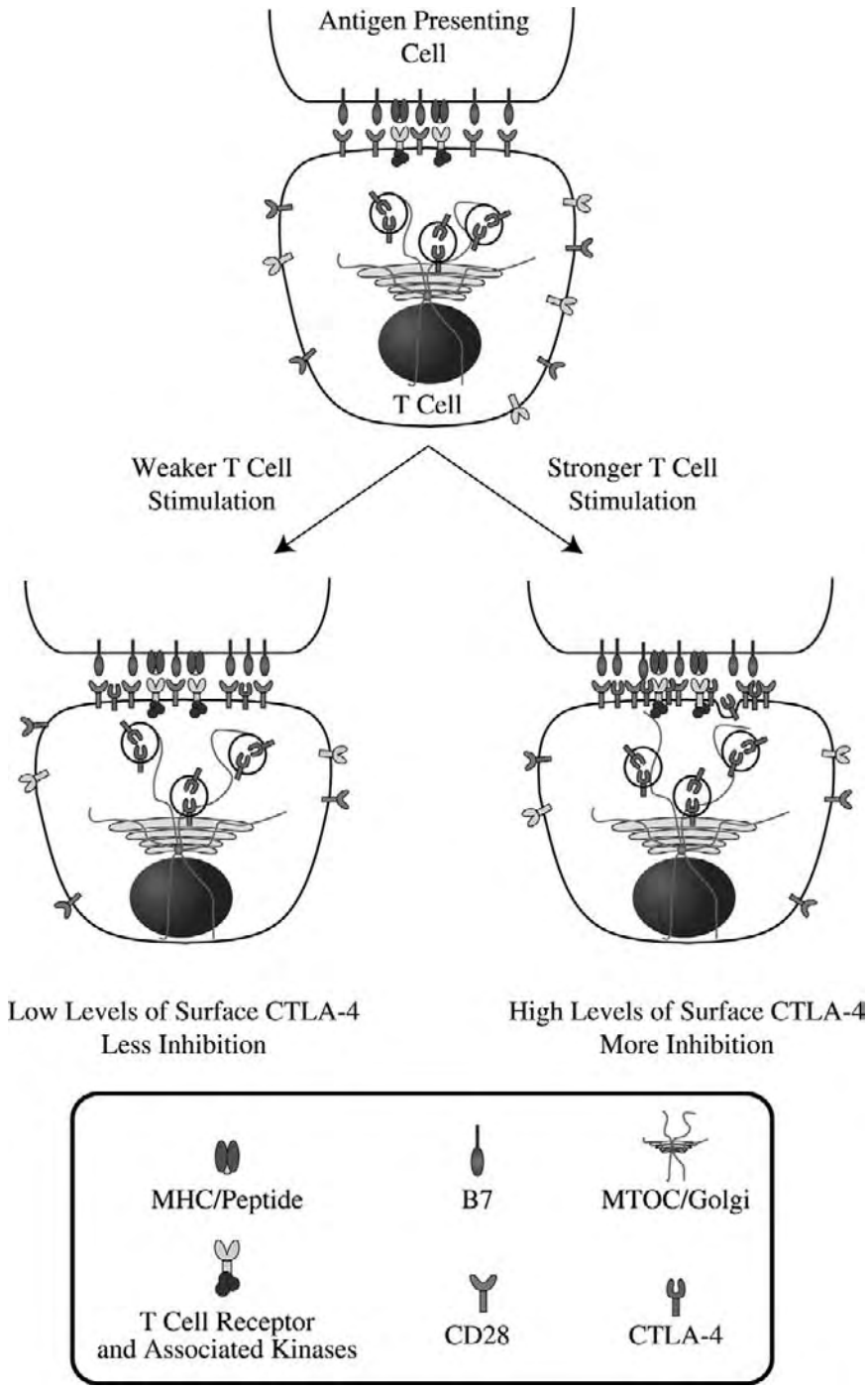


Figure 1 Protein trafficking of the T cell inhibitory protein CTLA-4 during antigen specific T cell-APC interactions. T cell antigen receptor signaling induces the rapid polarization of CTLA-4 toward the site of T cell-APC contact. This is likely caused by a reorientation of the microtubule cytoskeleton and its associated intracellular membrane compartments. Polarization of CTLA-4-containing vesicles enables CTLA-4 to be specifically targeted to the immunological synapse. Importantly, accumulation of CTLA-4 on the T cell surface at the site of contact with the APC is regulated by the strength of the TCR signal, with stronger signals being more efficient at localizing CTLA-4 to the cell surface. This trafficking property is not observed with CD28, a homologue of CTLA-4 which generates stimulatory signals. Because of these unique protein trafficking patterns, CTLA-4 may exert a greater inhibitory effect on T cells that receive stronger levels of stimulation and could thus function as a negative feedback control mechanism for TCR signaling.

weak stimulation, the cell can still respond, as these conditions will not efficiently recruit the inhibitory molecule to a functionally relevant site, yet a mechanism still exists to attenuate or terminate strong stimulatory signals so as not to overload the cellular response.

Mechanisms of CTLA-4-Mediated Negative Signaling

While the precise mechanism of CTLA-4-mediated inhibition remains unclear, several possibilities have been suggested. These include passive models in which binding

of CTLA-4 to B7 molecules can by itself inhibit T-cell activation and active models in which CTLA-4 functions by recruitment of accessory proteins that are capable of limiting the T-cell response. In both of these models, the key component regulating the ability of CTLA-4 to inhibit stimulatory signals is its localization within the cell. The passive models for CTLA-4-mediated inhibition include two distinct but related ideas. First, the finding that CTLA-4 binds to B7 with a much higher avidity than CD28 suggests that CTLA-4 may limit stimulatory signals by out-competing CD28 for ligand binding. This would effectively raise the levels of B7 on an APC required to fully activate a T cell. A second model for CTLA-4 function was recently proposed based on the solution of the B7.2:CTLA-4 crystal structure [25,26]. A single dimeric CTLA-4 molecule was shown to interact with two distinct B7 molecules. This binding mode, which is distinct from other immunoglobulin superfamily member receptor–ligand interactions in which the two chains of a dimeric receptor combine to form a monovalent ligand binding site, may explain the unusually high avidity of the B7:CTLA-4 interaction. Combining these data with evidence that B7 receptors may exist as noncovalent dimers on the surface of APCs [27] has led to a novel model for CTLA-4 function. CTLA-4 may form a lattice structure at the T-cell–APC interface consisting of repeated core units containing a single CTLA-4 dimer binding two distinct B7 dimers. This lattice structure may be sterically unfavorable for the formation of activating complexes at the T-cell–APC interface.

These models suggest that negative signaling by CTLA-4 does not require the specific interaction of accessory proteins with its cytoplasmic tail. This observation is supported by the demonstration that CTLA-4 can function to inhibit T-cell activation even after deletion of its intracellular tail, presumably by competing with CD28 for limited levels of B7 [28]. However, these and other studies have demonstrated that CTLA-4-mediated negative signaling can inhibit T-cell responses even when ligand is not limiting [29,30]. Thus, in addition to functioning through competition, CTLA-4 may also recruit proteins capable of downregulating the T-cell response. In support of this idea, CTLA-4 has been shown to bind the tyrosine phosphatase, SHP2, as well as the serine/threonine phosphatase, PP2A [31,32]. Recruitment of these phosphatases may explain the ability of CTLA-4 to reduce the tyrosine phosphorylation of the TCR complex itself, as well as several downstream effector molecules [28,29,30].

Conclusions

The ability of surface membrane receptors to relay extrinsic signals to the cell is often dependent upon their localization to functionally relevant sites on the cell surface. In activation pathways that are regulated by the convergence of positive and negative signals, differential localization of stimulatory and inhibitory receptors can be critical for regulating a balance between activation and inhibition. The localization patterns of CD28 and CTLA-4 exemplify this concept. Here, the stimulatory receptor is constitutively localized to a site where it is capable of binding ligand, but the inhibitory receptor is recruited from intracellular compartments only after activation. Intracellular retention of inhibitory receptors essentially gives stimulatory receptors a temporal advantage. Without this advantage, the threshold for stimulation may be too high. In general, depending on the potency of the signal, sequestration of inhibitory receptors away from the cell surface may be a requirement for initiation and/or maintenance of activation pathways. Upon activation, inhibitory receptors can then be recruited to the cell surface in a regulated manner where they can function to inhibit a cellular response.

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CHAPTER 65

Transmembrane Receptor Oligomerization

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Introduction

Signal transduction in eukaryotes is initiated from receptors that are activated by the binding of exogenous ligands. The majority of these receptors is localized in the plasma membrane and can be generally separated into several categories based on structure and signaling properties (see Table 1). Although there are many similarities among these families, the most common is the quaternary structure of the activated species. In most (if not all) cases, signaling requires that one or more types of monomers (protomers) assemble themselves in a complex, and the formation of this receptor complex is prerequisite for function. The active complexes can range from simple homodimers to heterotetramers; in some cases, higher order complexes may be required. However, what controls the oligomerization process and the role that ligand plays are, in most cases, poorly defined. While ligand is certainly required to complete the activation, it is less clear whether it is required to induce the formation of the receptor complexes themselves. Evidence supporting both induced and constitutive oligomerized states has been reported for many receptor types, and indeed both mechanisms may be utilized.

The formation of oligomers to generate active (or activatible) species is a common biological mechanism found throughout living systems. It is a primary example of protein–protein interactions, which are a major component of the developing field of proteomics, and it affords several basic physiological advantages [1], including regulatory, kinetic, and specificity opportunities of various types. For example, a simple receptor dimer can easily be viewed as binding its ligand with significantly higher affinity than a monomer due to increased surface binding area. A dimer would also have twice the signaling capacity and provide a simple on/off switch (assuming the monomer is inactive) by dissociating. In addition, the formation of heteromeric complexes offers another means to broaden the range of specificity in terms of both ligand binding and the signals produced. Furthermore, a heterodimer of a potentially active protomer with one that cannot be activated could produce a nonfunctioning complex (even though it can still bind ligands), thereby acting as a negative regulator of receptor signaling. This natural control mechanism has been extensively exploited in the research laboratory in recent years and is referred to as the "*dominant-negative*" strategy. The sections that follow provide a brief description of how oligomerization is utilized by the major cell transmembrane receptor classes.

Tyrosine Kinase-Containing Receptors

It has long been known that cell-surface receptors possessing intrinsic tyrosine kinase activity (RTKs) minimally require a dimeric state for their full activity. It has generally been accepted that RTK dimerization occurs as a result of ligand binding, (i.e., the receptors occur as mono-mers at the cell surface in the unstimulated state), and the ligand-bound monomer then recruits another monomer into the complex, allowing for the interaction of the kinase domains and their subsequent transphosphorylation. The initial phosphorylation events usually occur on the activation loop and help

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Table I

Principal receptor classes	Reported oligomeric states	Examples
Tyrosine-kinase-containing receptors (RTK)	Homodimer	EGFR, TrkA, FGFR, CSF1R
	Heterodimer	EGFR/ErbB2, PDGFRα/β
Cytokine receptors, type I	Homodimer	EPOR, GHR
	Heterotrimer	IL-2Rα/β/γχ, IL-4Rα/β/γχ
	Heterotetramer	IL-6R/gp130, IL-11/gp130
Cytokine receptors, type II	Heterotetramer	IFNγR1/2, IL-3R
Guanylyl-cyclase-containing receptors	Homodimer	ANPR
Serine/threonine-kinase-containing receptors	Heterotetramer	TβR1/2, BMPR1/2
TNF receptors	Homotrimer	TNFR1, Fas, CD40
	Homodimer	TNFR1
Heptahelical receptors (G-protein-coupled receptors)	Homodimer	δ-OpioidR, GluR
	Heterodimer	$GABA_BR1/2$, β_2AR/α_2AR

stabilize the "open" (active) conformation, thereby allowing access of adenosine triphosphate (ATP) and substrate to their respective binding sites within the kinase domain (see Section IIA, Protein Phosphorylation, for more detail). The activated kinases can then carry out further tyrosine phosphorylation events. Both chemical and physical evidence has been obtained to support this model, most of which depends on a lack of evidence for detecting receptor dimers except in cells exposed to stimulation. However, the growing body of evidence that at least some RTKs can exist as preformed dimers in the absence of ligand includes both direct [2–4] and indirect [5] measurements. There are indeed substantial reasons why this would be advantageous to cells. The presence of ligand-independent dimers could provide a precise regulation of the activation of RTKs. Preformed dimers would reduce the time necessary for widely dispersed monomers to associate, thereby reducing the time required to form the active dimeric structure. Additionally, a preformed dimer could present a higher affinity ligand binding site by allowing the interaction of the ligand with both receptors simultaneously. Finally, suppression (inhibition) of the kinase domains in such ligand-independent complexes would provide a means to suppress spurious signaling in the unstimulated state.

A natural example of a ligand-independent dimer is the insulin receptor (IR), which exists as a covalently bound heterotetramer consisting of two α and two β chains joined by disulfide bonds (see chapters 51 and 52). The α and β chains are actually derived from a single precursor, hence the unprocessed insulin receptor resembles the other RTKs except for the covalent links that ensure that the unliganded state will be dimeric. It is also clear that dimerization alone is insufficient for the activation of IR. Somehow, ligand binding to the receptor ectodomain modulates a conformational change of the endodomain even though the plasma membrane is transversed by a single membrane-spanning segment of approximately 20 to 25 residues in each protomer. Although the mechanisms by which this occurs remain largely unknown, it may be surmised that the two protomers (in this case, the protomer is an α/β chain unit) rotate relative to each other. A similar mechanism would be equally effective in the RTKs that are not covalently linked.

The active complexes of dimerized RTKs are stabilized by multiple contacts, such as interactions between monomers and ligand and between monomers. Interaction between monomers stabilized in a dimeric state has been shown to occur within the ectodomains, transmembrane domains, and endodomains of various receptors. For example, the crystal structure of a ligand-bound domain of TrkA shows direct contacts between the two domains as well as direct contacts to the ligand, nerve growth factor (NGF) (see chapter 48). In addition, regions within the ectodomains of fibroblast growth factor receptor (FGFR) and platelet-derived growth factor receptor (PDGFR) have been identified that mediate receptor–receptor interactions in the absence of ligand [6,7]. Furthermore, the transmembrane domains of ErbB family members self-associate in cell membranes [8], suggesting that the association may occur in the context of the full-length receptor provided that the ecto- and endodomains do not sterically hinder their association. Residues within the cytoplasmic domains of RTKs also are important for stabilization of dimers [9,10].

Cytokine Receptors

The cytokine receptors are comprised of numerous kinds of transmembrane receptors that are characterized by conserved patterns of amino acid residues in their ectodomains and the lack of enzymatic activity within their endodomains [11,12]. In lieu of intrinsic kinase activity, they associate with nonreceptor tyrosine kinases such as the Janus kinases (Jaks) or Src family kinases. Nevertheless, the active signaling complex induced by ligand is oligomeric and seems to

involve the juxtaposition of these nonreceptor tyrosine kinases. The quaternary structure of the cytokine receptor superfamily is both complex and varied, ranging from simple homodimers to heterotrimers, and can be separated into two subclasses, type I and type II, with the majority belonging to class I [11,12]. The class II receptors primarily consist of receptors for interferons (IFNs) and interleukin-10 (IL-10).

Type I Cytokine Receptors

The most-studied class I cytokine receptors are the growth hormone receptor (GHR) and erythropoietin receptor (EPOR) that function as homodimers (reviewed by Frank [13]). Recently, the unliganded and ligand-bound crystal structures of the receptor for erythropoietin have been described and provide substantial insight into the mechanisms of activation of this receptor [14,15]. EPOR appears to exist at the cell surface as an inactive dimer [14,15], and this state is mediated by the transmembrane domain [16]. The inactive dimer exists in a conformation in which the transmembrane domains (and presumably the endodomains) are separated by approximately 73 Å, thereby preventing the interaction of the associated Jaks [14,15]. Upon ligand binding, the separation of the transmembrane domains is reduced to 30 Å, which would likely be sufficient to allow the Jaks to transphosphorylate each other and thus become activated. The ligand-bound structure of GHR, also deduced from X-ray diffraction studies, suggests a similar mechanism of activation in that the endodomains appear to be brought into proximity, allowing activation of the associated Jaks [17]. It is not clear, however, whether the receptors exist in a predimerized state on the cell surface (either loosely or tightly associated) or whether ligand induces the dimerization. Neither is it known whether GHR requires a conformational change upon ligand binding for its activation, although if GHR is predimerized this would almost certainly be required for its activation. Interestingly, there is evidence to suggest that some GHRs, upon ligand stimulation, form covalent dimers through disulfide bridges [13].

Some type I cytokine receptors require three different subunits for their activity. The receptor for interleukin-2 (IL-2R) is one such case, as it has three subunits, α, β and γ (also known as the common γ chain [γc], as it is used by multiple receptors, including IL-4R). The β and γ receptors together are sufficient for signaling but do not possess sufficient affinity for ligand to be activated by normal levels of IL-2. IL-2Rα is not a member of the cytokine receptor superfamily, as it does not possess the conserved motifs within its ectodomain; however, when present in the complex with the β and γ subunits, it increases the affinity for ligand binding [18] and may induce higher order oligomerization [19]. The ectodomains of the receptor subunits have been shown to interact at the cell surface in the absence of ligand as α/β or β/γ pairs or α/β/γ heterotrimers [20], although the presence of preassociated, full-length IL-2R subunits remains controversial. In any case, the downstream signaling initiated by IL-2R subunits conforms to the common theme among many cell surface receptors: the juxtaposition of two catalytically active factors allows for their transactivation. With respect to IL-2R, the juxtaposed effectors appear to be Jak1 and Jak3 [21]. It has been shown, however, that the mere juxtaposition of the Jaks is insufficient for signaling and that the correct orientation of these entities is also required [22,23]. This observation supports the idea that the conformation of receptor endodomains is as important as their proximity within the active receptor complex in the activation process.

Analogous to the use of γc by IL-2R and IL-4R, the IL-6 and IL-11 receptors each require the common gp130 subunit for activity (reviewed in Taga and Kishimoto [24]). IL-6 cannot bind to gp130 alone, and IL-6R alone has a low affinity for IL-6. However, when IL-6R and gp130 are coexpressed, both high- and low-affinity binding sites for IL-6 are generated. IL-6 binds IL-6R on the cell surface or the soluble form of IL-6R (sIL-6R), which then recruits gp130 into the complex. The activity of gp130 requires its association to form homodimers as part of the IL-6/IL-6R/gp130 complex. The stoichiometry of the active receptor complex has been determined to be 2:2:2 [25,26], suggesting a twofold symmetry. IL-6R has a short cytoplasmic tail that is dispensable for its function, and gp130 provides all of the required cytoplasmic signaling motifs. Because gp130 has been shown to be constitutively associated with Jak1, Jak2, and Tyk2, the presumed mechanism of activation again involves a juxtaposition of these kinases that allows their transactivation and the phosphorylation of tyrosine residues in the C-terminal region of the endodomain of gp130.

Type II Cytokine Receptors

The type II cytokine receptors consist primarily of receptors for interferons and interleukin-10 (IL-10) [11]. The receptors for interferon-γ (IFNγR) and IL-10 (IL-10R) have a similar structure in that they are each comprised of two type 1 receptors and two type 2 receptors forming a heterotetramer (reviewed by Kotenko and Pestka [27]). As with many of the cytokine receptors, IFNγR subunits are constitutively associated with Jaks: IFNγR1 with Jak1 and IFNγR2 with Jak2. IFNγR1 dimers can bind IFNγ, but this complex is not functional even though the complex contains the associated Jak1. In contrast to the ligand-bound EPOR, which transposes the transmembrane domains within 30 Å in the active conformation, the 27-Å spacing between IFNγR1 molecules acts to prevent downstream signaling [28], exemplifying the importance of the orientation and/or conformation of the cytoplasmic domains. Indeed, it has recently been shown using FRET analysis [S. Pestka, personal communication] that the IFNγR1 and 2 heterotetramer (with the associated Jaks) is, contrary to commonly held opinion, preformed and that binding of IFNγ actually causes the intracellular domains (with their associated Jaks) to move apart. This new model suggests that there are likely inhibitory interactions that keep the Jaks inactive and that displacement is necessary to alleviate this or allow room for

substrates (e.g. STATS) to bind. The similarity of this model with that observed for FGFR3 is striking [5].

Guanylyl Cyclase-Containing Receptors

The receptors of this type are organized much like the RTKs in that they have an ectodomain, a single transmembrane-spanning domain, and an endodomain composed of a two interacting subdomains: a kinase-homologous regulatory domain that binds ATP and a guanylate cyclase domain that produces cGMP [29]. This group contains seven members, the best studied of which is GC-A, the atrial natriuretic peptide (ANP) receptor [30]. The three-dimensional structure of the ectodomain dimer has been solved by X-ray methods which revealed a bilobal periplasmic binding protein, similar to that found in several other proteins, including several DNA binding proteins [31]. Interestingly, these receptors clearly form unliganded dimers, and activation by ANP occurs through binding to and stimulation of these preformed structures; however, it is currently unclear how this activation is transmitted through the structure. Considering the overall similarity with the RTKs, it is likely that the preformed dimers are in a conformation that results in inhibition of the cyclases, and that binding causes a rotational motion that alleviates this, perhaps by the release of steric hinderances.

Serine/Threonine Kinase-Containing Receptors

The serine/threonine kinase receptors are typified by the receptors for transforming growth factor β (TGF-β), a dimeric ligand that exerts its effects through receptors composed of two different subunits designated type I and type II. Each possesses serine/threonine kinase activity. Both classes of receptor protomers are required for mediating the signaling response to ligand binding. The type II TGF-β receptor (TβR-II) exists as a constitutively active dimer and is responsible for the initial interaction with TGF-β, as the type I receptor (TβR-I) cannot bind TGF-β in the absence of TβR-II. Although the exact stoichiometry has not been elucidated, the minimal active signaling complex consists of TGF-β bound to two molecules of TβR-II and two of TβR-I. Two theories exist regarding the association and activation of this complex. The first involves the initial interaction of TGF-β with TβR-II. This complex then actively recruits TβR-I, leading to the phosphorylation and activation of this receptor. The second theory involves an inactive, preexisting (albeit weakly associated) heterotetramer of TβR-I and TβR-II that undergoes a conformational change upon ligand binding that alters the juxtaposition of TβR-I with TβR-II in such a way as to allow the phosphorylation and activation of TβR-I. In either case, ligand is required to induce or stabilize an active conformation that allows the phosphorylation of TβR-I by TβR-II.

The structure of TβR-II complexed with TGF-β, deduced from X-ray studies, has recently been described [32]. The structure supports the formation of a TGF-β/TβR-II/TβR-I heteropentameric complex in which TβR-I directly contacts both the ligand and TβR-II. These findings do not preclude either theory of receptor association, but they do provide evidence for direct interaction of TβR-I and -II that would be required for association of the receptors in the absence of ligand. It has been shown that TβR-I and -II can associate in the absence of ligand *in vitro* and when co-expressed in mammalian cells [33], two observations giving support for preexisting heterotetramers in the absence of ligand.

Tumor Necrosis Factor Receptors

The specific oligomeric nature of active tumor necrosis factor receptor (TNFR) family members has not been definitively proven. The ligands for these receptors are usually trimeric, suggesting that the functional oligomeric complex of the receptors may also be trimeric; however, the receptors may actually function as dimers. At least three different hypotheses have been proposed for the activation of TNFRs. The first, and most widely accepted, involves receptor trimerization. The earliest crystallographic studies of TNFR and TNFR1 bound to TNF-β demonstrate that one receptor molecule binds to each of the three monomer–monomer interfaces of the trimeric TNF ligand [34,35]. Interestingly, no direct contacts between any of the receptor monomers other than nonspecific crystal contacts were detected; however, the receptor endodomains presumably would be sufficiently close to allow interaction. TNF-receptor-associated factors (TRAFs), primary effectors of TNF-Rs, have been crystallized as trimers in association with domains of the TNFR family member CD40, and the structure of trimerized TRAFs supports a model of interaction with trimerized CD40. Furthermore, TRAF activity is greater when induced by trimeric forms of CD40 as compared to monomers or dimers [36]. Biochemical studies of TNFR also suggest that the receptors exist as trimers even in the absence of ligand [37]. There are numerous other studies supporting the notion that the active form of TNFR is trimeric.

Nonetheless, evidence also suggests that active TNFR is assembled as dimers [38]. First, biochemical studies of the ectodomain of TNFR2 determined that two or three TNFR2 molecules bind to each trimer of TNF-α or TNF-β ligand, suggesting that a receptor dimer may interact with the trimeric ligand [39]. CD27, a TNFR family member, functions as a disulfide-linked dimer, analogous to the insulin receptor. Chimeric receptors consisting of EPOR or PDGFR ectodomains inframe with the TNFR transmembrane, and cytoplasmic domains function as dimers, either constitutively (the EPOR chimera [40]), or ligand dependently (PDGFR [41]), indicating that two subunits are sufficient for signaling. Furthermore, secreted forms of TNFR have been purified as dimeric proteins. Most substantially, structural studies of crystallized ectodomains of TNFR1 in the absence of ligand indicated that TNFR could exist in at least

two different dimeric conformations [42]. These structural studies led to models of dimeric receptor activation.

Two different models exist for the activation of dimeric receptor complexes [43], and both models arise from crystallographic studies of unliganded ectodomains [42]. The first involves a conformational change via an axial rotation that would juxtapose the cytoplasmic domains [44]. The second is the so-called *expanding-network hypothesis* and involves the formation of higher order oligomeric complexes of dimeric receptors and trimeric ligand [42]. Even though the specific organization of receptor–ligand complexes has not been definitively determined, it is clear that the oligomerization of TNFR family members is required to allow signaling from these receptors.

Heptahelical Receptors (G-Protein-Coupled Receptors)

The heptahelical or G-protein-coupled receptors are one of the largest families in the human genome (and likely other mammalian genomes, as well), and with more than 1000 distinct entities they are the most used family of transmembrane signaling receptors. Not surprisingly, as a group, they form one of most popular targets for drug discovery. Their mechanism is well understood in general terms; that is, they interact with trimeric G-protein complexes, of which there are many, which in turn couple them to a wide variety of signaling effector systems, including adenylyl cyclase, the producer of cAMP, which was the first second messenger to be identified.

It has been generally believed that this class of receptors is monomeric and remains so during the binding of ligand and the activation of the trimeric G-protein complex; however, recent evidence suggests that this is not the case [45–47]. Indeed, these receptors appear to exist as homodimers, heterodimers, or even larger oligomers. The identification of these higher order structures has, of course, been aided by improved technology that has allowed detection of the oligomers both in solution and in viable cells [45,46]. The formation of heterodimers is a particularly interesting phenomenon as it allows a greatly increased range of ligand binding and subsequent responses. Apparently, even distantly related receptors have been observed to form complexes and, given the size of this family, the number of possibilities afforded by these interactions is enormous.

Concluding Remarks

In this brief overview, we have summarized much of the salient observations that support the view that oligomerization is a prevalent and perhaps universal requirement for transmembrane receptor function. There is clearly growing evidence, in part attributable to better technology, that many of these oligomeric structures form independent of ligand and that activation by ligand binding is not due to association of the receptor protomers but rather allows them to seek a different juxtaposition relative to each other, almost certainly at least in part by rotational motions. Such a model eliminates the diffusion control that ligand-induced dimerization (oligomerization) requires and provides additional opportunities for the regulation of signal flux by allowing inhibitory interactions in the unliganded complexes. The reader is directed to the many chapters in this Handbook describing individual systems for more specific details.

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PART II

Transmission: Effectors and Cytosolic Events

Tony Hunter

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PART II

Introduction

Tony Hunter

Signals received by receptors at the cell surface are transduced across the cell membrane and then propagated in the cytoplasm through a variety of mechanisms. We chose to subdivide Part II of the Handbook into sections according to the different mechanistic principles that are used to initiate and transmit receptor-activated signals in the cytoplasm, namely Protein Phosphorylation, Protein Dephosphorylation, Calcium Mobilization, Lipid-Derived Second Messengers, Protein Proximity Interactions, Cyclic Nucleotides, and G proteins. In addition, we included a section on Developmental Signaling, because of the increasing information about signaling pathways used in developmental processes. Space limitations precluded comprehensive coverage of the hundreds of cytoplasmic signaling molecules individually. Indeed, this type of information is much more effectively achieved through web-based molecule pages, and a number of databases of this sort are being developed. For this reason, we decided to use a thematic approach, focusing on systems, pathways and protein families. We realize that there are many important topics that are not covered, and we hope that these can be added during the development of future print and electronic editions of the Handbook. Much of the recent progress in our understanding of signaling has come from the development of new techniques and reagents, and, while not wanting this to be a methods book, we have included descriptions of particularly important recent technical developments that have already begun to have a major impact on research in most of the sections.

Protein phosphorylation and dephosphorylation is the main regulatory mechanism through which protein activity is regulated in eukaryotic cells, and the major classes of protein kinase and phosphatases are reviewed in these two sections. Some emphasis is given to the emerging concept that phosphorylation is frequently used to promote inducible protein–protein interactions through phosphoamino acid specific binding domains. Cognizant of the enormous impact of bioinformatics on the field of signal transduction, we have included articles on the genomic catalogues of protein kinases and phosphatases. Protein phosphatases were for a long time the poor cousins of the protein kinases. However, over the past decade protein phosphatases have come into their own, and the burgeoning numbers of protein phosphatases and the complexity of their regulation and substrate targeting justifies a complete section on protein dephosphorylation.

Cytoplasmic signaling is a highly organized in space and time, and the next frontier in intracellular signaling will be the elucidation of the spatial and temporal aspects of signaling at the single cell level. Calcium-mediated signal transduction is one of the best systems in which to study the spatiotemporal events of receptor induced signaling making use of fluorogenic calcium-binding dyes. The dynamic and oscillatory nature of calcium fluxes in the cytoplasm are surely a reflection of the dynamic nature of other signaling events in the cytoplasm. Articles describing the basis for the dynamic behavior intracellular calcium and the targets that propagate the calcium signal are collated in the section on calcium mobilization.

Lipid-derived second messengers have become increasingly important in signal transduction, particularly with the realization that a number of modular protein domains recognize differently phosphorylated forms of phosphatidylinositol, thus allowing these lipids to act as second messengers and recruit target proteins to distinct cellular membrane compartments. The early discovery of ligand stimulated synthesis of phosphoinositides and the finding that diacylglycerol derived through hydrolysis of PIP2 is a second messenger that activates protein kinase C were the harbinger of an increasing number of phospholipid-derived second messengers, which are either membrane anchored or soluble. In particular, the discovery of 3′ phosphoinositides has opened up a whole new area of signaling in which proteins are inducibly brought to the membrane through recognition by protein domains that specifically bind

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phospholipid head groups. These concepts are emphasized in this section.

Cyclic nucleotide mediated signaling is the oldest and best understood signaling system; cAMP continues to be the model for second messenger signaling, and the cAMP-dependent protein kinase remains the paradigm for the protein kinase superfamily. Nonetheless, new targets for cyclic nucleotides have been identified within the past few years, and this advance is described in this section, which also includes articles on biosensors that can be used to study cAMP-dependent signaling in real time in single living cells.

Signaling through heterotrimeric G proteins is also a venerable system, and it has become increasingly important with the proliferation of G protein subunit types, and the genomics revelation that G protein coupled receptors are by far the largest class of cell surface receptor. These switch-like proteins and their regulators and effectors have functions in a wide diversity of cellular processes that are reviewed in this section. The large family of small monomeric G proteins have become increasingly prominent. The small G proteins play key roles in propagating receptor signals to and from the cytoskeleton and in orchestrating cytoplasmic vesicular trafficking systems, nuclear import and export and cell cycle progression, and many of these are covered in this section.

A number of signaling pathways have been uncovered through genetic analysis of developmental processes in model eukaryotic organisms, and these pathways have revealed a number of new, highly conserved principles of signaling. Good examples are the Notch, Hedgehog and Wnt signaling pathways, whose main purpose is to regulate gene expression that is critical for development. All three pathways use regulated proteolysis; for instance, activation of the Notch pathway results in intramembrane cleavage of the Notch receptor releasing its cytoplasmic domain, which migrates into the nucleus and acts as a direct transcriptional regulator. This type of processing is now emerging for other types of receptor signaling system. The major signaling pathways used in development are reviewed in this section, with an emphasis on the developmental systems where they participate.

The concept that signal propagation in the cytoplasm involves freely diffusible second messengers and proteins has to some extent been supplanted with the idea that signal transmission may be more akin to a solid state system in which proteins are organized into transient and constitutive multiprotein complexes. Signaling through protein–protein interaction is an emergent theme, and both inducible and stable protein-protein interactions form the basis for many signal pathways. Indeed, signaling pathways that use induced protein–protein interaction as a primary signal initiation mechanism, such as the TNF receptor family, furnished a few principle of signaling that now impinges on many systems. This concept will be increasingly important to our understanding of signal propagation and specificity in the cytoplasm. Anchoring and scaffolding proteins that organize adjacent components of signaling pathways are a paradigm that is highlighted by articles in this section. Methods for studying protein–protein interactions *in vivo* and *in vitro*, and for analyzing protein complexes are also included.

If one looks forward to where cytoplasmic signaling will be in ten years time, we can foresee that a complete cellular parts list will be available, and that proteomic analysis will have told us the composition of multicomponent protein complexes and revealed all the possible protein–protein interactions that can occur. Cytoplasmic signaling is a highly organized and localized process and its often stochastic nature means that single cell analysis is vital for further progress. The development of high sensitivity biosensors to study the localization of active forms of signaling enzymes, such as protein kinases, to determine where and when protein–protein interactions occur and second messengers are generated is going to be needed in order to study spatiotemporal aspects of all types of signaling pathway in single living cells. Finally, one hopes it will be possible to use all this information to accurately model signaling networks and understand cooperative and inhibitory interactions between cytoplasmic signaling pathways.

Tony Hunter

SECTION A

Protein Phosphorylation

Tony Pawson

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Eukaryotic Kinomes: Genomic Cataloguing of Protein Kinases and Their Evolution

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Introduction

Ever since the discovery nearly 50 years ago that reversible phosphorylation regulates the activity of glycogen phosphorylase, there has been intense interest in the role of protein phosphorylation in regulating protein function. With the advent of DNA cloning and sequencing in the mid-1970s it rapidly became apparent that a large family of eukaryotic protein kinases exists, and the burgeoning numbers of protein kinases led to the speculation that a vertebrate genome might encode as many as 1001 protein kinases [1]. The importance of protein phosphorylation as a regulatory mechanism has continued to grow, and it is estimated that more than 30% of intracellular proteins can be phosphorylated at one or more sites. Phosphorylation not only regulates enzymatic activity through inducing conformational changes or through direct steric effects, but also modulates the function of structural proteins through conformational and charge effects. In addition, a major revelation has been the finding that protein-linked phosphates can act as binding sites for other proteins [2].

The first eukaryotic protein kinases to be identified in the 1950's (i.e., the casein kinases and phosphorylase kinase) were found to phosphorylate serine and/or threonine in their substrate proteins. For many years thereafter all the newly characterized protein kinases proved to be serine/threonine-specific, and it was not until the early 1980's that tyrosine-specific protein kinases were discovered. Subsequent molecular analysis showed that the catalytic domains of the serine/threonine- and tyrosine-specific protein kinases are in fact related in sequence, and belong to what is referred to as the eukaryotic protein kinase (ePK) superfamily. Members of the ePK superfamily all have a similar hinged bilobate catalytic domain structure. A few other types of protein kinase are known; these atypical protein kinases (aPKs) are either very distantly related to the ePK superfamily or have no sequence relationship at all. Nevertheless, the three-dimensional structures of the catalytic domains of several of the aPKs prove to be similar to that of the canonical ePK catalytic domain, even in cases when there is little or no detectable sequence similarity. In addition to Ser, Thr and Tyr, several other amino acids in proteins can be phosphorylated, including Lys, Arg, and His, but the provenance of the cognate protein kinases remains unclear. The prokaryotic two component protein kinases, commonly known as "histidine kinases" form yet another distinct family. In contrast to the ePKs, which transfer phosphate from ATP to protein in a concerted reaction, the histidine kinases use a phosphoenzyme intermediate, in which the phosphate from a phosphohistidine is donated to an acceptor protein, usually on an aspartate. This type of protein kinase is rare in eukaryotes, and, with exception of plants where a few exist, multicellular eukaryotes appear to have none of these protein

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kinases, apart from the unusual mitchondrial PDHK family members.

The ePK superfamily catalytic domain is characterized by a series of short sequence motifs, which define 11 subdomains, and serve as key elements in the catalytic core of the kinase domain [3,4]. These motifs in combination with the overall catalytic domain sequence can be used to identify genes that encode protein kinases through pairwise sequence alignments and hidden Markov model searches. Such analysis of complete genomic sequences and corresponding cDNA sequence information permits prediction of the number of ePKs encoded by a eukaryotic organism. Similar approaches can be used to identify members of the aPK families. Using this strategy, we have surveyed a series of sequenced eukaryotic genomes to define the protein kinase complement (kinome) of each organism [5–8]. In many cases, the assembled genomic sequences still have small gaps, and therefore a few protein kinase genes may be missing. Additional families of aPKs may also remain to be found by biochemical approaches In this regard, protein kinase activities that phosphorylate Lys, Arg, and His have been reported, but these have not been characterized molecularly, and it seems unlikely that there are large numbers of these enzymes.

A summary of the protein kinases in the budding yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, the insect *Drosophila melanogaster*, and the vertebrate *Homo sapiens* is provided in Table 1. A complete listing of the individual protein kinases, dendrograms, sequence alignments, etc., can be found at http://kinase.com/. The protein kinase classification we adopted in 1988 forms the basis for the major subdivisions of protein kinases, including the AGC, CMGC, CAM kinase and tyrosine kinase (TK) groups [3]. However, our more detailed and sophisticated genome-wide kinome analysis has led us to parse a number of protein kinases originally included in the large "Other" group into three additional groups, namely tyrosine kinase-like (TKL), STE and CK1. Within groups the protein kinases are divided into families (e.g., the MAP kinase family), which form major branches on the kinome tree; families can often be subdivided into several related subfamilies, which form twigs at the end of the branches (e.g., ERK, JNK and p38 MAP kinase subfamilies of the MAP kinase family). The sizes of protein kinase families/subfamilies are quite variable, with some families having more than 10 members, and others being represented by only a single protein kinase. Although most of the protein kinases fall into major groups, there are still a number of protein kinases that are outliers, either singly or as small families.

The Yeasts: *Saccharomyces cerevisiae and Schizosaccharomyces pombe*

The first eukaryotic genome sequence to be completed was that of the budding yeast S. cerevisiae. Out of ~6200 genes, our most recent estimate is that 130 encode protein kinases

Table I Protein Kinases in Budding Yeast, Nematodes, Flies and Humans

Group	Protein kinase families	Protein kinase subfamilies		Budding yeast kinases	Nematode kinases	Fly kinases	Human kinases
AGC	14	19		17	30	30	63
CAMK	17	33		21	46	32	74
CK1	3	3		4	85	10	12
CMGC	8	24		21	49	33	61
Other	37	38		38	67	45	83
Ste	3	13		14	25	18	47
Tyrosine Kinase-like	7	13		0	15	17	43
Tyrosine Kinase	30	30		0	90	32	90
RGC	1	1		0	27	6	5
Total ePK families	**120**	**174**	**Total ePKs**	**115**	**434**	**223**	**478**
Atypical - PDHK	1	1		2	1	1	5
Atypical - Alpha	1	2		0	4	1	6
Atypical – RIO	1	3		2	3	3	3
Atypical - A6	1	1		1	2	1	2
Atypical - Other	7	7		2	1	1	9
Atypical - ABC1	1	1		3	3	3	5
Atypical - BRD	1	1		0	1	1	4
Atypical – PIKK	1	6		5	5	5	6
Total families	**134**	**196**	**Total PKs**	**130**	**454**	**240**	**518**
			Total genes	**~6200**	**~19100**	**~13600**	**~31000**

(2.1% of all genes) [5,7]. Of these, 115 are conventional ePKs, and the rest are aPKs. None of the yeast protein kinases belongs to the metazoan tyrosine kinase group, even though there are yeast protein kinases, like Swe1p, that can phosphorylate tyrosine in their targets. The recently completed genome sequence of the fission yeast *S. pombe* predicts a similar number of protein kinase genes, but *S. pombe* also lacks genes encoding *bona fide* tyrosine kinases. The tyrosine kinases appear to have arisen concomitantly with multicellular organisms, and may have played a critical role in metazoan evolution as a result of their ability to facilitate intercellular communication. Interestingly, most of the protein kinase families present in budding yeast are also found in fission yeast, despite their great evolutionary distance, and, as will be discussed below, there are 7 families of protein kinases that are unique to the fungi.

Nematodes: *Caenorhabditis elegans*

The nematode *C. elegans* is a complex multicellular organism, which undergoes a deterministic developmental program. Out of the ~19,100 genes in the *C. elegans* genome, 454 (2.4% of all genes) encode protein kinases, of which 434 are ePKs [6,7]. A striking aspect of the nematode kinome is the emergence of the tyrosine kinase (TK) group. *C. elegans* encodes 90 tyrosine kinases (20% of all protein kinases), which are of receptor and nonreceptor types. Both types of tyrosine kinase arose early in metazoan evolution, being present in sponges and coelenterates. Compared to Drosophila, the relatively large number of tyrosine kinase genes is in part accounted for by the expansion of two families, the Fer nonreceptor tyrosine kinase (42 genes) and the Kin16 receptor tyrosine kinases (16 genes). Many of the major tyrosine kinase families are already evident in the nematode. Most of the major serine kinase families present in higher eukaryotes are already in place in *C. elegans*, with some families being greatly expanded (e.g., there are 85 CK1 genes in *C. elegans* compared with 12 in humans and 10 in *Drosophila*). Some of these expansions appear to be very recent, and are not seen in draft sequence from the related *C. briggsae.* Moreover, the lack of ESTs indicates that some members may be pseudogenes, although detailed sequence analysis is needed to establish this.

Insects: *Drosophila melanogaster*

The dipteran insect D. melanogaster has ~13,600 genes, which is significantly fewer than *C. elegans*, even though by most criteria the fly is a more complex organism. *Drosophila* has 240 protein kinase genes (1.8% of all genes), of which 223 are ePKs, with 32 tyrosine kinase genes (14% of all protein kinases) [7,9]. In fact, the percentage of protein kinase genes in *Drosophila* is very similar to that of *C. elegans*, if one trims away the highly expanded protein kinase families in *C. elegans*. Some new protein kinase families emerge in *Drosophila*, which have functions in immunity (e.g., JAK), morphogenesis (e.g., LIMK) and the nervous system (e.g., MuSK).

Vertebrates: *Homo sapiens*

The *Homo sapiens* genome, with ~31,000 genes, has a predicted total of 518 protein kinase genes (1.7% of all genes) [8]. Of these 478 are in the canonical ePK family (Table 1), and the others are divided between 9 small aPK families, which include the PIKK (PI3 kinase-like kinase), the PDHK (pyruvate dehydrogenase kinase) and alpha kinase (E2F kinase) families. There are 90 tyrosine kinase genes (16% of all protein kinases); several of the tyrosine kinase families present in *Drosophila* and *C. elegans* have undergone significant expansion in mammals (e.g. Eph receptor tyrosine kinases, where there are 14 members in humans, and only 1 in *Drosophila* and 1 in *C. elegans*). The total number of protein kinases is about half that predicted 15 years ago [1], but it is still a strikingly large number, comprising about 1.7% of all human genes. Moreover, the total number of protein kinase gene products is surely much greater than 518, due to the expression of alternatively spliced forms, which are known for many well-studied protein kinases. A few new protein kinase families arose during the evolution of vertebrates, including the Tie and Axl families of receptor tyrosine kinases, which play roles in angiogenesis and immune system homeostasis respectively. New serine kinase families are also present, such as Trio, which is involved in secondary myogenesis and neural organization. Our analysis of the mouse kinome is not as complete, but >95% of the human protein kinases have orthologues in the mouse, and it seems likely that the mouse kinome will be very similar indeed to the human kinome [8].

Our extensive analysis of the human kinome has revealed a number of other features [8]. Out of the 478 conventional ePK catalytic domains, 50 (~10%) are missing one or more of the 3 conserved catalytic residues (Lys72/Asp166/Asp184 in PKA C subunit) suggesting that most of them lack catalytic activity. These "kinase domains" may serve as docking platforms or scaffolds (e.g., ErbB3 and ILK), structural elements (receptor guanylyl cyclase kinase homology domains) and/or regulatory domains, which might bind and sense ATP levels. Many of these catalytically-dead protein kinases have been conserved throughout evolution (e.g., ILK and Derailed/RYK), implying that they serve a critical function in the absence of catalytic activity [9].

There are 106 predicted pseudogenes in the human genome that contain recognizable elements of the protein kinase catalytic domain (~20% of the total number of protein kinases), as defined by a lack of ESTs, reading frames with stop codons, and in many cases (75) a lack of introns, indicating that these genes underwent retrotransposition ("processed pseudogenes). For reasons that are unclear, some protein kinase families have a very high ratio of pseudogenes to functional genes (e.g., MARK 28:4). Since the

prediction of pseudogenes is not an exact science, some of these 106 genes may ultimately prove to have functional products, although possibly not active protein kinases.

The kinome analysis also has implications for human disease. Mutational activation/inactivation and overexpression of protein kinase genes is a frequent cause of hereditary and sporadic human disease. For instance, as many as half of the 90 tyrosine kinases have been implicated in cancer, through mutational activation or overexpression. For this reason, one might certainly expect mutation of some of the new protein kinase genes revealed by genomic analysis to be causal in human disease. Our analysis indicates that 80 protein kinase genes map to chromosomal disease loci, and these are candidate genes for the causative mutation. In addition, 164 protein kinase genes map to amplicons found in tumors.

Protein kinase catalytic domain function is often dependent on additional domains in the protein, which serve to regulate activity, localize, and recruit regulatory proteins/second messengers and substrates. The nature of these domains can provide insight into the functions of new protein kinases. About half the protein kinases are predicted to have additional domains, many of which are implicated in signaling processes. Of the tyrosine kinases, 25 have P.Tyr-binding SH2 domains that play a cardinal role in establishing tyrosine phosphorylation based signaling networks. In contrast, perhaps surprisingly, only one serine kinase contains a P.Ser/Thr-binding domain (an FHA domain in CHK2). In addition, 46 protein kinases have domains that interact with other proteins (e.g., SH3); 42 protein kinases have lipid interaction domains (e.g., PH) (present in both tyrosine and serine kinases); 38 protein kinases have domains linked to small GTPase signaling (present in both tyrosine and serine kinases); and 28 protein kinases have domains linked to calcium signaling (all are serine kinases). Generally, most members of a protein kinase family have the same constellation of ancillary domains, but there are some exceptions. A complete listing of additional domains found in human protein kinases is given at http:// kinase.com/.

Comparative Kinomics

A great deal can be learned about the evolution of the protein kinase superfamily through the comparison of the kinomes of different eukaryotes [7,8] (Figures 1 and 2). Fifty one protein kinase families are common to all eukaryotes; these serve cell essential functions; examples are Cdk, CAMK, PKA, MAP kinase, etc. Interestingly, most of the atypical protein kinase families exist in all four kinomes yeasts, but clearly were not selected for diversification during evolution. Seven families are unique to the budding and fission yeasts. These have functions commensurate with a fungal life style, such as cell wall biosynthesis and stress responses.

Among the metazoan kinomes analyzed to date there are 93 families in common, which presumably were present in the last common ancestor of nematodes, flies and vertebrates. Two major new groups of protein kinase are revealed in metazoa; tyrosine kinase (TK) (Figure 2) and TK-like. Many of these latter protein kinases are engaged in intercellular signaling, an activity vital for the development and viability of multicellular organisms. Tyrosine kinases rely on P.Tyr-binding domains for recruiting signaling proteins to transmit the signal, and the evolution of tyrosine phosphorylation based signaling may have depended on the development of SH2 domains. P.Tyr-specific tyrosine phosphatases are also a requisite, but members of the PTP family are already present in the yeasts, where their function is to dephosphorylate Cdc28/Cdc2 and the MAP kinases. Choanoflagellates, which are protists that can exist in multicellular colonies, possess at least one receptor tyrosine kinase, suggesting that tyrosine kinases may have evolved prior to the emergence of true metazoans, and indeed this may have been an essential step [11].

There are 8 protein kinase families present in humans and nematodes but not flies (e.g., Met/HGF receptor). These could have been present in the common ancestor, but lost as the insect lineage evolved. Fifteen protein kinase families are unique to *C. elegans*, and these may have evolved to serve specialized functions in the nematode. As discussed earlier, there are 18 families common to *Drosophila* and humans not found in *C. elegans*, which could have been lost in the evolution of nematodes from the common ancestor, or they could have evolved later. Finally, there are 13 protein kinase families unique to the human kinome, which presumably evolved hand in hand with the vertebrate lineage. As indicated, these serve functions in differentiation of novel cell types and tissue structures, particularly the vascular, immune and nervous systems. In terms of vertebrate kinome evolution, our ongoing analysis of the pufferfish (Fugu rubripes) genome sequence, and of the zebrafish (Danio rerio) when it becomes available, will surely be revealing. The various shared and unique protein kinase families in the yeasts, worms, flies, and humans can be explored online at http:// kinase.com/.

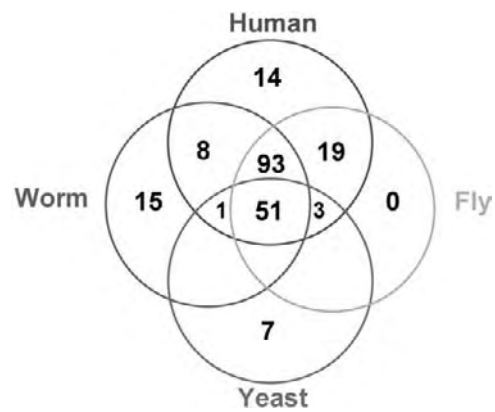


Figure 1 Occurrence of protein kinase families and subfamilies in the budding yeast, nematode, fly and human genomes illustrated by a Venn diagram. Comparison of 'orthology groups' across large evolutionary distances shows 51 distinct families/subfamilies conserved between all 4 kinomes, and 93 more in all three metazoan kinomes.

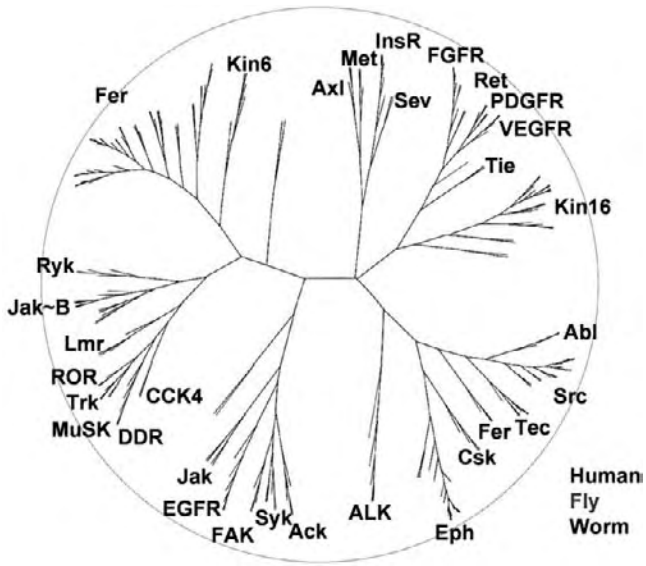


Figure 2 Trikinome dendrogram depicting the distribution of tyrosine kinases in nematode, fly and human. Most tyrosine kinases fall into distinct families (labeled), some of which are expanded greatly in worm (Fer, Kin6, Kin16), others in human (Eph, Src). As for other kinase groups, there are no significant expansions in fly. Of 30 tyrosine kinase families, 14 are present in all three genomes. Vertebrate-specific families include Tie, Axl and Lmr; coelomate-specific families (fly + human) are Jak, Syk, Tek, CCK4, MuSK, Ret, Sev and PDGFR/ VEGRFR, while Met and Trk are present in worm and human but not fly.

Interestingly, the flowering plant Arabidopsis thaliana, with ~25,500 genes, is predicted to have 1085 protein kinase genes [12], which is a significantly higher percentage of total genes (4%) than in vertebrates. None of the plant protein kinases belong to the tyrosine kinase family, and the greater number of protein kinases is in part accounted for by large families, several of which are unique to plants, such as a leucine-rich repeat (LRR) receptor serine kinases, which act as receptors for fungal pathogens and other environmental agents, and the calcium-dependent protein kinases (CDPK) (http://plantsp.sdsc.edu/). In addition, a partial genomic duplication in Arabidopsis may also contribute to the large number of protein kinase genes [13]. However, the total number of protein kinase genes in rice appears to be similar. The ability to respond rapidly and appropriately to ones external environment is obviously particularly important in sessile organisms like plants, and this selection pressure could account for the development and expansion of these new families of protein kinase.

Coda

The elucidation of the kinomes of both unicellular and multicellular eukaryotes provides us with rich insights into the evolutionary history of protein kinases, and also defines the precise number of protein kinases that can participate in phosphorylation reactions in a given eukaryotic cell type. On the other side of the coin, we have to remember that phosphorylation cannot regulate protein function unless there are protein phosphatases to remove the regulatory phosphate moieties. Genomics has also had a major impact on the world of protein phosphatases, and the number of protein phosphatases in the three major superfamilies (the 'phosphatome') has also burgeoned. In combination, the protein kinase and phosphatase genes account for nearly 2.5% of all genes in most eukaryotic species. The protein kinase catalogue also has profound implications for the understanding of the basis for human disease and for the development of small molecule drugs that target individual disease-causing protein kinases. Indeed, the first protein kinase inhibitors have recently been approved for the treatment of specific cancers. The existence of a thousand and one protein kinases may have seemed farfetched in 1987, but the human genome sequence has taught us that the enyzmes that regulate protein phosphorylation are indeed myriad and exceedingly complex.

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CHAPTER 67

Modular Protein Interaction Domains in Cellular Communication

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Introduction

Cellular regulation is largely accomplished by proteins acting in a coordinated fashion to transmit signals from outside and within the cell to produce a coherent output that guides the behavior of the cell. Intracellular signaling proteins are generally made up of modular domains that either have a catalytic function (such as kinase activity) or mediate the interactions of proteins with one another or with phospholipids, nucleic acids, or small molecules. These latter interaction domains typically fold in such a way that their N and C termini are juxtaposed in space, while their ligand binding site is located on the opposing surface; therefore, they are ideally configured for incorporation into a preexisting polypeptide while retaining their binding properties. Interaction domains play a critical role in the selective activation of signaling pathways through their ability to recruit target proteins to activated receptors and to regulate the ensuing assembly of signaling complexes. Such interaction domains can control not only the specificity of signal transduction but also the kinetics with which cells react to external and intrinsic stimuli, and they can thereby generate complex cellular behaviors. This chapter outlines these general themes; more detail is provided in a number of recent reviews [1–6].

Phosphotyrosine-Dependent Protein–Protein Interactions

The biological activities of protein kinases are, by definition, exerted through their ability to modify substrate proteins by phosphorylation, most commonly in eukaryotic cells on the hydroxyamino acids serine, threonine, and tyrosine. To understand how protein kinases regulate intracellular functions, it is critical to appreciate the mechanisms through which phosphorylation alters the biochemical properties of target proteins. One important consequence of phosphorylation is the creation of binding sites for modular interaction domains that recognize specific phosphorylated motifs. Such phospho-dependent protein–protein interactions induce the formation of heteromeric complexes that localize signaling proteins to their sites of action within the cell, juxtapose polypeptides that act within the same pathways, and induce conformational changes that regulate enzymatic activity. In addition, by binding a phosphorylated site within the same polypeptide chain, an interaction domain can mediate an intramolecular interaction, resulting in allosteric regulation.

The principal mechanism by which protein-tyrosine kinases engage downstream targets is through the ability of Src homology 2 (SH2) domains of cytoplasmic proteins to recognize specific phosphotyrosine-containing motifs on

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activated receptors. SH2 domains (containing ≈100 amino acids) have a conserved phosphotyrosine-binding pocket with an invariant arginine that anchors the phosphorylated tyrosine residue through a buried ionic bond (7–11). Recognition of the phosphotyrosine residue provides about half of the binding energy in the interaction of an SH2 domain with a phosphorylated motif; for this reason, SH2 domains generally bind phosphorylated sites with about 1000-fold higher affinity than their nonphosphorylated counterparts [12,13]. Because the dissociation constants of SH2 domains associated with optimal phosphorylated sites is commonly in the 0.5- to 1-μ*M* range, this means that phosphorylation effectively serves as a switch for the recruitment of SH2-containing proteins to activated receptors [14]. SH2 domains also recognize at least three residues C-terminal to the phosphotyrosine in a fashion that differs from one SH2 domain to another, and this discrimination provides an element of specificity in tyrosine kinase signaling [15–17]. For example, the SH2 domain of the Grb2 adaptor protein binds preferentially to pTyr–X–Asn motifs, whereas the SH2 domains of phosphatidyinositol 3′-kinase (PI3K) recognize pTyr–X–X– Met sequences. Activated receptor tyrosine kinases become autophosphorylated on sites that bind SH2-containing proteins (see Chapter 68), and the sequence contexts of these phosphorylation sites influence which SH2 proteins bind the receptor and which cytoplasmic signaling pathways are stimulated in the cell [18].

Both the affinity and specificity of SH2 domain interactions can be increased by the presence of two tandem SH2 domains in a single protein that recognize a bisphosphorylated ligand, as in the case of the ZAP-70 cytoplasmic tyrosine kinase binding to the signaling subunits of the T-cell antigen receptor [19]. SH2 domains are also often linked to other interaction modules, such as SH3 domains that typically bind Pro–X–X–Pro motifs [20,21], as in the case of the c-Src cytoplasmic tyrosine kinase. In the inactive state, when c-Src is phosphorylated on a C-terminal tyrosine residue, its SH2 and SH3 domains both make intramolecular interactions that repress kinase activity. However, upon dephosphorylation of the tail, both the SH2 and SH3 domains are liberated to bind other proteins, such as substrates for phosphorylation, through the recognition of both phosphotyrosine and proline-based motifs [22–25] (see Chapter 75).

Although most receptor tyrosine kinase targets have SH2 domains, these proteins otherwise have a diverse set of biochemical and biological functions. These include the regulation of small Ras-like GTPases, control of phosphoinositide metabolism, tyrosine phosphorylation and dephosphorylation, transcriptional regulation (STAT proteins), organization of the cytoskeleton, and protein ubiquitination (SOCS box proteins and RING domain proteins such as c-Cbl) (Fig. 1A). SH2-containing proteins, therefore, couple tyrosine kinases to a broad range of regulatory biochemical pathways within the cell. In addition, SH2 domains are often found in adaptor proteins that are composed exclusively of SH2 domains and other interaction modules, such as SH3 domains; these adaptors act as a bridge to physically link phosphotyrosine signaling to multiple targets [26].

In addition to SH2 domains, a quite distinct interaction module, the phosphotyrosine binding (PTB) domain, can bind proteins in a phosphotyrosine-dependent fashion [27]. The PTB domains of proteins such as Shc, IRS-1, and FRS2 recognize Asn–Pro–X–pTyr motifs in activated receptor tyrosine kinases, including the epidermal growth factor receptor, the insulin receptor, or the Trk nerve growth factor receptor [28–30]. Upon binding activated receptors, PTB proteins themselves become phosphorylated on multiple SH2 binding sites and recruit distinct sets of SH2-containing proteins. In this sense, they function as docking proteins to amplify and expand the range of receptor signaling.

It is evident from these observations that tyrosine kinase signaling requires a complex series of modular, phospho-dependent protein–protein interactions. Interestingly, SH2 domains are a recent evolutionary adaptation that arose only with the advent of multicellular organisms. Both tyrosine kinases and SH2 domains are absent from yeast but make a concomitant appearance in multicellular animals. The emergence of tyrosine kinases together with SH2 domains to mediate the effect of tyrosine phosphorylation represents an evolutionary step that likely facilitated intercellular signaling required for the formation of metazoan animals. The human genome encodes some 114 distinct SH2 domains, in 104 proteins; 49 PTB domains are found in 46 human proteins, although only a subset of these have phosphotyrosine-binding activity. Knowing the complete set of tyrosine kinases (see Chapter 65) and proteins with phosphotyrosine recognition domains, it should be feasible to establish the complete wiring circuitry of phosphotyrosine signaling.

Interaction Domains: A Common Theme in Signaling

Interaction domains are essential in signaling from many different types of cell-surface receptors, as well as in cellular events such as the cell cycle, protein and vesicle trafficking, targeted protein degradation, DNA repair, and control of the cytoskeleton. Thus, the SH2 domain serves as a prototype for a growing family of protein-interaction modules (Table 1), some of which specifically recognize posttranslationally modified motifs, in a fashion akin to the selective binding of SH2 and PTB domains to phosphotyrosine-containing sequences [1,31]. A number of domains can bind phosphothreonine/phosphoserine-containing motifs (i.e., 14-3-3, FHA, MH2, WD40, WW) and thereby mediate the effects of protein-serine/threonine kinases [32]. For example, in a series of interactions analogous to receptor tyrosine kinase signaling, the activated type I TGFβ receptor serine/threonine kinase becomes autophosphorylated within its juxtamembrane region, thereby creating binding sites for the MH2 domain of a regulatory (R-) SMAD protein, which recognizes pSer–X–pSer motifs [33,34]. Subsequent phosphorylation of the R-SMAD itself leads to binding to the MH2 domain of SMAD4 and translocation of the R-SMAD/SMAD-4 complex

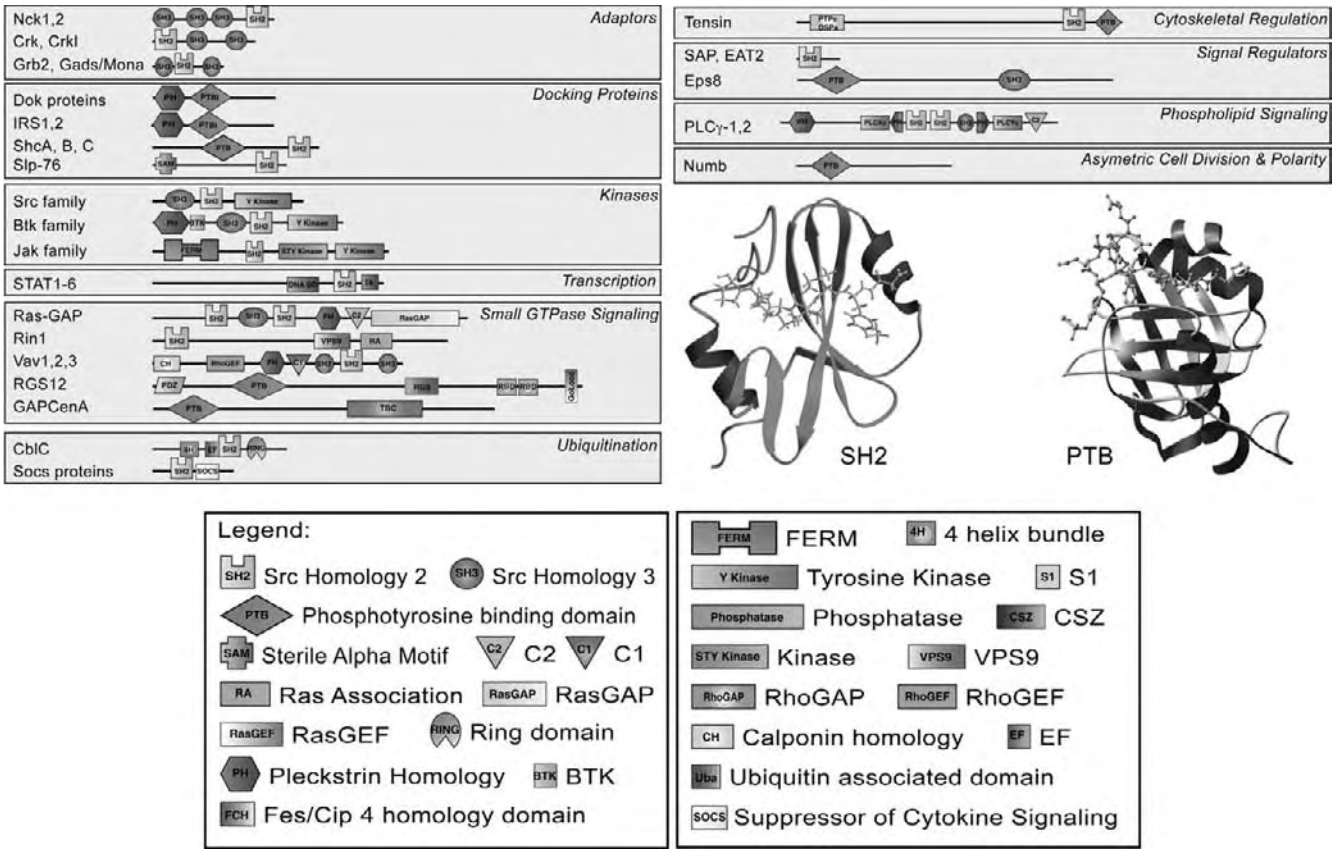


Figure 1 The modular nature of proteins containing SH2 or PTB/PID domains; a comparison of the modular protein domains and positional organization of a representative sample of the approximately 162 proteins that contain phosphotyrosine interaction modules and thus represent the key link between activated tyrosine kinases and cellular signaling cascades. The selected proteins demonstrate the variety of cellular functions and pathways in which the proteins containing these interaction modules are implicated. (Additional information on individual domains can be found at http://www.mshri.on.ca/pawson/research1.html and http://smart.embl-heidelberg.de/.) Ribbon diagrams show the SH2-C domain of phospholipase-Cγ bound to a specific phosphotyrosine-containing peptide (DNDpYPLPDPK) and the PTB domain of Shc bound to an HIIENPQpYFS peptide.

to the nucleus, where it acts to regulate gene expression (see Chapter 81) [35]. In contrast, bromo- and chromo-domains bind lysine-based motifs (notably in histones) in a fashion dependent on acetylation or methylation, respectively, of the lysine residue and thereby play an important role in chromatin organization and transcriptional control. Ubiquitin interaction motifs (UIM), a common feature of endocytic proteins, bind mono- or polyubiquitinated sites and appear to regulate protein trafficking to endosomes [36]. Other protein-interaction domains recognize unmodified peptide motifs, such as proline-rich sequences (SH3, WW, and EVH1 domains) [37] or the extreme C-terminal residues of target proteins (PDZ domains) [38].

A separate class of interaction domains (i.e., PH, FYVE, PX, ENTH, FERM, Tubby) recognizes specific phospholipids, particularly phosphoinositides, and therefore directs proteins to regions in the plasma membrane enriched for the appropriate phospholipid [39,40]. These phospholipid-binding domains mediate the effects of lipid kinases and phosphatases and function in synchrony with protein-interaction domains. For example, autophosphorylated receptor tyrosine kinases bind the p85 SH2-containing subunit of PI3K, thereby stimulating PI3K to produce PI-3,4,5-P_3. This phospholipid engages the PH domains of intracellular targets such as the serine/threonine protein kinases PKB/Akt and phosphoinositide-dependent protein kinase (PDK1), which consequently are recruited to the membrane, resulting in PKB activation. PKB, in turn, phosphorylates targets that include the pro-apoptotic protein BAD and the transcription factor FKHRL at Ser residues, which subsequently bind 14-3-3 proteins [32]. 14-3-3 binding represses the ability of the phosphorylated proteins to induce apoptosis by sequestering them in the cytoplasm away from their sites of action. Thus, a signaling pathway can be constructed from a series of protein and lipid kinases and a succession of phospho-dependent protein–protein and protein–phospholipid interactions.

Adaptors, Pathways, and Networks

As noted above, SH3 domains can be linked to SH2 domains to create adaptor proteins that connect a tyrosine kinase upstream signal to pathways that are engaged by the SH3 domains. The Grb2 family of adaptor proteins, for example, links tyrosine kinase signals to Ras and mitogen-activated protein (MAP) kinase pathways. Grb2 contains an

Table I Functions of Selected Protein Interaction Modules

Interaction module or domain	Example	Binding functions
Phospho-recognition		
SH2	Grb2 & Gads adaptors, Src family kinases, phospholipase C-γ	Phosphotyrosine
PTB	Shc, IRS-1, FRS2	Phosphotyrosine
FHA	Rad53	Phosphothreonine/ phosphoserine
14-3-3	14-3-3 proteins	″
WD40	Cdc4, β-TRCP (F-box proteins)	″
WW	Pin1	″
Proline recognition		
SH3	Src, Crk	Pro–X–X–Pro
WW	YAP, Nedd4	Pro–Pro–X–Tyr
EVH1	Mena, homer	Pro-rich
Gyf	CD2	PPPPGHR motif
Phospholipid recognition		
PH	PKB, mSos, phospholipase C-γ	$PI(3,4)P_2$, $PI(4,5)P_2$, $PI(3,4,5)P_3$
FYVE	SARA, Hrs, EEA1	PI(3)P
PX	p40phox, p47phox	PI(3)P
FERM	ERM, Radixin	$PI(4,5)P_2$
Tubby	TULP1, tubby	$PI(4,5)P_2$
Methylated or acetylated residue recognition		
Bromo	P/CAF	Acetylated lysine
Chromo	HP1	Methylated lysine
Other motif recognition		
SH3	Gads & Grb2 C-terminal SH3	Arg–X–X–Lys
PDZ	PSD-95	C-terminal motifs
PDZ	Neural nitric oxide synthase (nNos)	PDZ
SAM	—	SAM

SH2 domain flanked on either side by an SH3 domain. The SH2 domain of Grb2 binds preferentially to pTyr–X–Asn motifs on activated receptors or cytoplasmic docking or scaffolding proteins, while the N-terminal SH3 domain associates with a Pro–X–X–Pro motif on Sos, a Ras GDP–GTP exchange factor (GEF) (Fig. 1B) [41,42]. Genetic data from invertebrates and mammals has shown that this pathway is critical for RTKs to activate the Ras-MAP kinase pathway *in vivo*, leading to cell growth and differentiation [43–45]. The C-terminal SH3 domain of Grb2, however, has a different function, binding to the Gab1/2 docking proteins through an Arg–X–X–Lys motif [46–48]. Thus, Grb2 recruits Gab1/2 to activated RTKs such as the epidermal growth factor (EGF) receptor. The ensuing phosphorylation of Gab1 creates binding sites for additional SH2 proteins, particularly the p85 subunit of PI3K, resulting in the localized production of PI-3,4,5-P_3 and activation of survival pathways through the PH-containing serine/threonine kinases PKB and PDK1. Grb2 therefore coordinates the activation of two distinct signaling pathways (and in fact is implicated in several more). This suggests that intracellular signaling likely operates as a network, rather than as a simple linear scheme. A signaling network can therefore be established from the reiterated use of rather simple interaction modules.

Evolution of a Phospho-Dependent Docking Protein

As animals have become more complex, modular signaling proteins appear to have evolved through the acquisition of additional modules or binding sites. For example, Shc is a docking protein with a C-terminal SH2 domain and an N-terminal PTB domain flanking a central region containing a proline-rich section and an adaptin binding motif. The distinct binding properties of SH2 and PTB domains allow Shc to interact with multiple phosphotyrosine-containing motifs on cell-surface receptors. The prototypic Shc from *Caenorhabditis elegans* contains this basic organization but is simpler than its *Drosophila* or human counterparts [49]. *Drosophila melanogaster* Shc has an additional tyrosine-based motif in its central region, not present in the *C. elegans* protein, that can be phosphorylated and potentially act as an SH2-binding sequence [50]. In mammals, the situation has become significantly more complex. Mice and humans contain three distinct Shc genes (ShcA, ShcB, and ShcC), the latter two of which are largely restricted to the nervous sytem (Fig. 2A). Furthermore, mammalian Shc proteins have acquired yet an additional tyrosine phosphorylation site and thus have two motifs that upon phosphorylation bind Grb2 and potentially other SH2-containing proteins [51]. Finally, mammalian ShcA is expressed as three isoforms through alternative splicing; the largest form of these isoforms ($ShcA^{p66}$) contains an additional proline-rich N-terminal extension implicated as a factor in cellular response to oxidative stress and in longevity [52]. These data suggest that Shc proteins have multiple functions in signaling that have evolved through the acquisition of new phosphorylation sites and interaction motifs. The tyrosine phosphorylation sites of mammalian Shc allow this protein family to function as docking subunits of activated RTKs to enhance the range of receptor signaling (Fig. 1B). Shc most probably has additional functions as yet to be uncovered.

Multisite Phosphorylation, Ubiquitination, and Switch-Like Responses

An important issue in the design of cell signaling networks is how protein–protein interactions can be used to integrate signals and create all-or-none responses. A typical

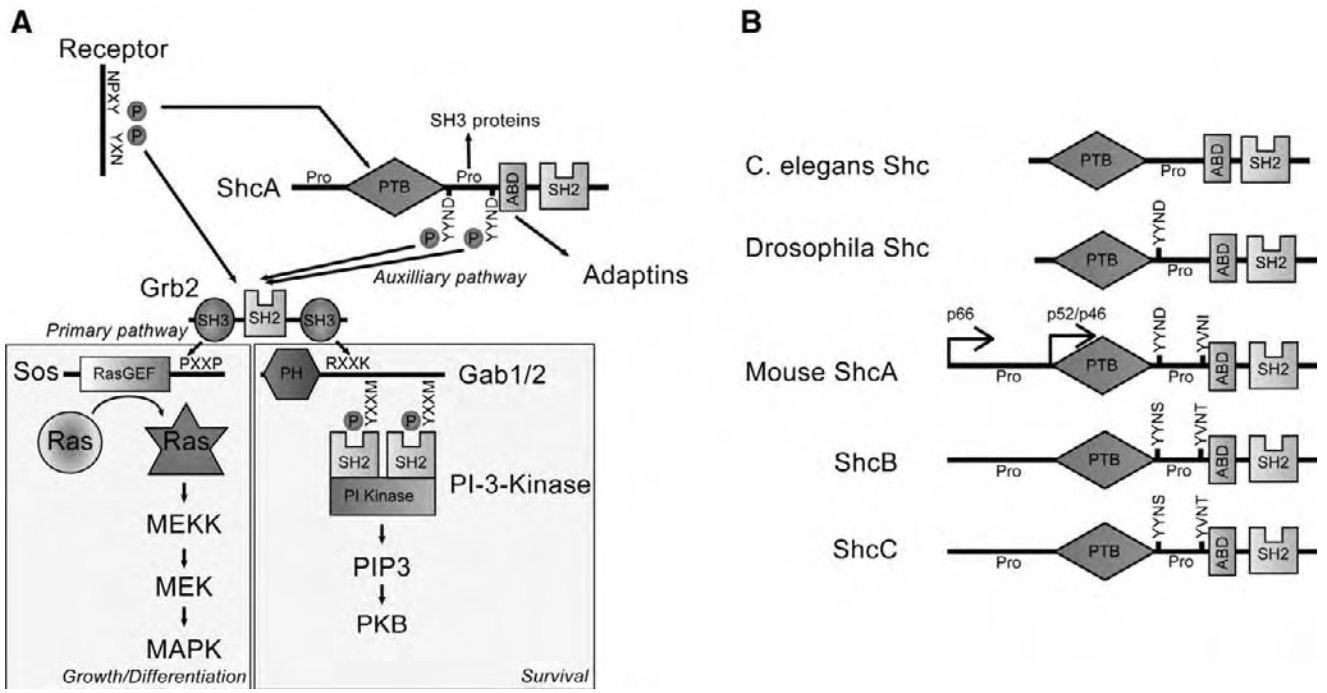


Figure 2 (A) The Grb2 SH3–SH2–SH3 adaptor couples a pTyr–X–Asn docking site to multiple downstream targets through a series of protein–protein and protein–phospholipid interactions. One core pathway to cell growth is assembled through the N-terminal SH3 domain of Grb2 interacting with Pro–X–X–Pro motifs on the Ras–GTPase-activating protein Sos leading to MAP kinase activation. A second core pathway to cell survival is linked through the C-terminal SH3 domain of Grb2 binding to an Arg–X–X–Lys motif within the scaffolding protein Gab1. Ancillary control over this pathway is generated by the ShcA docking protein. ShcA acts, in part, to extend or amplify the functional potential of a receptor to recruit binding partners that convey signals. (B) The Shc docking protein serves as a prototypic example of evolved complexity in signal transduction within a conserved modular architecture. Shc evolution extends the binding capacity of the protein from a primordial form containing an SH2 and PTB domain flanking a central region containing an adaptin-binding and proline-rich section. Shc has gained in complexity, concomitantly with evolution from simple to complex multicellular organisms, by gaining increasing numbers of tyrosine residues that act as binding sites for SH2 domains of other proteins such as Grb2. In mammals, Shc has expanded to a three-gene family with additional forms created by alternate splicing of the ShcA mRNA. ShcB and ShcC are predominantly localized in the brain of mammals, perhaps reflecting the requirement for additional complexity in the signal transduction cascades in this tissue.

enzymatic event, such as a kinase phosphorylating its substrate, or a simple binding event, such as an SH2 domain binding to a phosphorylated tyrosine site, conforms to Michaelis–Menton kinetics and therefore produces graded responses. In other words, the response to a given stimuls is initially linear and then tapers off in a hyperbolic manner (see Box 1). This contrasts with digital switches in which a certain amount of stimulus converts the system from zero to a complete response. Some signaling pathways have steps at which noise is filtered out, signals are integrated, and all-or none decisions are made [53]. This is particularly important in key decisions such as progression through the cell cycle, when the cell must exercise precise control to avoid catastrophic events such as initiating DNA replication prematurely. One mechanism by which a signaling cascade can create a switch-like response (referred to as an *ultrasensitive biological switch*), is through the requirement for multiple, independent phosphorylation events in order to sanction a requisite protein–protein interaction. Under these conditions, the response varies as a higher order of the kinase concentration, such that three independent phosphorylation events create a stimulus–response that responds to the third order of kinase concentration (modeled with a Hill coefficientof three). This has been observed biologically in a number of situations. In the maturation response of *Xenopus* oocytes, two independent phosphorylation events within the MAP kinase pathway set up conditions for an all-or-none activation of the ERK MAP kinase [54]. In a related example, degradation of the yeast cyclin-dependent kinase (CDK) inhibitor Sic1, a key event required for the G1 to S transition (or START in the cell cycle), requires six of nine serine/threonine phosphorylation sites on Sic1 to be phosphorylated by the CDK activity present in the G1 phase of the cell cycle in order for Sic1 to be targeted for ubiquitination and degradation. Phosphorylated Sic1 is bound by the WD40 repeat domain of an F-box protein, Cdc4, that serves as the substrate binding subunit of an E3 protein-ubiquitin ligase complex. Thus, Sic1 acts to monitor G1 CDK activity, setting a threshold for kinase activity that must be met in order for START to occur [55]. In this case, multisite phosphorylation, coupled with a simple binary interaction, creates an ultrasensitive response that ensures the orderly and timely transition into the S phase of the cell cycle [56,57]. In both *Xenopus* maturation factor response and yeast cell-cycle progression, additional factors may conspire to create extremely sharp switch-like responses.

A corollary to these observations is that phosphorylation of proteins on serine/threonine residues induces protein ubiquitination and destruction. The phosphorylated target is

Box 1: Modeling Ultrasensitivity with Stimulus–Response Curves

The shape of a given systems stimulus–response curve is a key aspect of the steady-state behavior of a signaling system. Typical Michaelis–Menten enzymes exhibit hyperbolic stimulus response curves (Fig. 3, $n^H = 1$). At very low stimulus levels, the response grows linearly with the stimulus. As the extent of the stimulus increases, the response to each quanta of stimulus becomes progressively smaller. In other words, a Michaelian (also referred to as graded or hyperbolic) system obeys the law of diminishing returns. A system that obeys Michaelian sensitivity requires an 81-fold increase in input stimulus to drive it from 10% to 90% maximal activation. By contrast, some systems can achieve sigmoidal stimulus–response curves, and this can be well approximated by the Hill equation:

$$y = x^{nH}/(EC_{50} + x^{nH})$$

where n^H is the Hill coefficient. In such a system, the first increments of stimulus produce little response, but once the system does begin to respond, it reaches its maximal response rapidly. The higher the Hill coefficient, the more switch-like the response becomes. This is represented graphically in Fig. 3 for Hill coefficients (n^H) of 1, 6, and 40.

Biologically, this has several significant outcomes. By incorporating an ultrasensitive biological switch into a signal transduction pathway, a cell can filter out noise in a system as small amounts of stimuli will fail to yield any consequential response. Such a switch could also be used to allow precise control over key decisions in which an exact degree of stimuli rapidly creates a complete response. It also sets a threshold for the signal, allowing the system to effectively collect inputs and precisely monitor when these exceed the threshold level set to convert the system to a complete response. In this last case, the signals are integrated at the level of the switch.

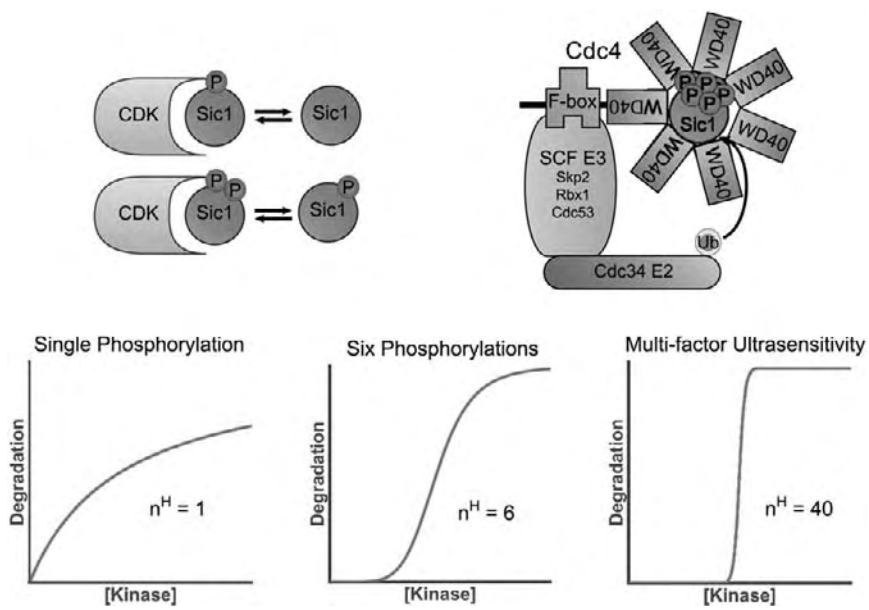


Figure 3 Multiple, independent phosphorylation events of Sic1 by a cyclin-dependent kinase (CDK) create the basis for an ultrasensitive biological switch for the onset of DNA replication in yeast. Phosphorylation of the CDK inhibitor Sic1by the Cln1/2-Cdc28 kinase on at least six independent sites is required for the productive interaction of Sic1 with the WD40 repeat region of the Cdc4 F-box protein. Binding of Sic1 to Cdc4 allows the ubiquitination of Sic1 by the SCF E3 ubiquitin protein ligase complex and subsequent degradation of Sic1. The requirement for multisite phosphorylation results in a sigmoidal stimulus-response curve with a Hill coefficient (n^H) of six, forming the basis for an ultrasensitive biological switch. Additional factors in the biological milieu of the cell likely conspire to significantly improve the degree of ultrasensitivity, as indicated by the curve shown with a Hill coefficient of 40. By contrast, a single phosphorylation event, or simple protein–protein interaction is inherently Michaelian in nature with $n^H = 1$.

recognized by the phospho-dependent interaction domain of an E3 protein-ubiquitin ligase and is thereby recruited into a ubiquitination complex [58].

Summary

Protein phosphorylation frequently induces specific protein–protein interactions mediated by specific phosphotyrosine- or phosphoserine/threonine-binding domains. These phospho-dependent interaction domains are important for the specificity of signal transduction downstream of cell-surface receptors and serve as the prototype for a large family of binding modules that control many aspects of cellular organization.

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CHAPTER 68

Structures of Serine/Threonine and Tyrosine Kinases

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Introduction

Eukaryotic protein kinases that phosphorylate serine/threonine or tyrosine residues constitute a large family of enzymes that are critical components of cell signaling and regulatory pathways [1]. The nature of the amino acid that is phosphorylated defines the two major classes of protein kinases in eukaryotic cells: serine/threonine (Ser/Thr) kinases and tyrosine (Tyr) kinases [2]. Despite the differences in their substrate specificities, Ser/Thr and tyrosine kinases are very closely related in terms of the structure of their catalytic domains. Ser/Thr kinases are found in all eukaryotes and function in a broad range of signaling pathways, including those that control transcription or the regulation of metabolic pathways. Tyrosine kinases are a later evolutionary offshoot of the family and are predominantly found in multicellular animals, where they play important roles in intercellular signaling.

The human genome is estimated to encode several hundred distinct protein kinases [3,4] that function as molecular switches, in which the signaling state of the switch is related to the level of enzymatic activity of the kinase domain. The activity of protein kinases can be regulated by other signaling molecules using a number of different mechanisms [5]. The most common regulatory mechanisms include protein localization, ligand-coupled allosteric activation or inhibition, and reversible conformational changes at the catalytic site of the kinases that are controlled by phosphorylation or dephosphorylation. The improper activation of protein kinases can be highly disruptive to the cell, and as a consequence protein kinases are emerging as an extremely important set of targets for drug development [6,7].

Structures of Protein Kinases

The first crystal structure of a protein kinase to be determined was that of the catalytic domain of cyclic-AMP-dependent kinase, also known as protein kinase A (PKA) [8,9]. PKA is a ubiquitously expressed Ser/Thr kinase involved in several different signaling pathways. The overall fold of the catalytic domain, first seen for PKA, is highly conserved among all Ser/Thr and tyrosine protein kinases. Additional sequences and domains that are located both C-terminal and N-terminal to this structurally conserved catalytic domain account for most of the functional diversity among different protein kinases.

The structure of the catalytic domain of PKA, co-crystallized with adenosine triphosphate (ATP)-Mg and an inhibitor peptide molecule, is shown in Fig. 1 [8,9]. The protein kinase domain is composed of two lobes with an overall length of roughly 275 residues. The N-terminal lobe, alternatively referred to as the N lobe or the small lobe, contains an anti-parallel β-sheet and one important α-helix (helix C), while the C-terminal lobe (or large lobe) is primarily α-helical in composition.

This structure is an example of a protein kinase that is in a catalytically competent conformation and serves as a

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model for the structure of active protein kinases. The two substrates of the phosphorylation reaction, ATP and the polypeptide phosphate acceptor, both bind in a cleft formed between the N lobe and the C lobe. The highly charged chemical groups of the ATP and two associated Mg^{2+} ions are coordinated by a collection of highly conserved Lys, Asn, Asp, and Phe residues (Fig. 2). A recent crystal structure of PKA with a transition state analog supports an in-line mechanism of phosphate transfer from ATP to a target Ser-containing substrate [10]. This mechanism is believed to be conserved among protein kinases, consistent with the highly conserved kinase fold.

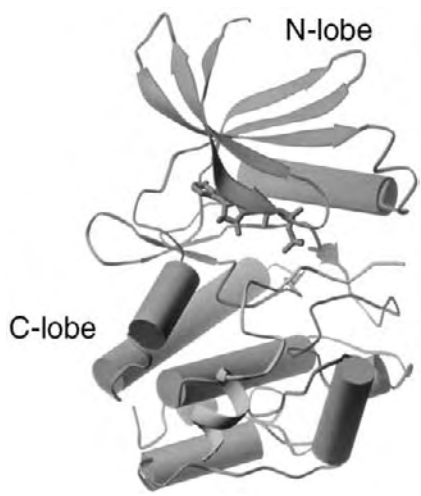


Figure 1 Structure of the Ser/Thr kinase PKA complexed with ATP–Mg^{2+} and a specific inhibitor peptide (PDB code 1ATP) [8,9]. ATP is gray, the peptide inhibitor PKI is yellow, α-helix C is pink, and the activation loop is red.

The structure of the catalytic domain of Lck [11] provides an example of the catalytically active conformation of a tyrosine kinase enzyme. Despite the absence of both nucleotide and substrate, the conformations of key catalytic residues are primed for catalysis, in line with the conformations of homologous residues seen in the structure of PKA. The active form of the kinase domain of Lck is phosphorylated on Tyr 394, which lies in the activation loop of the kinase domain, a segment of roughly 15 to 20 residues located between the N and C lobes of the kinase domain. Phosphorylation on one or more residues in the activation loop is a signature of the activated state of many protein kinases. In active Lck, phosphorylated Tyr 394 forms a salt bridge with Arg 387, thereby stabilizing the conformation of the activation loop and locking the enzyme in a catalytically active state.

Structures of Inactive Protein Kinases

The modulation of catalytic activity in protein kinases is achieved by a diverse range of mechanisms. The structures of the active states of kinases that are constrained to be similar by the chemical requirements for catalysis of phosphate transfer. In contrast, the structures of inactive states of protein kinases show considerable diversity [5]. In this review, we present examples of the inactivation mechanisms of two different tyrosine kinases for which structure determinations have been carried out in both the active and inactive states of the enzymes. The first is the insulin receptor kinase (Irk), and the second is the Src family of tyrosine kinases.

The structures of the kinase domains and the extracellular ligand binding domains of several receptor protein

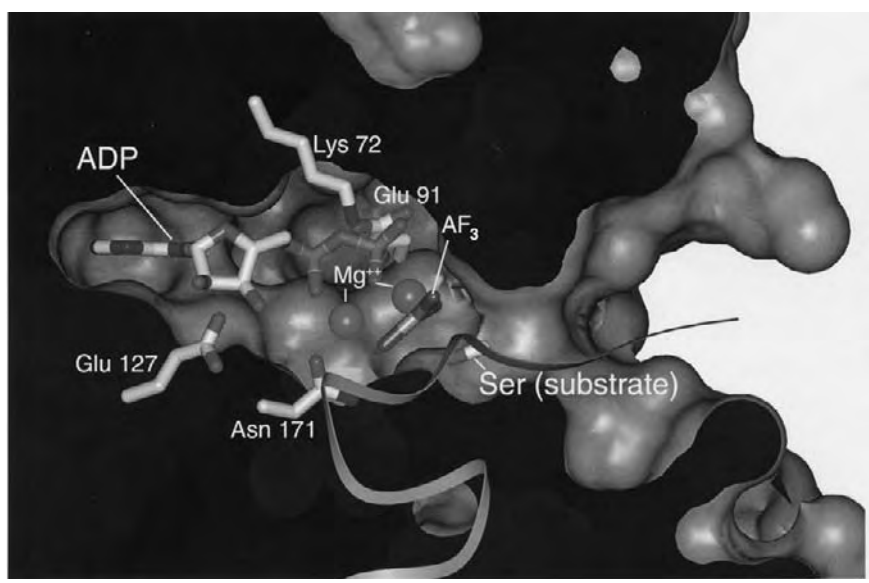


Figure 2 Active site of a crystal structure of PKA trapped in a transition state intermediate conformation bound to ADP–AlF_3 and substrate peptide (PDB code 1L3R) [10]. Charged sidechains that make key interactions with the ATP and Mg^{2+} ions are shown: Lys 72, Glu 91, Glu 127, Asn 171, and Asp 184. The serine acceptor on the peptide substrate is also indicated.

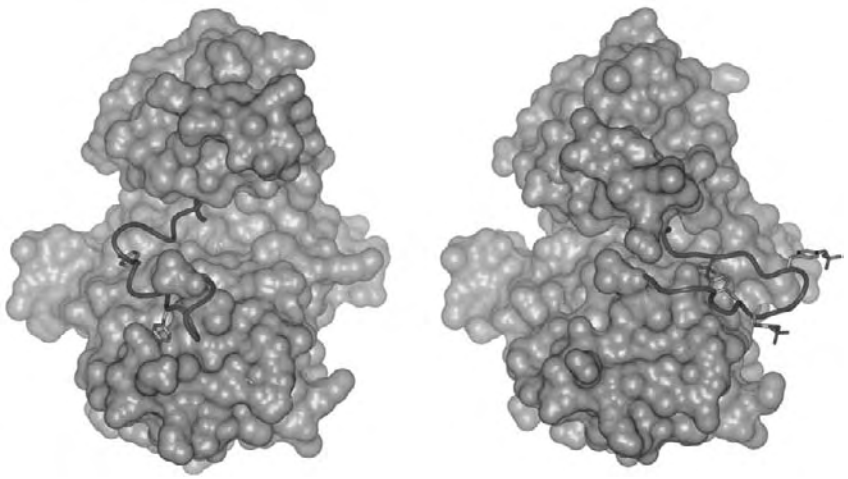


Figure 3 Structures of the catalytically inactive (left) and active (right) forms of the kinase domain of Irk (PDB codes 1IRK and 1IR3, respectively) [15,16]. The active state is stabilized by the phosphorylation of three tyrosine residues in the activation loop.

kinases have been solved in recent years (for reviews, see references [12] and [13]). Receptor tyrosine kinases contain a variety of extracellular ligand binding domains in the N-terminal extracellular region, followed by a short membrane-spanning region, and finally a catalytic tyrosine kinase domain in the C-terminal cytoplasmic portion of the proteins. Ligand-induced alterations in the oligomeric state of the receptor or in the conformation of oligomeric forms of the receptor can stimulate phosphorylation of the kinase domains, facilitating the propagation of a downstream cytoplasmic signal [14].

Structures of catalytically active and inactive states of the catalytic domain of insulin receptor kinase are shown in Fig. 3 [15,16]. Activation of this receptor involves the phosphorylation of three tyrosine residues located in the activation loop. The effect of this chemical modification is to induce a major conformational change in the structure of the loop. In the inactive state, the activation loop is found in a relatively compact buried conformation that sterically blocks access to the protein active site and further attenuates catalytic activity by causing conformational changes within the catalytic center. The addition of three negatively charged phosphate groups to the tyrosine residues in the loop destabilizes the buried conformation of the activation loop and favors a more solvent exposed conformation that opens up the enzyme active site for the entry of a peptide substrate.

The Src family kinases comprise a closely related family of nine distinct nonreceptor tyrosine kinases that function downstream of membrane-associated proteins in intercellular signaling pathways. Src kinases possess two peptide binding domains, the SH2 and SH3 domains, that are located upstream of the catalytic domain. In addition to having a single Tyr phosphorylation site (Tyr 416 in chicken c-Src numbering) in the activation segment that serves to activate the kinase when phosphorylated, Src family kinases also possess an important regulatory segment that is located immediately after the kinase domain (Fig. 4) [17].

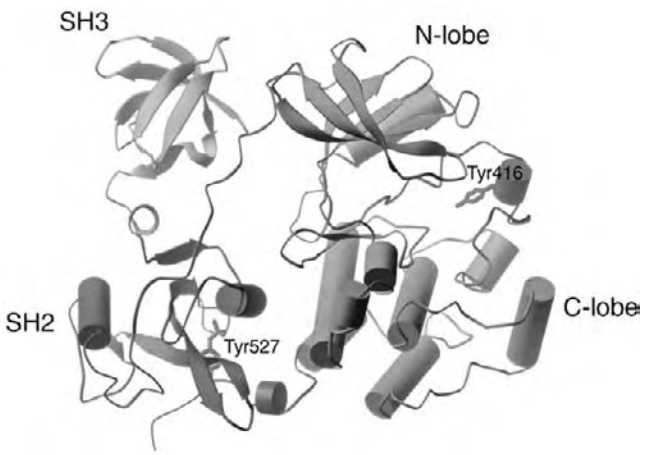


Figure 4 Crystal structure of inactive Hck (PDB code 1QCF) [18]. The two peptide binding domains (SH3 and SH2) bind intramolecularly to form an assembled inactive conformation. Tyr 416 in the activation loop (red) is dephosphorylated. Tyr 527 in the C-terminal tail of the protein (red) is phosphorylated and bound to the SH2 domain (green).

The crystal structures of inactive Src kinases have been determined [18,19]. The crystal structure of the catalytically inactive state of the Src family member Hck is shown in Fig. 4 [18] (the structure of inactive c-Src is very similar [19]). The downregulated state of Src-family kinases is characterized by phosphorylation on Tyr 527 in the C-terminal tail, with Tyr 416 in the activation loop unphosphorylated. A distinguishing structural feature of these kinases is that upon inactivation these proteins adopt a closed and assembled state in which the SH2 domain binds intramolecularly to phosphorylated Tyr 527. The assembled state also finds the SH3 domain, located immediately upstream of the SH2 domain, bound intramolecularly to a type II polyproline helix that is part of the connector between the SH2 domain and the kinase domain. Displacement of either of these two intramolecular interactions via external ligands will stimulate activation

of the kinase, demonstrating that these intermolecular docking domains function as protein localization handles as well as playing a role in the allosteric inactivation of the kinase.

Members of the Src family of Tyr kinases share roughly 75% sequence identity with each other in the SH3, SH2, and catalytic domains. The structure of the activated state of the Src family member Lck can thus be contrasted with the structures of the inactive state of Hck and c-Src to highlight specific conformational changes that occur in this family of kinases upon inactivation. The two most dramatic changes include the closing down of the activation segment to sterically block the peptide–substrate binding cleft, and the nearly 45° rotation of α-helix C to bring a catalytically important Glu residue (Glu 310 in chicken c-Src) out of alignment such that it can no longer interact with a conserved lysine residue that coordinates ATP. While the former mechanism is similar to the inactivation mechanism found in both Irk and PKA, the specific misalignment of α-helix C is tightly coupled to the unique assembled closed state that the three domains Src family kinases adopt upon inactivation and is an example of diversity found in the inactivation mechanism of distinct protein kinases.

Summary

In conclusion, the structures of Ser/Thr and tyrosine kinases are strikingly similar when these enzymes are released from inhibitory interactions. In contrast, the mechanisms by which kinases are inhibited are numerous, and these distinct regulatory mechanisms result in quite different conformations for the inactive states of kinases. The unexpected diversity in the structures of inactive kinases provides routes to the acquisition of specificity by small-molecule inhibitors of kinase function [20].

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CHAPTER 69

Protein Tyrosine Kinase Receptor Signaling Overview

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Introduction

Protein tyrosine kinase (PTK) receptors constitute an important class of transmembrane receptors that transduce signals regulating cell growth, differentiation, survival, and migration. PTK receptors are also conserved in lower species, and much of our knowledge about their functional properties comes from studies of *Drosophila* and *Caenorhabditis elegans*. Several PTK receptor genes that have been inactivated in mice have revealed the important functional roles of individual PTK receptors in different organs at various stages of the development. Overactivity of PTK receptors has been implicated in a number of diseases, particularly cancer, and several of the PTK receptors were first identified as transforming oncogene products. This chapter reviews the general principles for PTK receptor structure, activation mechanism, and regulation.

PTK Subfamilies

In the human genome, 58 genes encode PTK receptors [2,36]. Each receptor consists of an extracellular ligand-binding part, a single transmembrane domain, and an intracellular part with an intrinsic kinase domain. Based on their overall structures, the PTK receptors can be placed into 20 subfamilies (Fig. 1). Individual subfamilies are characterized by specific structural motifs in their extracellular parts (e.g., Ig-like domains and fibronectin type III domains). Moreover, the sequences of the kinase domains are normally more similar within the subfamilies than between the subfamilies. The major families are briefly introduced below (for reviews, see Fantl *et al.* [8] and Schlessinger [29]).

The epidermal growth factor (EGF) receptor was the first PTK receptor to be identified. The four members of the family are important for the morphogenesis of epithelial tissues. Members of this family are often amplified or activated through mutations in human malignancies.

The three members of the insulin receptor family are disulfide-bonded dimers that undergo cleavage during processing to generate α- and β-subunits. In addition to the well-known metabolic effects mediated by the insulin receptor, this family mediates important survival signals.

The platelet-derived growth factor (PDGF) family members are characterized by 5 Ig-like domains in the extracellular domain and by the presence of an intervening sequence that splits the kinase into two parts. PDGF receptors are of particular importance for the development of the connective tissue compartments of various organs, as well as for the development of smooth muscle cells of blood vessels. The related receptors for stem cell factor and colony-stimulating factor 1 (CSF-1) are implicated, for example, in the development of hematopoietic cells, germ and neuronal cells, and macrophages.

The vascular endothelial cell growth factor (VEGF) receptor family members have seven Ig-like domains extracellularly and are primarily expressed on endothelial cells; thus, they are implicated in vasculogenesis, angiogenesis, and lymphangiogenesis.

The fibroblast growth factor (FGF) receptor family members are characterized by three Ig-like domains extracellularly, although splice variants with only two Ig-like domains have been described. Like the VEGF receptors, FGF receptors are expressed on endothelial cells and are implicated in angiogenesis; however, these receptors are also expressed in other cell types and have important roles in the embryonal development of several organs and tissues.

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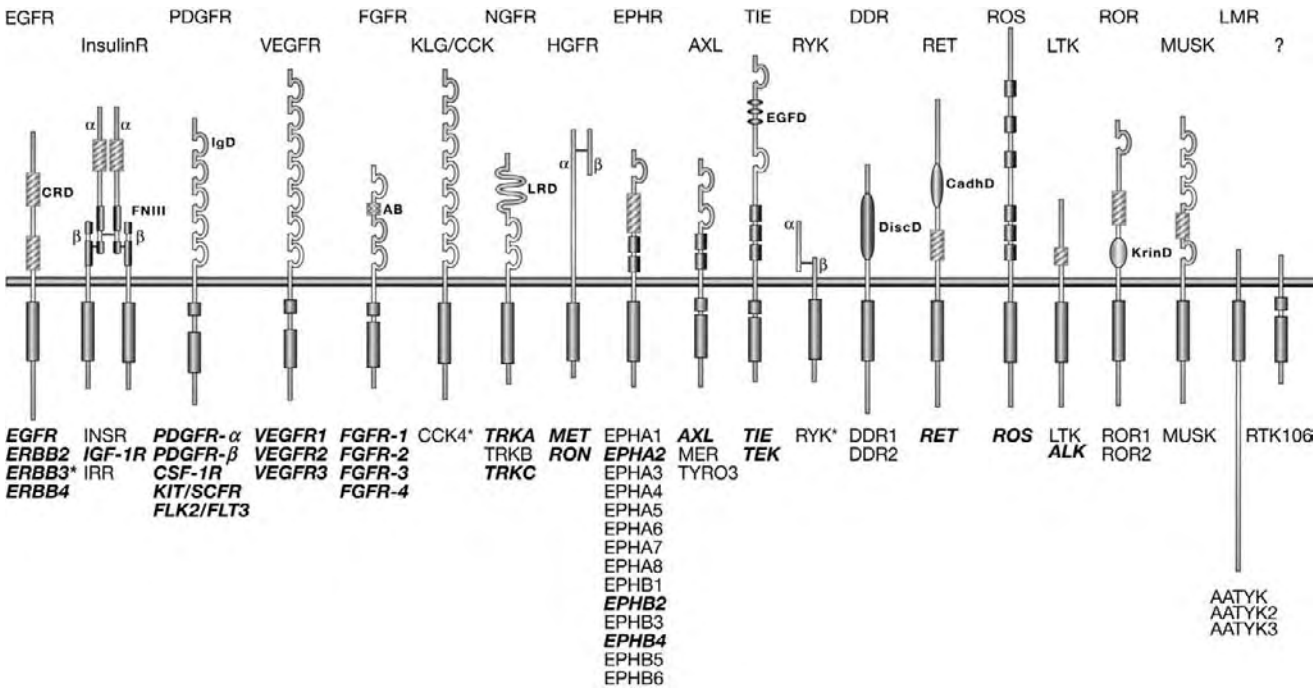


Figure 1 Organization of human PTK receptors in 20 subfamilies of structurally related receptors. The designations of the members in each family are given below each schematic figure. Receptors implicated in malignancies are written in italics and bold. An asterisk after the name indicates that the PTK receptor has an inactive kinase domain. (From Blume-Jensen, P. and Hunter, T., *Nature*, 411, 355–365, 2001. With permission.)

Members of the neurotrophin receptor family (TrkA, B, and C) bind members of the NGF family of neurotrophins and have important functions during the development and maintenance of the central nervous system.

The two members of the hepatocyte growth factor (HGF) receptor family (Met and Ros) undergo cleavage of their extracellular domains after their syntheses. They have important roles in regulation of cell motility and in organ morphogenesis during embryonal development.

The Eph receptor family, the largest of the PTK receptor subfamilies, has 14 members. Eph receptors are expressed in the nervous system and also in endothelial cells; thus, they are implicated in neuronal guidance and angiogenesis. Interestingly, one class of their ligands, ephrin-Bs, are also transmembrane molecules expressed on the surface of cells; binding of ephrin-Bs to Eph receptors leads not only to activation of the Eph PTK receptor but also to initiation of signaling events at the intracellular part of the ephrin molecules [31].

The remaining PTK subfamilies generally consist of single members and are generally less well characterized. Interestingly, one of these families, the DDR family, has collagens as ligands [33,38], thus exemplifying the observation that PTK receptors mediate signals not only from soluble or membrane-associated growth factors but also from the surrounding extracellular matrix.

Of note is that three examples of PTK receptor family members have mutations in their kinase domains, rendering them devoid of kinase activity (Fig. 1); however, they may still have important roles in signaling (see later discussion).

In general, each subfamily binds a family of structurally related ligands. The specificity is not always absolute within the subfamilies; several receptors bind more than one ligand and several ligands bind more than one receptor. In contrast, high-affinity interactions of individual ligands with more than one subfamily of PTK receptors, or of individual PTK receptors with more than one class of ligands, have not been observed.

Mechanism of Activation

Ligand-Induced Receptor Dimerization

Protein tyrosine kinase receptors are activated by ligand-induced dimerization in all cases that have been investigated [29]. This brings the receptor kinase domains close to each other, which results in autophosphorylation in *trans* within the intracellular parts of the receptors. The autophosphorylation occurs on tyrosine residues located within or outside the kinase domain of the receptor.

There are, however, many different modes whereby ligand binding induces receptor dimerization [13]. Some ligands are disulfide-bonded dimers, such as PDGFs and VEGFs; the binding of these ligands leads to formation of a symmetric complex consisting of two receptors and one dimeric ligand [39]. In contrast, ephrins are monomeric molecules; after binding of two ephrin molecules to Eph receptors, a dimeric receptor complex is formed in which each ephrin molecule contacts two receptors and each receptor contacts two ligands [15]. Ligand binding to the EGF receptor causes

a conformational change allowing direct receptor-receptor interaction which stabilizes dimerization [9a,24a]. There are also examples of accessory molecules helping to stabilize a dimeric complex; FGFs are monomeric molecules that interact with receptors at a 1:1 stoichiometry, and receptor dimerization is induced by binding of heparin or heparan sulfate to the complex of FGF and receptor [30]. Finally, members of the insulin receptor family are already disulfide-bonded dimers before ligand binding; binding of ligand presumably induces a conformational change that allows receptor autophosphorylation and activation.

Although receptor dimerization is likely to be necessary for activation of PTK receptors, it is not always sufficient. Evidence suggests that the orientation of the two intracellular domains in the receptors relative to each other is important [19]. Moreover, there are indications that the initial dimerization of EGF receptors may be followed by further oligomerization, which may be necessary to obtain a fully active receptor [4].

Homo- and Heterodimerization

In the classical case of ligand-induced dimerization of PTK receptors, two identical receptors form a homodimer; however, two related receptors from the same subfamily may also form a heterodimer. Examples from the PDGF [14] and EGF [41] receptor subfamilies show that heterodimeric receptors may have quantitatively or qualitatively different signaling capacities.

There are also examples of heteromeric complexes between individual PTK receptors and unrelated receptors. Examples include interactions between PTK receptors from one subfamily with PTK receptors from another subfamily (e.g., PDGF and EGF receptor have been shown to interact [27]). In addition, PTK receptors have been shown to bind to integrins, an interaction that has been shown to enhance integrin signaling [10]. In some cases, interactions with non-kinase receptors enhance the affinity for ligand binding (e.g., a long splice form of VEGF binds to its PTK receptors with higher affinity if the receptor neuropilin is also part of the complex [34]).

Activation of the Receptor Kinase

Ligand-induced PTK receptor dimerization leads to autophosphorylation of the receptors in *trans* within the complex. The autophosphorylation serves two important roles: (1) it causes activation of the kinase domain and (2) it creates docking sites for downstream SH2-domain-containing signaling molecules. Autophosphorylation may lead to activation of the kinase via several different mechanisms, of which more than one may apply for individual PTK receptors [2]. Tyrosine residues exist within the activation loops of kinases; after phosphorylation, these residues cause the loop to swing out and open up the active site of the kinase [17]. Because most PTK receptors are phosphorylated in this region, this is likely to be a common mechanism for activation of the kinase domain. However, members of the EGF receptor family are not autophosphorylated in the activation loop. In these receptors, it is possible that the activation loops do not efficiently inhibit the kinase of the receptors. Instead, it has been proposed that the long C-terminal tails of these receptors block the active site of the kinase, an inhibition that may be relieved by autophosphorylation and a conformational change of the C-terminal tail, as has been shown for the PTK receptor Tek [32]. Finally, the recent elucidation of the three-dimensional structure of the Eph receptor revealed that in the inactive receptor the juxtamembrane domain forms a helical structure that distorts the small lobe of the kinase domain and prevents access to the active site of the receptor; after autophosphorylation in the juxtamembrane domain, this loop moves away and opens up the active site of the receptor [40].

Docking of SH2 Domain Signaling Proteins

The SH2 domain is a protein module that folds to form a pocket into which a phosphorylated tyrosine residue fits [25]. Genes encoding 87 SH2-domain-containing proteins with a total of 95 SH2 domains are present in the human genome [36]. They interact with phosphorylated tyrosine residues in a specific manner that is directed mainly by the three to six amino acid residues downstream of the phosphorylated tyrosine residue.

As an example, Fig. 2 illustrates the interaction between the autophosphorylated PDGF β-receptor and different SH2-domain-containing molecules. One class of SH2 domain proteins has intrinsic enzymatic activity (e.g., the tyrosine kinase Src, phospholipase Cγ, the tyrosine phosphatase SHP-2, and the GTPase-activating protein (GAP) for Ras. The respective enzymatic activities are induced by binding of the SH2 domain to the receptor or by tyrosine phosphorylation induced by the receptor kinase; alternatively, the enzyme is constitutively active and, by binding to the receptor, may simply be brought to the inner leaflet of the cell membrane, where the next component in the signaling chain is located.

Other SH2 domain proteins are devoid of intrinsic enzymatic activity and serve as adaptors that connect the activated receptors with downstream signaling molecules. Adaptors often have additional domains that mediate interactions with other molecules, such as the SH3, PTB, and PH domains [25]. Examples of such adaptors include Nck, Crk, and Shc, as well as Grb2, which forms a complex with Sos, a nucleotide exchange molecule for Ras, and the regulatory subunit p85, which forms a complex with the catalytic subunit p110 of phosphatidylinositol 3′-kinase (PI3-kinase).

The interaction between the activated and autophosphorylated receptor and individual SH2-domain-containing molecules initiates signaling pathways that lead to growth stimulation, survival, migration, and actin reorganization. The signaling capacity of a receptor is thus dependent on which SH2 domain proteins it can dock. Differential autophosphorylation may also be the mechanism by which

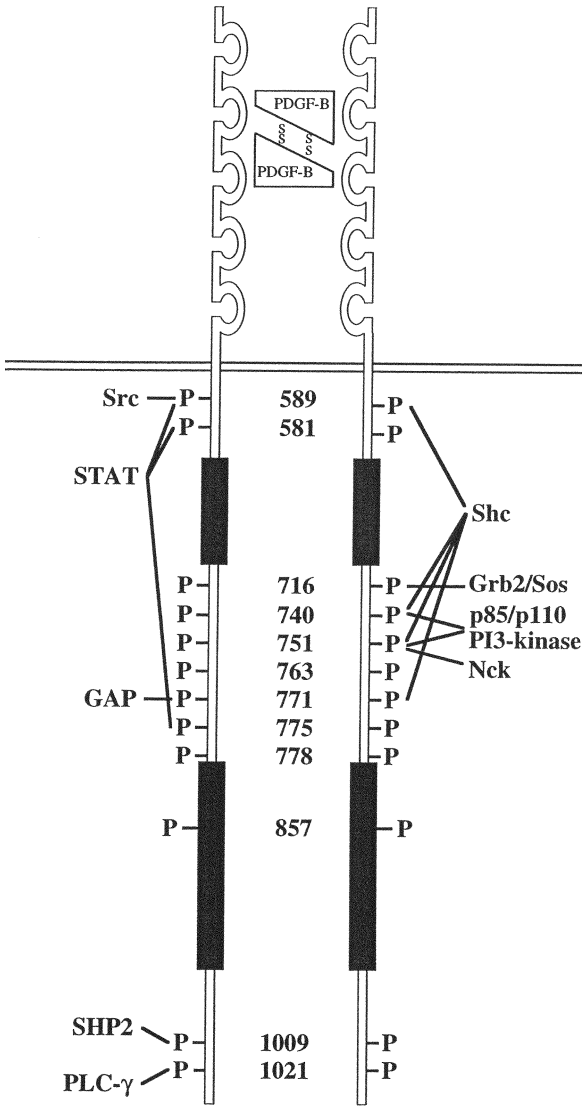


Figure 2 Schematic illustration of a complex between PDGF-BB and two PDGF β receptors. Known autophosphorylated tyrosine residues (P) and their numbers in the receptor sequence are indicated, as well as their interactions with SH2-domain-containing signaling molecules. Signaling molecules with intrinsic enzymatic or transcription factor activity are to the left, and adaptors to the right. Note that it is not known how many SH2 domain proteins can bind simultaneously to a dimeric receptor complex.

heterodimeric receptor complexes acquire unique signaling properties [14]. It should be noted that one member of the EGF receptor family, ErbB3, is devoid of kinase activity, yet in heterodimeric configuration with other members of the family it has a potent signaling capacity due to its ability to provide docking sites for SH2 domain proteins [41].

Inhibition of Phosphatases

The phosphorylation events performed by PTK receptors are counteracted by dephosphorylation by specific tyrosine phosphatases. Recent studies have shown that in order for efficient signaling via PTK receptors, tyrosine phosphatases must be inactivated [35,37]. This may be done by transient, specific oxidation of a cysteine residue in the active site of phosphatases, induced after PTK receptor activation in a PI3-kinase-dependent manner [1].

Regulated Intramembrane Proteolysis

Although activation of cytoplasmic signaling pathways by docking of SH2 domain signaling proteins is a major mode of signaling via PTK receptors, an alternative mechanism was recently revealed. The EGF receptor family member ErbB4 was shown to undergo regulated proteolysis in two steps. First, the extracellular domain is cleaved off by a metalloprotease, then another protease, γ-secretase, cleaves within the transmembrane domain and liberates the intracellular domain of ErbB4 for translocation to the nucleus, where it potentially can regulate transcription directly [24]. A similar situation may prevail for the EGF receptor [23]. Although regulated intramembrane proteolysis is a well-established signaling mechanism for another receptor type (i.e., Notch), its general importance in PTK receptor signaling remains to be elucidated.

Control of PTK Receptor Activity

Receptor Internalization and Degradation

After ligand-induced receptor activation, PTK receptors are often accumulated in coated pits and thereafter internalized in endosomes [5], where they are deactivated by several different mechanisms. Upon acidification of the milieu inside the endosomes, the ligand may dissociate from the receptor, which then monomerizes, becoming dephosphorylated by tyrosine phosphatases and then being recycled back into the membrane. Alternatively, the ligand–receptor complex is degraded after fusion of the endosomes with lysosomes. Moreover, PTK receptors have been shown to become ubiquitinated after activation. The ubiquitination may be mediated by interaction of the activated receptor with the ubiquitin ligase Cbl and may trigger degradation also in proteasomes [20,22].

Control of PTK Receptor Signaling

There are several examples of mechanisms that control PTK receptor signaling. When pathways that stimulate certain cellular responses are initiated, signals that inhibit the same responses are often induced. Examples include Ras activation by the PDGF receptor; at the same time as Ras is activated (i.e., converted to its GTP-bound form by the actions of the Grb2/Sos complex), it is also inactivated (i.e., converted to the GDP-bound form by RasGAP). The net effect on Ras activation by the PDGF receptor is thus dependent on the stoichiometry in phosphorylation of the tyrosine residues that can bind Grb2/Sos and RasGAP; evidence suggests that this balance can differ, for example, between homo- and heterodimeric receptor complexes [6].

Other examples of such mechanisms are the tyrosine phosphates SHP-1 and -2, each of which has two SH2 domains through which they can bind to several PTK receptors. The binding to tyrosine-phosphorylated residues activates the

enzymatic activities of SHP-1 and -2, which may then counteract signaling by dephosphorylating the receptor or its substrates. It is an interesting possibility that SHP-1 and -2, or other tyrosine phosphatases, may dephosphorylate individual tyrosine residues with different efficiency and thereby modulate signaling not only quantitatively but also qualitatively [42].

To complicate the issue even further, evidence indicates that SHP-2 and possibly RasGAP, in addition to their negative modulatory role in signaling, also influence signaling by serving as adaptor molecules providing a bridge between the PTK receptor and downstream signaling molecules.

Another mechanism for feedback control of signaling is via activation of protein kinase C (PKC). The classical members of the PKC family are activated by Ca^{2+} and diacylglycerol, which are produced downstream of phospholipase Cγ. For instance, the receptors for EGF, insulin, HGF, and stem cell factor are phosphorylated by PKC in such a way that inhibits the tyrosine kinase activities of the receptors [3].

Cross-Talk Between Signaling Pathways

In addition to examples of negative modulation of one signaling pathway on another, as discussed for RasGAP and SHP-1 and -2, components in certain signaling pathways have been found to activate components in other signaling pathways. Examples include Ras and PI3-kinase, which can form a physical complex and activate each other mutually [16,26].

The cross-talk between signaling pathways downstream of PTK receptors may be the reason why the effects of activating one receptor or another in the same cell are rather similar, as illustrated, for example, by the use of microarray analysis of 3T3 cells after activation of PDGF or FGF receptors [7]. Even though there are several indications that the signaling capabilities of PTK receptors overlap extensively, it is likely that qualitative and quantitative differences in signaling capacity occur between PTK receptors, particularly in the situation (common *in vivo*) when the availability of ligand is limiting and only a small fraction of the receptors on the cell surface is activated.

In addition, cross-talk in signaling occurs via PTK receptors and other receptor types. Cytokine receptors, for example, which do not have any intrinsic kinase domain but interact with cytoplasmic tyrosine kinases of the JAK family, exert much of their signaling via activation of members of the STAT family [18]. However, STATs are also activated by certain PTK receptors [29]. Moreover, classical signaling pathways downstream of PTK receptors, such as Ras, PI3-kinase, and phospholipase Cγ, are also activated after ligation of cytokine receptors [28] or integrins [10].

Protein serine/threonine kinase receptors, which mediate growth inhibitory signals, activate SMAD molecules, which after translocation into the nucleus act as transcription factors. The MAP kinase Erk, which is activated by PTK receptors via Ras, has been shown to phosphorylate and inhibit SMADs [21], providing one example of how PTK-receptor-induced signals can modulate in an inhibitory manner signaling by other receptors.

Another example of cooperation between different receptor types is the finding that the mitogenic activity of certain seven-transmembrane-spanning G-protein-coupled receptors occur by transactivation of the EGF receptor [11].

PTK Receptors and Disease

Given the importance of PTK receptors in the control of cell proliferation and migration, it is not surprising that overactivity of PTK receptors occurs in cancer and other diseases that involve excess cell proliferation, such as inflammatory and fibrotic conditions and psoriasis. About half of the PTK receptors are implicated in various human malignancies (Fig. 1) [2]. Often, the receptors are constitutively activated by amplification or mutational events. Several mutations of PTK receptors cause constitutive dimerization (1) by mutations affecting disulfide bonding in the extracellular parts of the receptors, thus causing the formation of covalent dimers, mutations of other residues in the transmembrane, or juxtamembrane domains that promote dimerization; or (2) by formation of fusion proteins between the kinase domains of the receptors and proteins that normally occur as dimers or oligomers. The end result is a constitutively active kinase that drives cell growth.

Another mechanism of activation of PTK receptors seen in disease is overproduction of the corresponding ligand. If a cell produces a growth factor for which it has the corresponding receptor, autocrine stimulation of growth may result. Alternatively, the growth factor may stimulate cells in the environment in a paracrine manner, which is relevant in tumor progression. Tumor-derived factors (e.g., VEGFs and FGFs) act on angiogenic PTK receptors and cause vascularization of the tumors, which is a prerequisite for tumor growth [9]. Likewise, other growth factors produced by tumor cells (e.g., PDGFs) may stimulate the formation of tumor stroma, which is important for the balanced growth of tumors [43].

Given the importance of PTK receptors for serious diseases, clinically useful PTK receptor antagonists are warranted. Several types of antagonists are currently used clinically or are in clinical trials for cancer, including a monoclonal-antibody-recognizing ErbB2 and low-molecular-weight selective inhibitors of various tyrosine kinases [12]. It is likely that PTK receptor antagonists will be important tools in the treatment of cancer and possibly other diseases characterized by an excessive cell growth.

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CHAPTER 70

Signaling by the Platelet-Derived Growth Factor Receptor Family

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Introduction

The platelet-derived growth factor (PDGF) family causes cellular responses by engaging its cell-surface receptors. These receptors are tyrosine kinases, which initiate many signaling cascades that result in cell proliferation, motility, and survival. This chapter reviews current understanding of the signaling enzymes that serve as the intracellular effectors of the PDGF receptors. In addition, it discusses the impact of integrins, phosphotyrosine phosphatases (PTPs), and cell cycle on PDGF-induced signaling pathways and subsequent cellular responses.

Platelet-Derived Growth Factors, Their Receptors, and Assembly of the PDGF Receptor Signaling Complex

Platelet-Derived Growth Factor Isoforms

At present, four genes encoding different platelet-derived growth factor chains are known: A, B, C, and D. Biologically active PDGFs exist as disulfide-bonded homodimers designated AA, BB, CC, and DD. A and B chains form a heterodimeric PDGF AB (Fig. 1). The history of the discovery of PDGFs dates back to as early as 1974, when mitogenic activity of whole blood serum was linked to the presence of platelets [1]. PDGF AB was the first to be purified and biochemically characterized a few years later, followed by PDGF BB and AA [2–5]. Cloning of PDGF A and B cDNAs and determination of the structure of corresponding genes were completed in the 1980s [6–8].

The newest members of the PDGF family, PDGF C and PDGF D, were found only recently by searching the database of human expressed sequences [9–12]. PDGFs C and D have a two-domain structure with an N-terminal CUB domain and C-terminal PDGF/vascular endothelial growth factor (VEGF) (core) domain, separated by a hinge region (Fig. 1). No heterodimers involving C or D chains have been detected. The unique feature of these two new PDGFs is the requirement for proteolytic cleavage of the CUB domain upon secretion in order to achieve biological activity. Thus, latency may be the reason why these growth factors were not originally detected by functional assays.

Core domains of PDGF C and PDGF D have more structural similarity to each other than to PDGF A and B, sharing about 43% of identical amino acids [11,12]. PDGFs A and B are even more closely related, with 60% identity [13]; only 25 to 35% amino acid identity is found when A and B are compared to C and D [9,11].

Another ligand related to the PDGF/VEGF family, Pvf1, was recently discovered in *Drosophila* [14]. It is 29% identical to human PDGF A and regulates migration of border cells to oocytes during oogenesis. There is evidence that at least two more proteins with PDGF motifs may exist in *Drosophila*.

Platelet-Derived Growth Factor Receptors

PDGFs exert their biological functions by binding to two isoforms of PDGF receptors, α and β, with different degrees of affinity. Both receptors are composed of extracellular, transmembrane, and intracellular parts. The extracellular part consists of five immunoglobulin-like domains that are involved in ligand binding (domains I–III) [15,16] and receptor dimerization (domain IV) [17]. The intracellular kinase domain of the PDGF receptors is split in two by an approximately 100-amino-acid insert (Fig. 1) [18].

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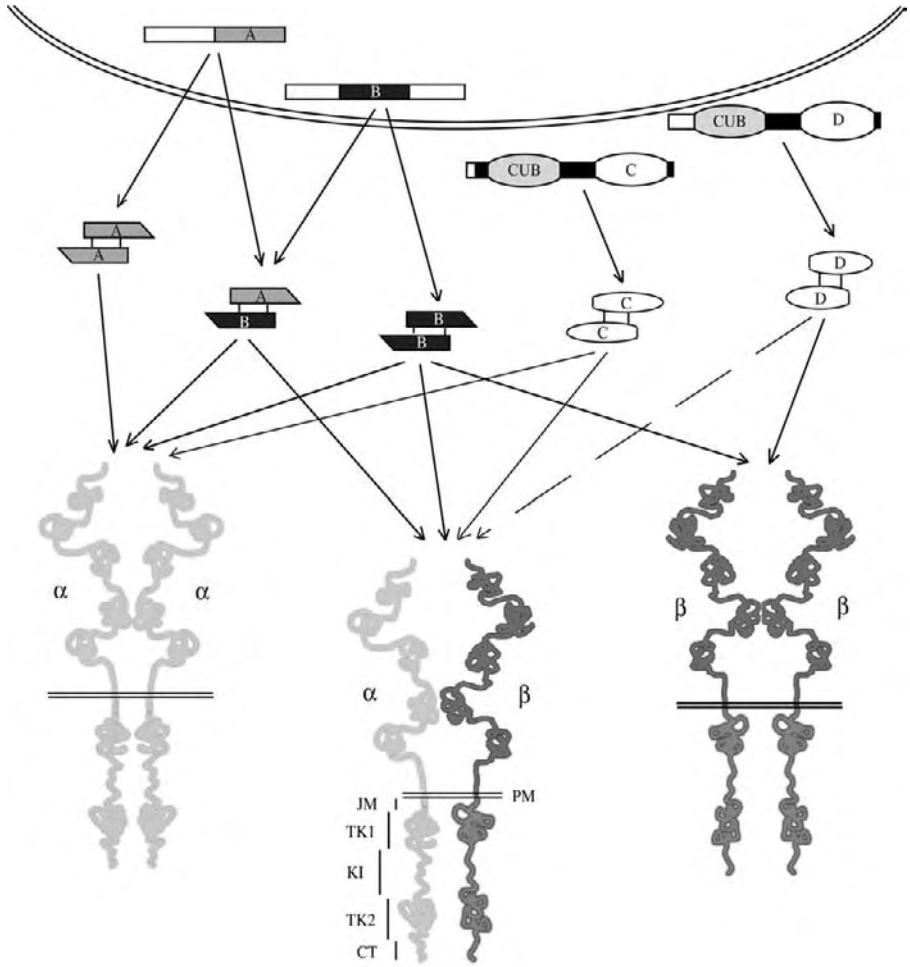


Figure 1 Platelet-derived growth factors and their receptors. PDGFs A and B are synthesized as precursors that are proteolytically processed before secretion. PDGFs C and D are secreted in latent form and activated by proteolytic cleavage of the CUB domain. All PDGF isoforms dimerize prior to the proteolytic cleavage (not shown in the figure). Specificity of binding of mature active PDGFs to their receptors is shown; dashed arrow indicates that controversy exists with regard to the ability of PDGF DD to cause the formation of a heterodimeric receptor (reference [11] versus [12]). Domain structure of the PDGF receptor α subunit (identical to that of the β subunit) is presented. The extracellular part of the receptor consists of five Ig-like domains. PM, plasma membrane; JM, juxtamembrane domain; TK1 and TK2, proximal and distal parts of tyrosine kinase domain, respectively; KI, kinase insert; CT, carboxyl-terminal tail.

PDGF A chain binds specifically to α-receptor, and PDGF B can bind both α and β receptors [19,20]. PDGF C was originally described as a ligand for PDGF α, but not β receptor [9]; however, later it was shown to bind PDGF β receptor in cells expressing both α and β isoforms [10]. PDGF D has been reported to be a β receptor ligand [12], but its ability to bind α receptor in α/β-expressing cells remains controversial [11,12]. Interactions of PDGFs with α and β receptors are summarized in Fig. 1. A bivalent dimer, PDGF molecule binds to two receptor subunits, causing them to dimerize. Upon dimerization, PDGF receptors become rapidly phosphorylated on multiple tyrosine residues. One of them (regulatory tyrosine) is located in the second part of the kinase domain and is important for receptor kinase activity (Tyr857 in β receptor [21] and, by homology, Tyr849 in α receptor). Most of the other phosphorylation sites lie in noncatalytic parts of the receptor (Fig. 2). The exact sequence of events during receptor activation and formation of signaling complex is unknown. By analogy to other receptor tyrosine kinases (RTKs), it seems likely that ligand binding and subsequent dimerization of the receptor induce a conformational change that facilitates transphosphorylation of regulatory tyrosine residues within the dimer. Importantly, the regulatory tyrosine lies within the activation loop, the region that is conserved among receptor tyrosine kinases [22]. In the insulin receptor, the position of the activation loop is regulated by phosphorylation of homologous residues Tyr 1059, 1061, and 1062 [23]. In the nonphosphorylated state, the activation loop blocks the catalytic site, whereas upon ligand-induced phosphorylation it moves away, providing access to the substrate. It is possible that the events that follow transphosphorylation of the PDGF receptors are similar to those described for the insulin receptor, although there is no direct evidence for this in the absence of crystal structure of the PDGF receptors.

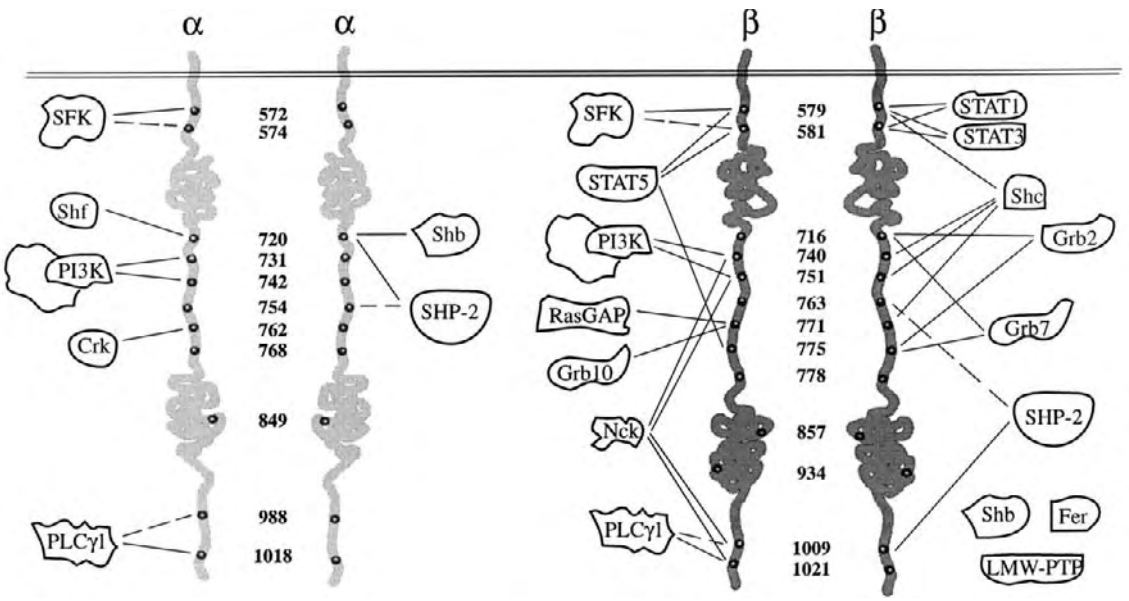


Figure 2 Intracellular domains of αα and ββ homodimers of the PDGF receptor. Phosphorylated tyrosine residues are shown (dark circles). Numbers indicate their positions in the human PDGF receptor sequence. Association of signaling proteins with phosphorylation sites of the activated PDGF receptors is shown by solid lines (major binding sites) or dashed lines (additional sites). Proteins binding with high affinity (e.g., PI3-kinase, PLCγ1, RasGAP) occupy one or two phosphotyrosine residues on the receptor, whereas low-affinity binding (e.g., Nck, Shc, or Shb binding to β receptor) involves multiple sites. Shb was found to bind to most of the phosphorylated tyrosines on the PDGF β receptor [61]. Fer and LMW-PTP bind to as-yet-unidentified sites on the PDGF β receptor.

As discovered recently, the juxtamembrane domain of the EphB2 receptor is involved in autoinhibition of the receptor kinase. The inhibition is relieved by ligand-induced phosphorylation of juxtamembrane tyrosine residues [24]. It is tempting to speculate that a similar regulatory mechanism exists for the PDGF β receptor, as mutation of the corresponding juxtamembrane tyrosines 579 and 581 of the PDGF β receptor inhibits PDGF-dependent activation [25]. Interestingly, this form of regulation may not be operative in the PDGF α receptor, even though these tyrosines and surrounding amino acids are conserved. Mutation of these tyrosine residues has little effect on the activation of the PDGF α receptor [41].

An additional PTP-linked mechanism that is likely to contribute to receptor activation has been proposed recently. When PTP activity in cells was blocked, phosphorylation of most tyrosine residues on the monomeric PDGF β receptor was increased to the level comparable to that of ligand-stimulated receptor, showing that PTP action might contribute to keeping non-activated receptor in unphosphorylated state. In a cell-free system, ligand-bound dimerized β receptor was found to be less susceptible to dephosphorylation by PTPs than monomeric receptor, indicating that receptor dimerization might protect it from dephosphorylation by PTPs. However, phosphorylation of the regulatory tyrosine (Tyr857) was strictly dependent upon ligand binding, i.e. it could not be induced by blocking PTP activity [26]. As known from earlier studies, catalytic activation (significant change of enzymatic parameters) of purified β receptor kinase is caused by PDGF binding and dimerization [27]. Therefore, the initial elevation of the receptor kinase activity, which involves phosphorylation of Tyr857, appears to require PDGF-induced dimerization and changes in the receptor's tertiary structure. "Phosphatase protection" caused by receptor dimerization may contribute to maintenance of its activated (phosphorylated) state.

Proteins Associated with the PDGF Receptors and PDGF-Driven Signaling Pathways

A total of 13 tyrosine residues in the β receptor and 11 in the α receptor are phosphorylated upon PDGF stimulation [19,30]. As discussed earlier, some of these phosphorylation events may be involved in stabilizing a catalytically active conformation of the receptor.

Phosphorylation of most of the tyrosines within PDGF receptors results in the creation of docking sites for a variety of proteins, many of which in turn are phosphorylated upon association with the receptors (Fig. 2) [19,20]. These proteins include enzymes (e.g., PI3-kinase, phospholipase C γ1 [PLCγ1], SHP-2, RasGAP, or Src family kinases [SFKs]), adaptor proteins (e.g., Grb2, Grb7, Shc, Shb, Nck, or Crk) linking the receptor to signaling proteins further downstream, or transcription factors (members of STAT family). Some enzymes may have adaptor function; for example, SHP-2 is able to recruit Grb2 via its phosphorylated C terminus [28]. Association is mediated in most cases by SH2 domains of these proteins [29] and is remarkably specific, each phosphorylation site having its own binding partners. This specificity is based on the ability of SH2 domains to

differentially recognize amino acid sequences following the phosphorylated tyrosine. Recruitment of other signaling molecules to the complex is facilitated by SH3, phosphotyrosine binding (PTB), or PH domains present in many of the receptor binding proteins.

It is yet uncertain whether the PDGF receptor is solely responsible for phosphorylating both itself and its substrates. Because other tyrosine kinases are present in the PDGF receptor signaling complex (e.g., SFK), their involvement in phosphorylation is possible and has indeed been confirmed. Using a specific inhibitor for Src family kinases, Blake *et al.* [43] showed that some proteins, including c-Cbl and protein kinase Cδ (PKCδ), were SFK substrates, whereas others (PLCγ) were not. However, this approach leaves out other possible players (JAKs, FAKs, or yet unidentified kinases) and can be complemented by other methods. One possible strategy involves introducing a single amino acid substitution into the adenosine triphosphate (ATP) binding site of the kinase of interest so that it acquires the unique ability to bind a bulkier synthetic ATP analog. In cells depleted of regular ATP and loaded with the analog, the mutant kinase will be the only one capable of phosphorylating its substrates [44].

Assembly of the PDGF receptor complex initiates a number of signaling pathways leading to cellular responses (Fig. 3). Some of the pathways are redundant and converge on the same cellular effect. For instance, chemotaxis can be driven by PI3-kinase via Rac and different PKC isoforms [30], as well as by Grb2/Sos1/Ras [33] via p38 [34] and possibly Rac. There is evidence of cross-talk between these

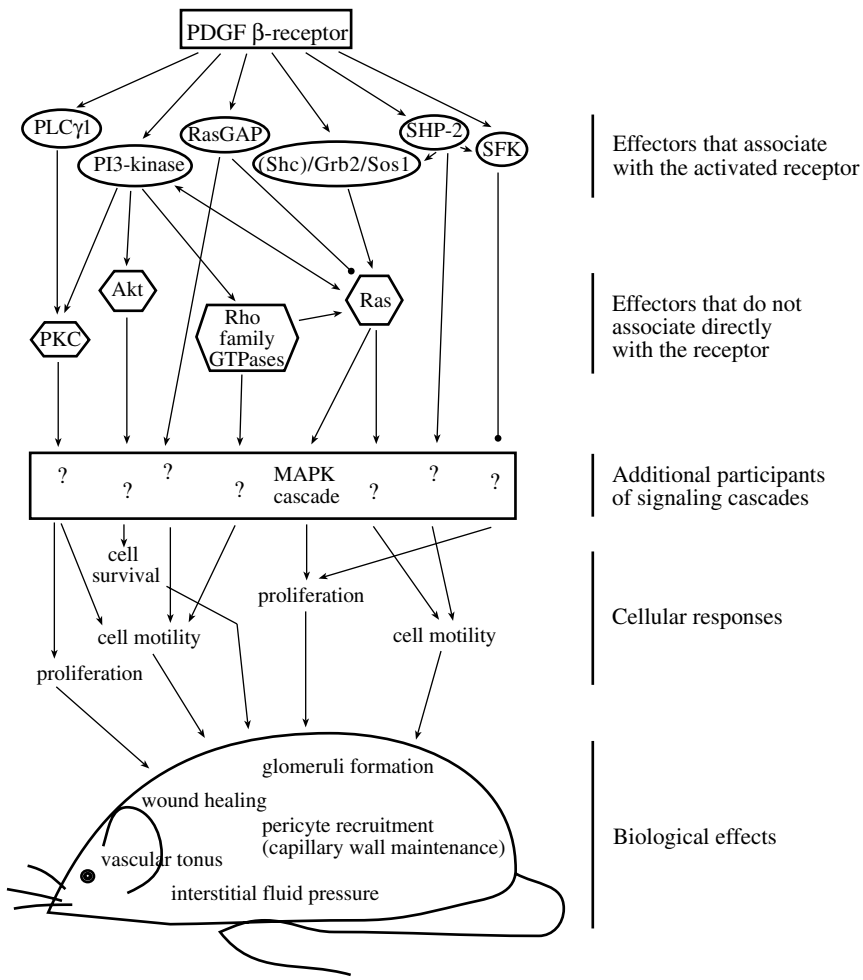


Figure 3 Major events initiated by the PDGF β receptor and culminating in biological responses. Upon ligand binding, activated PDGF β receptor recruits enzymes and adaptor proteins that start a number of distinct signaling pathways, some of which are shown on the figure. Note that not all of them are initiated by the same receptor dimer or in the same cell. Arrows represent either physical or functional interactions along the pathways. Inhibitory effect of RasGAP is shown with -•. At present, only proteins that directly associate with the PDGF receptor and their immediate downstream effectors are best characterized. The least defined part of the signaling cascade is the one that bridges the receptor-proximal events with proteins that carry out cellular responses. PDGF is necessary but not sufficient to achieve biological effects. In a living organism, other growth factors and hormones, as well as cell–cell and cell–matrix interactions within tissues and organs are indispensable.

pathways, as Ras and PI3-kinase are able to interact and activate each other [35,36]. PLCγ-dependent activation of sphingosine kinase [37,38] and PKC and can also be important for motility response. RasGAP, a negative regulator of Ras, has been shown to inhibit chemotaxis [39]. However, RasGAP has also been shown to have a positive impact on motility that is most likely independent of Ras [40]. Another chemotaxis pathway is initiated at SFK binding sites in the juxtamembrane domain of the PDGF α receptor, as determined by mutating these tyrosines to phenylalanine residues [41]. However, it is not yet clear whether it is Src family kinases or other signal transduction molecules binding to the same sites that are responsible for cell migration, as in triple SFK knockout cells PDGF-AA-dependent cell migration was intact [42]. Other positive mediators of PDGF-induced cell motility include SHP-2, which can work as adaptor molecule for Grb2/Sos1, and LMW-PTP, which acts presumably by inactivating p190RhoGAP and, consequently, activating Rho and causing cytoskeletal rearrangements [30].

In addition to chemotaxis, other PDGF-induced cellular responses include proliferation, differentiation (in certain cell types), and protection from apoptosis, as well as rapid Ca^{2+} fluxes and cytoskeletal rearrangements [19,20]. It is necessary to note that αα and ββ homodimers of the PDGF receptor cause different, although partially overlapping, cellular responses [20], due to the differences in their ability to bind signaling proteins (Fig. 2). Heterodimeric α/β receptor is believed to have unique signaling properties due to altered phosphorylation pattern of both the α and β subunits [31,32].

Some Aspects of Regulation of the PDGF Receptor-Initiated Signaling

Until recently, it has not been entirely clear whether each receptor molecule carried a complete set or a selected subset of phosphorylated tyrosines and corresponding downstream signaling proteins. By now, a considerable amount of data indicates that signaling output of the PDGF receptor complex is a result of interaction of various factors. Both intracellular and extracellular conditions may contribute to selective activation of some pathways and suppression of others. The sections that follow provide a brief discussion of some important aspects of this regulation, such as the influence of extracellular matrix (ECM) interactions, the input of PTPs, and how the cell-cycle stage determines which PDGF-driven pathways will have an effect on cells continuously treated with PDGF.

PDGF Receptor Signaling and Cell-Cycle Progression

To drive quiescent cells out of G_0 and through one round of the cell cycle, continuous treatment with PDGF for at least 8 to 10 hours is required [45]. It has been shown that prolonged exposure to PDGF induces two distinct peaks of PI3-kinase activity, one within minutes and another after a few hours' delay [46]. Only the second peak was found to be critical for cell-cycle progression, whereas the early increase of activity was required only for an immediate response to PDGF such as chemotaxis. Ras activation, induced by growth factors, remained elevated throughout G_0/late G_1. However, injection of neutralizing antibodies at different time points showed that the requirement for active Ras was not continuous but was restricted to at least two distinct phases of this transition [47]. These findings indicate that Ras and perhaps PI3-kinase use different effectors depending on the cell-cycle stage.

It still remains an open question as to how this complex interplay of early and late events induced by PDGF is linked to the cell-cycle machinery. According to recent findings, continuous treatment can be substituted by two separate pulses of the growth factor [48], the first getting cells out of G_0 and into early G_1, and the second pushing them through late G_1 and into the S phase. Early elevation of c-*myc* and sustained activation of the Erk pathway can replace the first pulse of PDGF but are not sufficient to drive the cell through late G_1. To complete cell-cycle progression, a properly timed second peak of PI3-kinase activity (second pulse of PDGF or just the addition of PI3-kinase lipid products) is required. It is hypothesized that early c-*myc* and Erk activation triggers expression of new proteins for which interaction with the late PI3-kinase products is critical for S phase entry. This two-step mechanism would prevent mitogenesis in response to a single accidental spike of PDGF. Consequently, mitogenesis is possible when the growth factor is present continuously or released in pulses frequent enough to ensure proper timing of early and late events in G_1 (which would reflect either normal physiological requirement or a serious disorder).

PTPs and Effect of Intracellular Reactive Oxygen Species (ROS)

As is the case for other cellular proteins, the phosphorylation level of the PDGF receptor and its substrates is a balance of kinase and phosphatase activities. LMW-PTP binds to activated PDGF receptor within the first five minutes after PDGF stimulation. It has been shown that this PTP selectively interferes with two pathways initiated by the receptor: Src-dependent induction of c-*myc* and STAT1/3-mediated c-*fos* expression. LMW-PTP association with the receptor prevents binding and activation of Src [49]. Because STAT1 and 3 use the same binding sites on the PDGF receptor as Src does, it is possible that LMW-PTP counteracts STAT1/3 signaling the same way (i.e., by competition for receptor binding).

Another PTP directly associated with the PDGF receptor upon its stimulation is SHP-2, which can have both negative and positive effects on signaling. SHP-2 is able to dephosphorylate selectively tyrosines 771 and 751 on the β receptor, potentially turning off RasGAP and PI3-kinase pathways [50]. On the other hand, a recent report indicates that SHP-2 is involved in growth-factor-dependent activation of

PI3-kinase [51]. Also, SHP-2 was reported to serve as an adaptor for Grb2 binding [28], thus initiating Ras-mediated signaling. SHP-2 has been implicated as a positive regulator in the stimulatory effect of integrins on PDGF receptor signaling.

The activity of PTPs is widely believed to be regulated by intracellular redox status. Elevation of ROS content would lead to reversible oxidation of catalytic cysteine residues of PTPs and consequently to their inhibition, whereas a reducing environment results in PTP activation. An increase of intracellular ROS is an early PDGF-dependent event mediated by PI3-kinase [52] and Rac, and it was shown to be important for mitogenic signaling [53]. It is likely that this event helps to prevent the negative input of (receptor-associated) PTPs on early stages of signaling. In contact-inhibited cells that do not respond mitogenically to PDGF, ligand-induced PDGF receptor phosphorylation is diminished [54] due to elevated PTP activity [55,56], which in turn has been linked to dramatically decreased ROS content in these cells [57]. LMW-PTP, in particular, can be regulated by ROS [58] and may contribute to reduced PDGF receptor activation in these cells. These data suggest that changes in the cellular redox environment determine whether PTPs will counteract PDGF receptor signaling.

Input of Integrins

The PDGF receptor is capable of initiating both positive signals (e.g., through SFK, PI3-kinase, or PLCγ1) and negative signals (RasGAP) with relation to the mitogenic response. Because proper attachment to matrix is critical for cell proliferation, ECM-dependent engagement of integrins may be expected to affect signaling output of the PDGF receptor in such a way that positive signals prevail. Indeed, plating cells on vitronectin, an αvβ3 ligand, leads to increased PDGF-dependent mitogenicity and chemotaxis. Along with this, a small, highly phosphorylated fraction of PDGF β receptors becomes associated with αvβ3 integrin after PDGF stimulation [59]. Another set of data shows that activation of integrins leads to increased association of SHP-2 with the receptor, and, as a consequence, to dephosphorylation of the RasGAP binding site and decreased RasGAP binding [60]. This is followed by prolonged activation of Ras and Erk. Thus, integrins can alter the recruitment of signaling enzymes to the PDGF receptor in favor of increased mitogenic signaling. It is also noteworthy that, due to its substrate specificity, SHP-2 (a member of the PTP family generally consisting of RTK antagonists) acts under these conditions as a positive component of the PDGF receptor signaling by inactivating a negative regulatory pathway at the receptor level.

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CHAPTER 71

EGF Receptor Family

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Introduction

Receptor tyrosine kinases (RTKs), transmembrane molecules with intrinsic tyrosine kinase activity, couple binding of growth factor ligands to the intracellular signaling pathways that regulate diverse processes such as cell division, differentiation, and survival. The receptor for the epidermal growth factor (EGFR) represents a prototypical RTK and was the first cell-surface signaling protein characterized by molecular and genetic methods [1]. The EGFR or ErbB family of RTKs is now known to be comprised of four closely related members: EGFR (also known as ErbB1 or HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4), which are widely expressed on cells of epithelial, mesenchymal, and neuronal origin. Analyses of the function of ErbBs have revealed their critical developmental role in inductive cell fate determination in mammals (see Table 1).

The EGFR pathway is evolutionarily conserved in invertebrates, with a single ErbB homolog, LET-23, binding to a single ligand, LIN-3, to induce development of the vulva and other organs in *Caenorhabditis elegans*. In *Drosophila melanogaster*, the interactions of a single ErbB homolog, DER, with its four ligands regulate several stages of development including oogenesis, embryogenesis, and wing and eye development. In higher organisms, a complex network composed of the four ErbB receptors and ten ligands has evolved. This layered information processing module allows for the combinatorial interactions of ligands, receptors, effectors, and transcription factors and provides a high degree of signal diversification with multiple levels of output control [2].

Domain Structure of ErbBs

ErbB receptors contain an extracellular ligand binding domain with two cysteine-rich regions and a single hydrophobic transmembrane domain. The intracellular portion consists of a tyrosine kinase domain and a carboxy-terminal tail containing tyrosine autophosphorylation sites. ErbB2 does not appear to bind growth factor ligands directly, while ErbB3 is devoid of intrinsic kinase activity. All ErbB family ligands include an EGF-like domain consisting of three disulfide-bonded intramolecular loops and are generated upon the regulated proteolytic cleavage of glycosylated transmembrane precursors. Binding of these ligands, the specificity of which is depicted in Fig. 1, involves two non-consecutive portions of both the ectodomain of the receptor and the ligand molecule, suggesting bivalent interactions. An essential feature of transmembrane signaling is the ability of all ligands to promote homo- and heterodimers of ErbBs. Although the resulting three-dimensional structures have not been solved, modeling based on other RTKs predicts 2:2 ligand/receptor complexes stabilized by a long loop of each receptor forming the interface of the dimer. Dimerization of ErbBs is essential for receptor auto- or transphosphorylation on tyrosine residues, leading to the initiation of signaling by serving as docking points for signaling effectors containing SH2 and phosphotyrosine binding (PTB) domains.

Subcellular Localization of ErbB Proteins

The localization of LET-23 at the basolateral face of precursor epithelial cells is important for vulval development in *C. elegans*. In mammalian skeletal muscle, the clustering of ErbB2, ErbB3, and ErbB4 at the postsynaptic membrane of neuromuscular junctions is required for efficient binding to neuron-derived neuregulins and subsequent induction of acetylcholine receptor synthesis. In polarized epithelial cells, most EGFR and ErbB2 molecules are localized to the basolateral face where signaling can be initiated by

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Table I Phenotypes of Mice Deficient in ErbB Proteins

$EGFR^{-/-}$	Strain-dependent phenotypes (embryonic or perinatal lethality): death *in utero* due to defects in placental spongioblasts; after birth: progressive neurodegeneration and abnormalities in multiple organs, including brain, skin, lung, eyes, GI tract, and hair follicles.
$ErbB2^{-/-}$ (lethal E10.5)	Insufficient heart development, with trabeculae malformation in ventricles. Introduction of transgenic ErbB2 into myocardial cells results in perinatal lethality and nervous system defects, including lack of Schwann cells, defects in motor and sensory neurons and neuromuscular junctions.
$ErbB3^{-/-}$ (lethal E13.5)	Defects in valve formation in heart development and nervous system defects, including neural crest defects, lack Schwann cells, degeneration of peripheral nervous system.
$ErbB4^{-/-}$ (lethal E10.5)	Insufficient heart development and trabeculae malformation in ventricles and central nervous system defects, including hindbrain innervation.

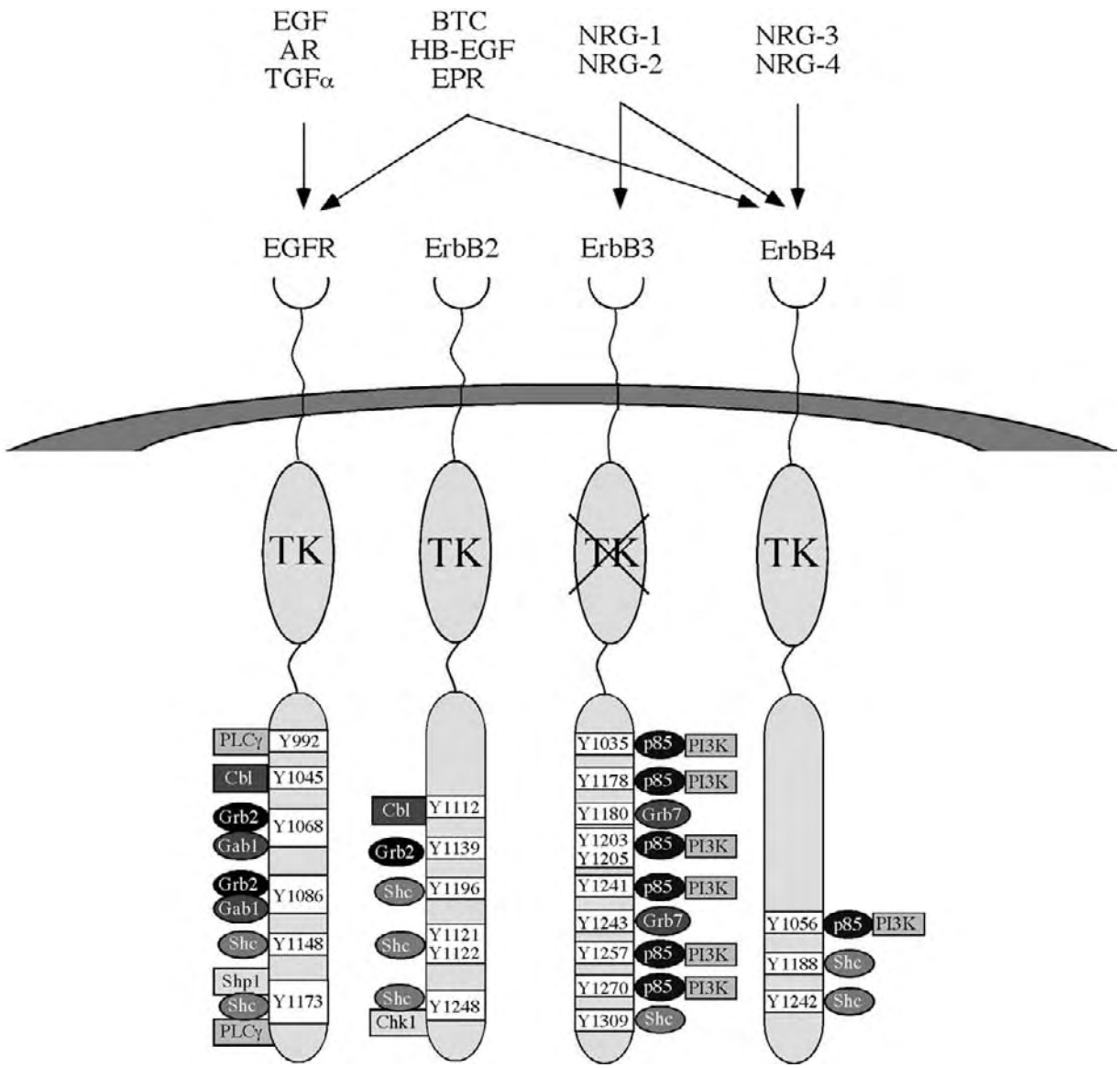


Figure 1 ErbB ligands, receptor tyrosine kinases, and signal transduction pathways. Ten growth factor ligands bind ErbB RTK: EGF, amphiregulin (AR), and transforming growth factor-α (TGF-α) bind EGFR; betacellulin, heparin-binding EGF-like growth factor (HB-EGF), and epiregulin bind both EGFR and ErbB4; the neuregulins (also called Neu differentiation factors; NDFs) NRG-1 and NRG-2 bind ErbB3 and ErbB4; and NRG-3 and NRG-4 bind ErbB4. Ligand binding induces receptor homo- or heterodimerization and activation of tyrosine kinase activity. Phosphorylation of tyrosine residues within ErbBs enables the recruitment of SH2 and PTB domain-containing proteins. The binding sites for such molecules are indicated and include adaptor proteins such as Grb2, Shc, Grb7, and Gab1 and enzymes such as PLCγ, Chk kinase, SHP-1 phosphatase, p85 subunit of PI3K, and Cbl, a ubiquitin ligase.

stroma-derived ligands. Three PDZ-containing proteins (Lin-2, Lin-7, and Lin-10) are implicated in the regulation of LET-23 localization. In addition, the PDZ-containing proteins PSD-95 and Erbin, as well as their binding partner PICK1, are important for ErbB2 targeting to and retention and clustering at specific cell surface sites in mammalian cells.

In resting cells, a large proportion of EGFR, as well as ErbB2 and ErbB4, is localized in caveolae, which are membrane microdomains enriched in caveolin proteins, glycosphingolipids, and cholesterol. Caveolae are thought to regulate signaling through the preassembly of signaling molecules and have also been implicated in non-clathrin-mediated internalization. Caveolin-1 interacts with ErbBs and inhibits their catalytic activity. In response to ligand, both EGFR and ErbB4 migrate out of caveolae, which may remove the inhibitory effect of caveolin. Interestingly, the localization of ErbB2 in caveolae is unchanged upon stimulation [3]. In addition, some signaling pathways induced through ErbBs, notably the Ras-MAPK pathway, were observed to occur in caveolae. The significance of the caveolar retention of ErbB2 as well as the function of caveolae in regulating signaling and endocytosis of ErbB RTK remain to be fully elucidated.

ErbB-Induced Signaling Pathways

Signaling pathways induced through ErbBs are dictated by their pattern of autophosphorylation, as the specificity of SH2 and PTB domain binding is conferred by amino acids surrounding the tyrosine phosphorylation site. Thus, individual ErbBs couple to distinct subsets of signaling proteins, as depicted in Fig. 1. All ErbB ligands and receptors couple to activation of the Ras–MAPK pathway through recruitment of Grb-2 and/or Shc and the Grb-2-bound exchange factor Sos. Activation of phosphatidylinositol 3-kinase (PI3K) is differentially induced: ErbB3 contains six putative binding sites for the SH2 domain of the PI3K p85 regulatory subunit, while ErbB4 contains one binding site, and EGFR and ErbB2 couple to PI3K indirectly through adaptor proteins such as Gab1 and c-Cbl [4]. Thus, ErbBs induce the proliferative and survival signals resulting from the activation of PI3K and its downstream effectors, such as Akt and $p70^{S6}$ kinase, with differing potencies and kinetics. Other signaling effectors are recruited only to some ErbB family members, such as the recruitment of PLCγ to EGFR and ErbB2. Subsequent PLCγ activation results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate and the generation of the second messengers diacylglycerol and inositol trisphosphate, leading to activation of protein kinase C (PKC) and increases in intracellular calcium concentrations.

Signaling through EGFR has been more extensively characterized than other ErbB family members and is known to involve multiple additional pathways. c-Src is activated upon stimulation with EGF and phosphorylates two of the tyrosine residues within EGFR implicated in mitogenesis. Furthermore, c-Src phosphorylates and activates STAT family transcription factors, cytoskeletal proteins, and proteins involved in endocytosis. The FAK and Pyk2 kinases are implicated in EGF-induced cell migration and link EGFR to integrin signaling pathways. The JNK pathway is also activated by ErbBs, likely through the adaptor protein Crk, Dbl family exchange factors, and the small GTPases Rac1 and Cdc42. Further, EGFR and ErbB4 have recently been detected in the nucleus of cells, where EGFR may function as a transcription factor [5,6].

EGFR can also integrate signals through other cellular stimuli, notably by G-protein-coupled receptor (GPCR) agonists which may require EGFR-induced signaling for MAPK activation [7]. Transactivation of EGFR may involve its phosphorylation by non-receptor tyrosine kinases such as FAK, Pyk2, or Src. Phosphorylation may lead to the catalytic activation of EGFR or may enable the receptor to function as a scaffold for the recruitment of signaling molecules as outlined above. In addition, activation of PKC by GPCRs may in turn result in the activation of membrane metalloproteinases, resulting in the generation of the cognate ligands of EGFR and its subsequent direct activation [8].

Negative Regulatory Pathways

The principal route of signal attentuation is the downregulation of surface receptor levels through ligand-induced receptor endocytosis. Receptor dimers are internalized through clathrin-coated regions of plasma membrane that invaginate to form endocytic vesicles. These vesicles mature into early and late endosomes, while their internal pH increases and they accumulate hydrolytic enzymes leading to receptor degradation. EGFR is one of the most extensively studied receptors with respect to endocytosis. Ligand-induced phosphorylation and coupling of EGFR to proteins involved in the endocytic machinery, such as Eps15, are critical for accelerated endocytosis. Further, phosphorylated EGFR recruits c-Cbl, a ubiquitin ligase that directs the receptor to lysosomal degradation through polyubiquitination [9,10]. In contrast to the rapid internalization and degradation of EGFR, sorting in the early endosome or multivesicular body leads to the recycling of other ErbBs back to the cell surface, thus potentiating signaling. Moreover, as c-Cbl does not bind ErbB3, this receptor demonstrates impaired ligand-induced polyubiquitination and downregulation relative to EGFR.

Other negative regulatory pathways serve to limit signaling through ErbB proteins [11]. Ras-GAP is recruited to and activated by EGFR and inhibits the MAPK pathway by accelerating the GTPase activity of Ras. Studies in *D. melanogaster* demonstrate that Argos is a soluble EGF-like molecule that inhibits ligand binding to DER, while Kekkon1 is a transmembrane protein that interacts with DER and interferes with ligand activation. Further, the cytosolic protein Sprouty binds c-Cbl and inhibits activation of the Ras–MAPK pathway. In *C. elegans*, the Ark-1 kinase was shown to inhibit LET-23/EGFR signaling in a SEM5/Grb-2-dependent manner. Mammalian homologs of Kekkon (Lig-1), Sprouty (Spry1-4),

and Ark-1 (ACK-1) have been identified but their functions remain to be fully characterized.

Specificity of Signaling Through the ErbB Network

The specificity of signaling through ErbB receptors is regulated at multiple levels. The distinct organ and developmental stage-specific expression profiles of ErbB ligands regulate biological responses throughout development and adulthood. The ligand influences ErbB homo- or heterodimer formation, as well as the identity of the phosphorylation sites within individual ErbBs [12]. Ligand affinity influences signal strength and duration, although low-affinity ligands may not induce normal receptor downregulation and degradation thereby functioning more potently, as was shown for low-affinity virally encoded ligands [13]. Further, the pH stability of the ligand-receptor interaction influences receptor trafficking; the pH-resistant interaction of EGFR with EGF targets the receptor to the lysosome, whereas TGFα and NRG-1 dissociate from their receptors in early endosomes, thus favoring receptor recycling and signal potentiation.

Dimerization of ErbBs adds an additional level of signal diversification. Homodimeric receptors combinations are less potent and mitogenic than heterodimers. The critical role of heterodimers is most clearly demonstrated in mice with targeted deletions in individual ErbBs. The defective heart formation phenotype of both ErbB2 and ErbB4 knockouts demonstrate that ErbB4 homodimers cannot functionally substitute for ErbB4–ErbB2 heterodimers. Although ErbB2 does not bind directly to ligands, it is the preferred heterodimer partner for other ErbBs. ErbB2-containing heterodimers are the most potent complexes due to an increased affinity of ligand binding, decreased ligand dissociation, decreased rate of endocytosis, and increased receptor recycling [2].

ErbB Proteins and Pathological Conditions

Due to their widespread expression and signaling potency, ErbB molecules are involved in a variety of physiological processes (e.g., myelination, implantation, wound healing, mammary development, angiogenesis) and pathological states (airway inflammation, asthma, ulcers, and other gastrointestinal tract diseases). However, the best studied is the oncogenic aspect of the ErbB network in human malignancies. ErbBs were first implicated in cancer upon the characterization of an aberrant form of EGFR encoded by the avian erythroblastosis tumor virus. EGFR and ErbB2 have since been implicated in various forms of human cancers. Abnormal activation of these receptors occurs through overexpression, gene amplification, constitutive activation of mutant receptors, or autocrine growth factor loops. ErbB2 has been used as a prognostic marker as its overexpression is associated with shorter overall and relapse-free survival of patients with breast or ovarian cancer [13]. Further, ErbB molecules serve as therapeutic targets in cancer treatment. Herceptin/Trastuzumab® and Cetuximab® are EGFR- and ErbB2-specific monoclonal antibodies in use in the treatment of breast cancer patients, and ErbB-specific tyrosine kinase inhibitors are in clinical trials. Also under examination are derivatives of the antibiotic geldanamycin, which block the growth of ErbB2-overexpressing cells by inducing ErbB2 degradation subsequent to binding the ErbB2 chaperone Hsp90. ErbB receptors are similarly implicated in other hyperproliferative disorders such as coronary atherosclerosis and psoriasis. Conversely, signaling through ErbBs may promote wound healing. Thus, a more complete understanding of the ErbB network may allow for the development of therapeutic strategies that may be of clinical benefit in a variety of pathological conditions.

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CHAPTER 72

IRS-Protein Scaffolds and Insulin/IGF Action

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IRS-Proteins: The Beginnings

After the discovery of the insulin receptor tyrosine kinase, many groups searched for insulin receptor substrates (IRS-proteins) that might regulate downstream signaling [1,2]. The first evidence for an IRS-protein came from phosphotyrosine antibody immunoprecipitates that revealed a 185-kDa phosphoprotein (pp185) in insulin-stimulated hepatoma cells [3]. This phosphoprotein seemed to be biologically important because it was phosphorylated immediately after insulin stimulation, and catalytically active insulin receptor mutants that failed to phosphorylate it were biological inactive [4]. Purification and molecular cloning of pp185 revealed one of the first signaling scaffolds, and the first insulin receptor substrate (IRS1) [5]. IRS1 contains many tyrosine phosphorylation sites that are phosphorylated during insulin and IGF1 stimulation and bind to the Src homology-2 domains in various signaling proteins [6,7]. The interaction between IRS1 and p85 activates the class 1A phosphatidylinositide 3-kinase (PI 3-kinase), revealing the first insulin signaling cascade that could be reconstituted successfully in cells and test tubes [8].

IRS-Proteins and Insulin Signaling

Insulin and IGF1 receptors, like the receptors for other growth factors and cytokines, are composed of an extracellular ligand-binding domain that controls the conformation and activity of the intracellular tyrosine kinase [9,10]. Unlike most receptor tyrosine kinases that are activated upon ligand-induced dimerization, insulin and IGF1 receptors exist as inactive covalent dimers composed of two extracellular α-subunits and two transmembrane β-subunits. Insulin and IGF1 bind between the two α-subunits, thus inducing a conformation change that promotes tyrosine autophosphorylation on the cytoplasmic side of the adjacent β-subunits [11,12]. Autophosphorylation occurs in three distinct regions of the β-subunits, including the regulatory loop, the juxtamembrane region, and the C-terminus. Phosphorylation of three tyrosine residues in the regulatory loop activates the tyrosine kinase by opening the catalytic domain to facilitate entry of adenosine triphosphate (ATP) and peptide substrates; autophosphorylation of the NPEY motif in the juxtamembrane region creates a binding site for IRS-proteins and other substrates that have similar phosphotyrosine binding (PTB) domains. The role of phosphorylation in the C terminus is poorly understood.

The mammalian insulin receptor is biologically inactive without its substrates, suggesting that most signals are generated through complexes that are assembled around tyrosyl-phosphorylated scaffolds, including IRS1 and its homologs; Shc, APS, and SH2B [13]; and Gab1/2, Dock1/2, and cbl [14–20]. Although the role of each of these substrates merits attention, work with transgenic mice reveals that many insulin responses, especially those that are associated with somatic growth and carbohydrate metabolism are mediated largely through IRS1 and IRS2 [21]. IRS-proteins are composed of multiple interaction domains and phosphorylation motifs [22]. At least three IRS-proteins occur in mice and people, including IRS1 and IRS2, which are widely expressed, and IRS4, which is limited to the thymus, brain, kidney, and β-cells [23]. Rodents also express IRS3, which is largely restricted to adipose tissue and displays activity similar to

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IRS1; however, an IRS3 ortholog might not occur in people [24]. Disruption of the genes for IRS3 or IRS4 in mice is uninformative [25,26]. By contrast, mice lacking IRS1 are small and those without IRS2 are infertile and develop diabetes [5,27]. The *Drosophila* genome contains a single IRS-protein called Chico that promotes growth and regulates metabolism in flies (Fig. 1) [28], while a functional IRS-protein is not expressed in *Caenorhabditis elegans*. However, the insulin and IGF receptor orthologs in flies and worms contain a significant C-terminal extension that is absent from mammalian orthologs and contains potential tyrosine phosphorylation sites that can recruit PI 3-kinase without IRS-proteins [29].

IRS-Protein Structure and Function

Alignment of the amino acid sequences of the IRS-proteins reveals important similarities and subtle differences (Fig. 1). IRS1–4 and Chico contain an NH_2-terminal pleckstrin homology (PH) domain adjacent to a PTB domain. The structures of the PH and PTB domains are remarkably similar [30], and both facilitate recruitment of IRS-proteins to the activated insulin and IGF1 receptors; deletion of the PH and PTB domain almost completely prevents phosphorylation of the C-terminus. The PTB domain binds to the phosphorylated NPEY motif in the β-subunit of the insulin or IGF1 receptor [31–33]. At ordinary expression levels, deletion of the PTB domain reduces that ability of insulin to promote tyrosine phosphorylation of IRS1 or IRS2; however, overexpression of the insulin receptor restores phosphorylation and signaling, suggesting that the PTB domain enhances coupling but is not essential for signaling [31].

The PH domain also promotes interaction between IRS-proteins and physiological levels of insulin receptors. Like the PTB domain, the PH domain is not required in cells overexpressing the insulin receptor [31]. However, the mechanism of coupling employed by the PH domain is not understood. Some, but certainly not all, PH domains bind to membrane phospholipids which provides membrane targeting [34]; however, the PH domain in IRS-proteins has a relatively low affinity for phospholipids. The PH domains can be exchanged between IRS-proteins without noticeable loss of bioactivity, but chimeric IRS-proteins composed of heterologous PH domains fail to be phosphorylated by the insulin receptor [35]. Because IRS-protein PH domains do not bind to the insulin receptor, other proteins of membrane lipids might be involved. Yeast two-hybrid screens reveal a few potential binding partners, including nucleolin or a novel protein called PHIP [35,36].

The tyrosine phosphorylation sites in the COOH-terminal end of each IRS-protein recruit and regulate various downstream signaling proteins (Fig. 1). IRS1 and IRS2 have the longest tails, which contain 20 potential tyrosine phosphorylation sites; however, only a few sites have been formally identified, and little information is available on the phosphorylation sites in IRS3 and IRS4 (Fig. 1). Many of the tyrosine residues cluster into common motifs that recruit or activate enzymes (PI 3-kinase, SHP2, fyn) or adapter molecules (Grb-2, nck, crk, Sh2b) (Fig. 1). Grb2 and possibly SHP2 couple Grb2/SOS to IRS-proteins, which promotes the ras → raf cascade [37]. All IRS-proteins contain multiple p85 binding motifs that recruit PI 3-kinase, which is the best-studied insulin-signaling pathway.

In addition to the tyrosine phosphorylation sites, sequence alignment of IRS-proteins reveals several conserved motifs that might be binding sites for other cellular proteins. One of the best characterized is the binding site for the c-Jun N-terminal kinase (JNK), which resembles the sites in the JNK-interacting proteins (JIP1 and JIP2) [38,39]. This site occurs in IRS1, IRS2, and Chico but has only been validated in mouse and human IRS1 [40]. During stimulation by proinflammatory cytokines or by insulin, JNK binds to IRS1 and promotes phosphorylation of Ser^{312} in human IRS1 (Ser^{307} in rat/mouse IRS1) [41]. Phosphorylation of this serine residue inhibits binding of the PTB domain to the phosphorylated NPEY motif in the activated insulin receptor [40]. JNK also phosphorylates IRS2, but the homologous site is absent and the inhibitory mechanism is poorly described.

IRS1 and IRS2 contain several putative 14-3-3 binding sites (Fig. 1). 14-3-3 proteins are highly conserved acidic scaffold proteins that bind to specific amino acid sequence motifs. In some cases, serine phosphorylation of the target motif directs the binding of a 14-3-3 isoform, which might inhibit signaling by sequestering the complex to an inappropriate subcellular compartments [42]. IRS1 and IRS2 contain several serine phosphorylation motifs that bind to 14-3-3ε or 14-3-3ζ might inhibit signaling [43,44]. Some of these sites are in the PTB domain, which might inhibit coupling to the activated insulin receptor. Recent evidence suggests that 14-3-3 binding might displace serine-phosphorylated IRS-1 from particular structures, thus reducing PI 3-kinase activity [45]; however, a role for 14-3-3 binding in IRS-protein regulation remains to be validated in animal models. Whether 14-3-3 binding regulates IRS2 function is unknown.

Work with transgenic mice reveals that IRS1 and IRS2 have distinct signaling potential even though the structures of the two proteins and their functions in cell-based assays are rather similar; however, certain differences do exist that might establish specificity. For example, IRS2 contains a unique region of undefined structure that binds to the phosphorylated regulatory loop of the insulin receptor kinase, called the kinase-regulatory-loop-binding (KRLB) domain. The discovery of this interaction was unexpected, as it maps to the portion of the COOH-terminal region between amino acid residues 591 and 786 that contains tyrosine phosphorylation sites (Fig. 1). Two tyrosine residues in the KRLB domain at positions 628 and 632 are crucial for this interaction. Phosphorylation of tyrosine residues in the KRLB domain by the insulin receptor inhibits the binding to the receptor, suggesting that a novel mechanism regulates the interaction of the insulin receptor and IRS-2 and may be able to distinguish the signal of IRS2 from IRS1 [46].

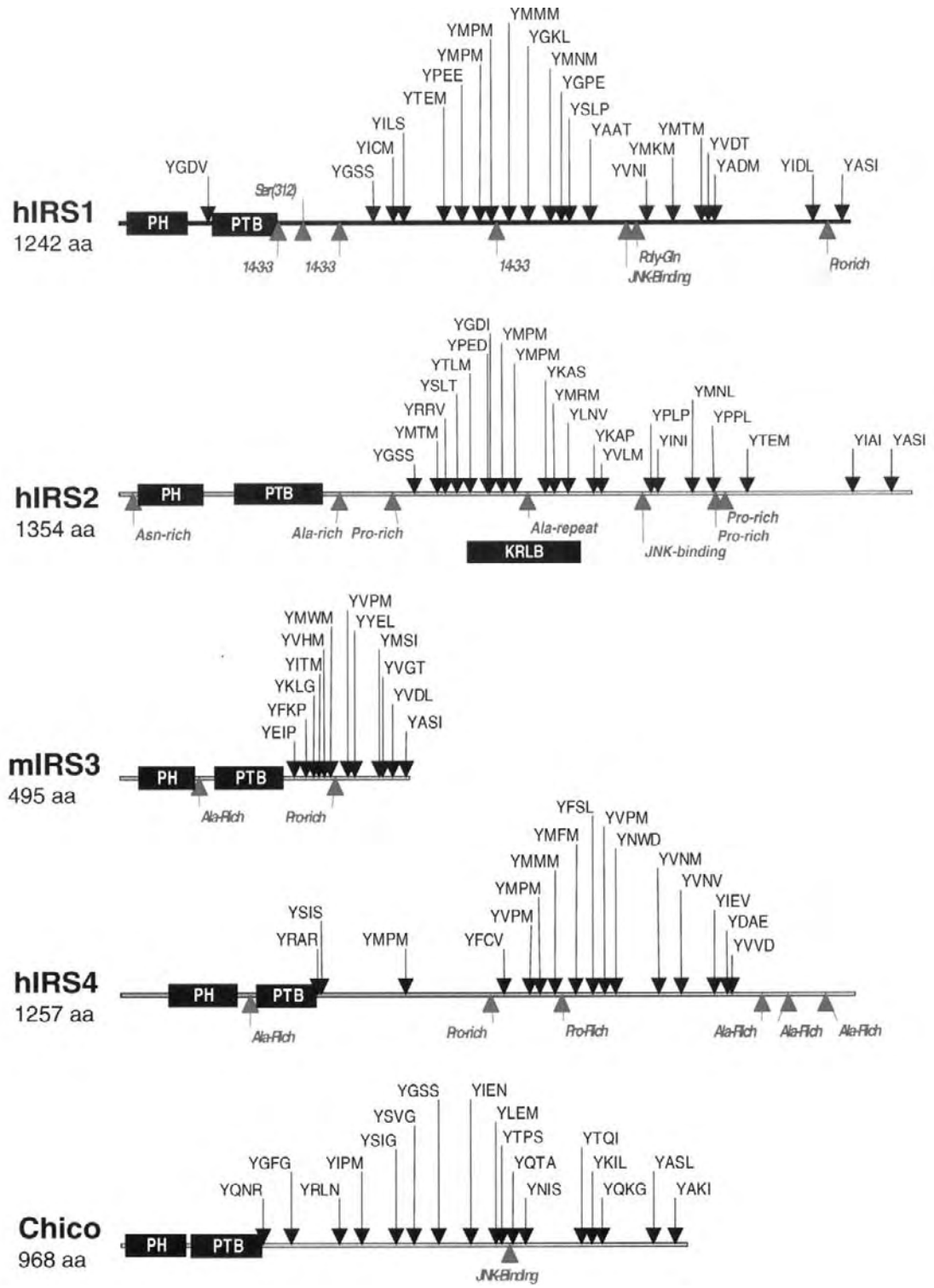


Figure 1 IRS-protein structures; a comparison of important sequence features of human IRS1, IRS2, and IRS4, mouse IRS4, and *Drosophila* Chico. The relative positions of the pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains are indicated. The relative positions of potential tyrosine phosphorylation sites are also shown, as are other known interaction motifs, including sites that bind the N-terminal c-Jun kinase (JNK) 14-3-3 proteins. Alanine- and proline-rich motifs are also highlighted.

PI 3-Kinase Cascade

IRS-proteins couple insulin and IGF receptors to various signal pathways, including the PI 3-kinase → protein kinase B (PKB)/AKT cascade and the Grb2/SOS → $p21^{ras}$ cascade (Fig. 2). Activation of class 1A PI 3-kinase is required for many insulin responses, including the stimulation of glucose uptake, glycogen synthesis, and gene transcription [47]. PI 3-kinase is composed of a catalytic and a regulatory subunit. Catalytic subunits encoded by Pik3ca (p110α), Pik3cb (p110β), and Pik3cd (p110δ) associate noncovalently with a regulatory subunit encoded by Pik3r2 (p85β), Pik3r3 ($p55^{PIK}$), or the alternatively spliced Pik3r1 (p85α, p55α, and p50α) [48]. Pik3r1 and Pik3r2 are ubiquitously expressed; p85α is usually more abundant than p85β, whereas Pik3r3 displays a restricted pattern of expression [49]. Each regulatory subunit contains a p110-binding region flanked by an SH2 domain, and p85α and p85β have an NH_2-terminal SH3-domain that is replaced by short unique sequences in p55α, p50α, and $p55^{PIK}$. The importance of these structural differences is unknown.

During insulin stimulation, phosphorylated YMXM motifs in IRS-proteins occupy both SH2 domains in the regulatory subunits which directly activates the PI 3-kinase [8]. Products of the PI 3-kinase, including phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, recruit and activate the PDK(1/2) → PKB (1/2/3) cascade (Fig. 2). During colocalization at the plasma membrane, PDKs phosphorylate and activate PKB isoforms, which control various biological processes, including glucose transport, protein synthesis, glycogen synthesis, cell proliferation, and cell survival in various cells and tissues (Fig. 2) [14,50,51].

The role of IRS-proteins in PI 3-kinase signaling is complex, as IRS1 and IRS2 play distinct regulatory roles in liver and muscle. Basal PI 3-kinase activity in muscle and liver is

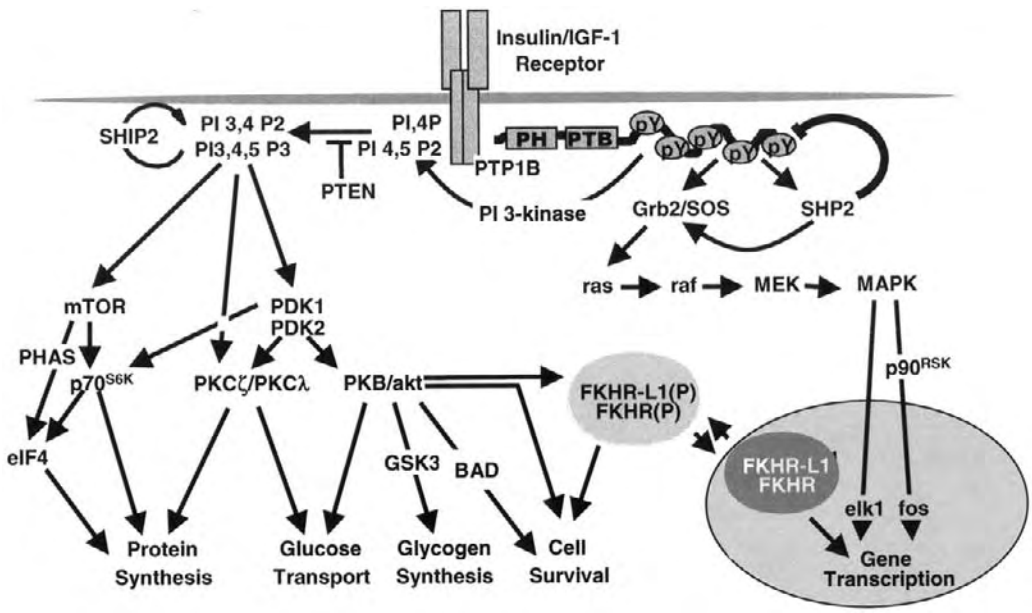


Figure 2 IRS-proteins coordinate downstream signaling pathways; the activation of intracellular signaling pathways by insulin is shown. The diversity of insulin action in various tissues is partly explained by the different signaling pathways activated by the hormone. The two main limbs that propagate the signal generated through the insulin receptor are the insulin receptor substrate/phosphatidylinositol 3-kinase (IRS/PI3K) pathway, and the Ras/mitogen-activated protein kinase (MAPK) pathway. Activation of the receptors for insulin and IGF1 results in tyrosine phosphorylation of the IRS-proteins. The IRS-proteins bind PI 3-kinase, Grb2/SOS, and SHP2. The GRb2/SOS complex mediates the activation of $p21^{ras}$, thereby activating the ras → raf → MEK → MAP kinase cascade. SHP2 feeds back to inhibit IRS-protein phosphorylation by direct dephosphorylation of the IRS-protein but might also transmit an independent signal to activate MAP kinase. The activated MAP kinase phosphorylates $p90^{rsk}$, which itself phosphorylates c-*fos*, thus increasing its transcriptional activity. MAP kinase also phosphorylates ELK1, also increasing its transcriptional activity. The activation of PI 3-kinase by IRS-protein recruitment results in the generation of $PI3,4P_2$ and $PI3,4,5P_3$ (antagonized by the action of PTEN or SHIP2). In aggregate, $PI3,4P_2$ and $PI3,4,5P_3$ activate a variety of downstream signaling kinases, including mTOR, which regulates protein synthesis via PHAS/$p70^{s6k}$/EIF4. These lipids also activate alternate PKC isoforms and PDK isoforms. The PDKs activate protein kinase B (PKB), which appears to mediate glucose transport in concert with the atypical PKC isoforms. PKB also regulates GSK3, which may regulate glycogen synthesis, and a variety of regulators of cell survival. PKB-mediated BAD phosphorylation inhibits apoptosis, and phosphorylation of the forkhead proteins results in their sequestration in the cytoplasm, in effect inhibiting their transcriptional activity. Abbreviations: AKT, product of the *akt* protooncogene; GAP, guanosine-triphosphatase-associated protein; GLUT4, glucose transporter 4; GRB-2, factor receptor binding protein 2; GSK3, glycogen synthase kinase 3; MAPKK, MAPK kinase; PDK, PI-dependent protein kinase; PKC, protein kinase C; SOS, son-of-sevenless.

elevated upon disruption of IRS2, which reduces the relative increase during insulin stimulation (Fig. 3). By contrast, without IRS1, basal PI 3-kinase activity is reduced significantly while insulin-stimulated activity is preserved, so the relative activation by insulin is increased. Apparently, IRS1 has a greater impact on basal PI 3-kinase activity and IRS2 has it greatest impact on insulin-stimulated activity. Thus, IRS2 might be the principle regulator of the metabolic insulin actions in liver and muscle [52].

The PI 3-kinase regulatory subunits also influence the signal specificity and signal strength. Complete disruption of the Pik3r1 gene that eliminates p85α, p55α, and p50α causes death of newborn mice [53]. By contrast, complete disruption of Pik3r2 is not lethal [48]. Interestingly, mice lacking p85β display improvised insulin sensitivity. These results suggest that the strength of the IRS → PI 3-kinase signal might be determined, at least in part, by the stoichiometry between the regulatory and catalytic subunits. Because the number of regulatory subunits generally exceeds that of the catalytic subunits, a competition apparently exists for IRS-protein binding sites between catalytically competent heterodimers and catalytically deficient regulatory monomers [48,54]. This hypothesis is supported by the finding that the selective reduction of p85α increases insulin sensitivity [48]. Although the stoichiometry between IRS-proteins and PI 3-kinase regulatory subunits is important for signal strength, other mechanisms might also contribute to feedback inhibition through direct inhibition of IRS-protein function.

IRS-Protein Signaling in Growth, Nutrition, and Longevity

The disruption of IRS-proteins in mice and flies reveals their role of coordinating multiple biological processes, including growth, nutrition, and fertility. The framework relating IRS-proteins to these biological processes might be easier to establish in *Drosophila*, because fewer signaling protein are involved. Deletion of Chico, the *Drosophila* IRS-protein, causes female sterility as well as reduced somatic growth and increased lipid storage [55]. Moreover, specific mutations of the binding sites for the *Drosophila* PI 3-kinase adapter p60 completely abrogates Chico function in growth control; however, mutating the consensus binding site in Chico for Grb2/Drk, which regulates the *ras* cascade, does not interfere with growth [28]. The important role of the PI 3-kinase cascade is further supported by the finding that growth is restored in Chico mutants by reducing the level of the PTEN ortholog, confirming that the PI 3-kinase cascade is a critical pathway for growth regulation [28].

In mice, the PI 3-kinase → PKB cascade also regulates growth, but the control appears to be parsed between IRS1 and IRS2. Mice lacking IRS1 are about 50% smaller, where IRS2$^{-/-}$ mice are nearly normal size; however, mice lacking alleles of each gene reveal a role for IRS2 in growth (Fig. 4). Growth curves based on daily weights from birth to 30 days

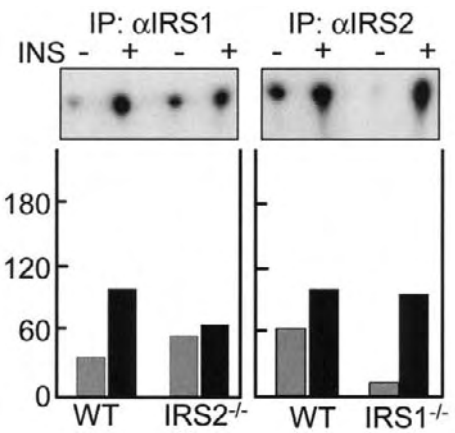


Figure 3 Phosphorylation of IRS1 or IRS2 and activation of PI 3-kinase in mouse liver. Wild-type mice, or mice retaining expression of IRS2 (IRS1$^{-/-}$ mice) or IRS1 (IRS2$^{-/-}$ mice) were stimulated with insulin as previously described [56]. Both IRS1 and IRS2 are tyrosine phosphorylated during insulin stimulation; however, the activation of the PI 3-kinase is distinctly different. In IRS2$^{-/-}$ liver, IRS1 barely mediates insulin-stimulated activation of the PI3K because the basal activity is relatively high. By contrast, in Irs1$^{-/-}$ livers, Irs2 strongly promotes insulin-stimulated activation of the PI 3-kinase because the basal activity is relatively low. This difference has been detected in 6-week-old mice when the circulating insulin levels are approximately equivalent.

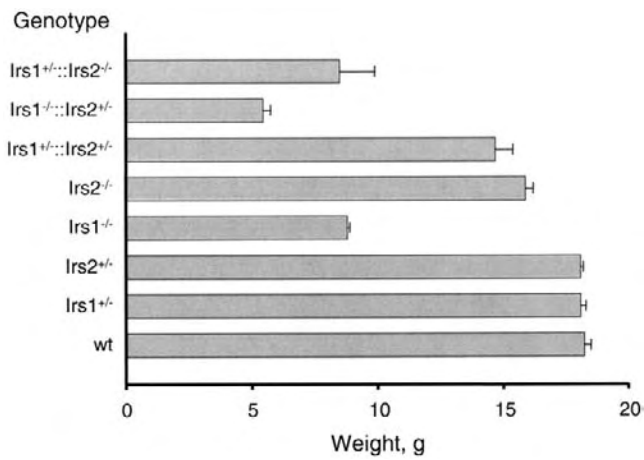


Figure 4 Growth characteristics of progeny of IRS1$^{+/-}$×IRS2$^{+/-}$ intercross; mean weights ±SEM of mice of the various genotypes on a C57Bl/6×129SV background at 30 days of age. Data are from 170 liters with a total of at least 4 animals per genotype [98].

of age reveal that Irs1$^{+/-}$ mice, Irs2$^{+/-}$, and IRS2$^{-/-}$ mice are normal compared to controls [56]. Compound heterozygous mice (IRS1$^{+/-}$×IRS2$^{+/-}$) weigh 25% less than wild-type animals, whereas IRS1$^{+/-}$×IRS2$^{+/-}$ mice were of similar size to IRS1$^{-/-}$ animals (Fig. 4). By contrast, IRS1$^{-/-}$×IRS2$^{+/-}$ are nearly 75% smaller than normal throughout their life. IRS1$^{-/-}$×IRS2$^{+/-}$ mice are among the smallest viable mice that have been generated from a variety of knockout strategies aimed at components of growth factor signaling pathways and demonstrate that a single copy of IRS-2 is sufficient to allow viability of very small mice, but only for a year. It is important to emphasize that owing to multiple system failure

in $Irs1^{-/-} \times Irs2^{+/-}$ mice, longevity is markedly reduced. The popular conclusion based on work with invertebrates that partial inhibition of insulin and IGF signaling extends life span must be applied to mammalian system with caution [57]; however, application of this hypothesis to specific tissues might be another story.

Interleukin-4 and IRS2 Signaling

IRS2 was originally identified as a tyrosyl-phosphorylated protein called 4PS in interleukin-4 (IL-4)-stimulated cells [58]. 4PS was also stimulated by insulin and insulin-like growth factor 1 (IGF1), and IL-4- or insulin-insensitive 32D cells lacking 4PS were rescued by expression of IRS1 [59]. Although 4PS was found to comigrate with IRS1 during sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), IRS1 antibodies failed to immunoprecipitate it, suggesting that 4PS was a distinct protein [60]. The cloning of 4PS revealed a novel protein with important similarity to IRS1 (Fig. 1), so its name was changed to IRS2 even before its central importance to insulin action and secretion was realized [61].

The story of IRS2 reveals an unexpected and possibly important molecular link between immune system function and nutrient homeostasis. IL-4, a multifunctional cytokine made by T helper type 2 (Th2) cells, basophils, mast cells, and NK1.1 T cells, influences the viability, proliferative capacity, and differentiation of B and T cells, as well as other cell types [62]. Cellular responses to IL-4 are mediated through the IL-4 receptor alpha chain (IL-4Rα) functioning as a heterodimer with the common gamma chain (γc) [63]. During IL-4 stimulation, the PTB domain of IRS2 binds to the phosphorylated NPAY motif in the activated IL-4Rα chain, promoting IRS2 tyrosine phosphorylation and signaling [64]. Other proteins in addition to IRS2 are also phosphorylated in response to IL-4R stimulation, such as JAK1 and JAK3, which phosphorylate IRS2 and other cellular proteins including FRIP, SHIP, SHP2, and the transcription factor STAT6 [65]. STAT6 plays an important role during IL-4 signaling by directly regulating gene expression that controls differentiation and cell growth [66]; however, IRS2 also plays an important role in the mitogenic response of T lymphocytes to IL-4 and in the development of Th2 cells *in vitro* [65].

In addition to IL4, other cytokines promote IRS1 or IRS2 tyrosine phosphorylation, including IL-7, IL-9, and IL-13; growth hormone; prolactin and leukemia inhibitory factor (LIF); and interferon [14]. In particular, comparisons between IL-4 and IL-9 reveal unique signaling [67]. IL-4 and IL-9 receptors share the common IL-2Rγ chain, so the signaling mechanism that establishes cytokine specificity and redundancy are not well understood. However, unlike IL-9, IL-4 receptors utilize IRS-protein in unique ways. IL9-Rα does not contain an NPXY motif to engage the PTB domain of IRS1 or IRS2, so it couples through the pleckstrin homology. Unlike IL-4 signaling, IL-9 does not promote SHP2 binding and does not require PKB activation for cell proliferation [67]. Thus, IRS-protein scaffolds provide a common interface between various membrane receptor, but mediate distinct signals depending on the cell context and the type of receptor involved.

Heterologous Regulation of IRS-Protein Signals

Infection, traumatic stress, obesity, inactivity, and aging are common physiologic causes of insulin resistance that contribute to various metabolic disorders, including diabetes. Experiments with transgenic mice and clinical investigation reveal that insulin resistance associated with diabetes is usually accompanied by downregulation of IRS-protein expression and function [68]. A common mechanism explaining the occurrence of acute or chronic insulin resistance in people is difficult to identify. Mutations in the insulin receptor are an obvious source of life-long insulin resistance, but these are rare and not necessarily accompanied by β-cell failure [69–72]. Recent experiments with transgenic mice teach us that dysregulation at many steps in the signaling cascade (especially regulatory interactions) might lead to insulin resistance. Elevated activity of protein or lipid phosphatases, including PTP1B, SHIP2, or PTEN, might be a clinically relevant cause of insulin resistance. Inhibition of these phosphatases by gene knockout or by chemical inhibitors increases glucose tolerance, suggesting that specific phosphatase inhibitors might be useful treatments for diabetes [73–75]; however, modulation of the activity of shared signaling proteins might result in undesirable phenotypes, including activation of signals that promote cancer.

Various cytokines or metabolites promote serine phosphorylation of the IRS-proteins which inhibits signal transduction and causes insulin resistance. Adipose-derived cytokines, especially tumor necrosis factor alpha (TNFα), inhibit signaling by serine phosphorylation of IRS1/IRS2 [76–78]. The signaling cascades regulated by TNFα are complex and involve many branch points, including the activation of various serine kinases and transcription factors that promote apoptosis or proliferation [79]. Inhibition of IκB kinase (IKKβ) with high doses of salicylates reverses hyperglycemia, hyperinsulinemia, and dyslipidemia in obese rodents by sensitizing the insulin signaling pathway [80,81]. Although no physical interaction occurs between IRS-proteins and IKKβ, salicylates increase insulin-stimulated tyrosine phosphorylation of the IRS-proteins in the liver, suggesting that IKKβ might indirectly inhibit insulin receptor function or its coupling to the substrates [82].

A more direct mechanism to inhibit IRS-protein function might involve activation of the c-Jun N-terminal kinase (JNK) [83–85]. JNK is a prototype stress-induced kinase that is stimulated by many agonists during acute or chronic inflammation. JNK phosphorylates numerous cellular proteins, including IRS1 and IRS2, Shc and Gab1 [86]. A role for JNK during insulin action is compelling, as both IRS1 and IRS2 contain JNK-binding motifs, originally identified

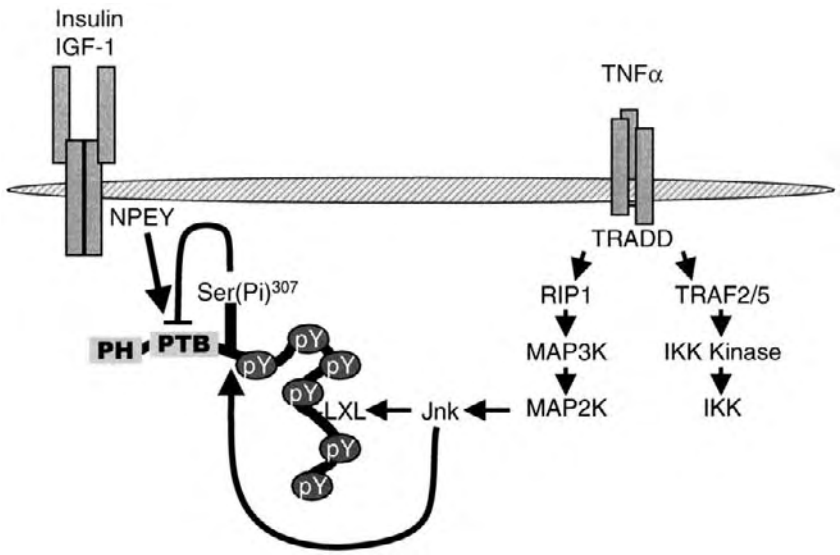


Figure 5 TNFα-induced inhibition of Irs-protein signaling. TNFα binding to TNFR1 results in recruitment of TRAF2/5, RIP1, and FADD through the adapter protein TRADD. TRAF2/5 and RIP1 appear to lead to activation of the protein kinases JNK and IKK. Activated JNK associates with IRS-1 and the JNK-binding LXL motif and promotes phosphorylation of Ser^{307}. Phosphorylation of Ser^{307} inhibits PTB domain function and inhibits insulin/IGF-stimulated tyrosine phosphorylation and signal transduction. Abbreviations: FADD, Fas-associated death domain protein; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; RIP1, receptor-interacting protein 1; TNFα, tumor necrosis factor α; TNFR1, TNF receptor type 1; TRAF2, TNF-receptor-associated factor 2.

in the JNK-interacting proteins JIP1 and JIP2. This motif mediates the specific association of JNK with IRS1, which promotes serine phosphorylation on the COOH-terminal side of the PTB domain, which inhibits recruitment to the insulin receptor (Fig. 5). Insulin also promotes the binding of active JNK to IRS-proteins, suggesting that it might mediate negative feedback control (Fig. 5).

Degradation of IRS-proteins is also regulated and might contribute to inhibition of insulin signaling. Prolonged insulin stimulation substantially reduces IRS1 and IRS2 protein levels in multiple cell lines, which is blocked by specific inhibitors of the 26S proteasome [87]. These results suggest that proteasome-mediated degradation of IRS2, rather than inhibition of transcription and/or translation of IRS2, determines protein levels and activity of IRS2-mediated signaling pathways [74]. Consistent with this idea, insulin stimulates the ubiquitination of IRS2 [88]. Reduction of IRS2 by ubiquitin/proteasome-mediated proteolysis in mouse embryo fibroblasts lacking IRS1 dramatically inhibits the activation of AKT and ERK1/2 in response to insulin/IGF1; strikingly, proteasome inhibitors completely reverse this inhibition. The activity of the ubiquitin/proteasome system is elevated in diabetes, which might promote degradation of the IRS-proteins and exacerbate insulin resistance [89,90].

IRS2 and Pancreatic β-Cells

Peripheral insulin resistance is a well-known component of Type 2 diabetes, but it clearly is not enough, as clinical experience and work with many transgenic mice reveal [91]. However, a compelling molecular link to diabetes emerges from the finding in mice that inhibition of the IRS2 branch of the insulin/IGF signaling system impairs the capacity of the pancreatic β-cells to compensate for insulin resistance [21,56]. Not only do $IRS2^{-/-}$ mice develop peripheral insulin resistance, but they also eventually fail to sustain compensatory insulin secretion [56]. The convergence of peripheral and islet defects around the IRS2 branch of the insulin/IGF signaling pathway reveals a common pathway to diabetes.

In mice, IRS1 and IRS2 contribute to peripheral insulin response, and there is no reason to suspect different roles for these proteins in people [27,56,92]. IRS1 exerts its greatest effect on metabolism by regulating insulin signals in muscle and adipose tissue, whereas it plays a lesser role in mediating the effects of insulin on liver metabolism [56,93–97]. But, $IRS1^{-/-}$ mice never become diabetic because they develop lifelong compensatory hyperinsulinemia. The amount of functional β-cell mass increases throughout the life of $IRS1^{-/-}$ mice, and the β-cells continue to detect changes in the serum glucose levels [93,98]. By contrast, $IRS2^{-/-}$ mice display dysregulated lipolysis, peripheral glucose uptake, and hepatic gluconeogenesis and ultimately develop diabetes due to β-cell failure [99]. Although $IRS2^{-/-}$ mice are nearly normal at birth and up to weaning, $IRS2^{-/-}$ mice develop hyperinsulinemia as young adults; males die at 12 weeks of age and females by 30 weeks of age [100]. As the disease progresses, pancreatic islet size invariably decreases and β-cell function fails [98].

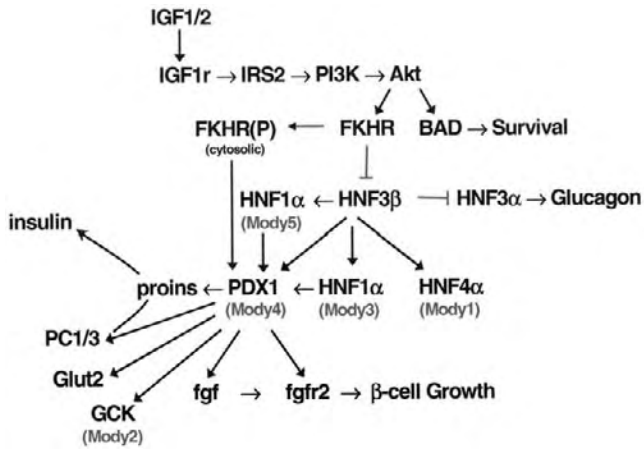


Figure 6 A potential pathway linking IRS2 signaling to the expression and function of the homeodomain transcription factor PDX1. The diagram shows the relation between the MODY genes, especially PDX1, and the IRS2 branch of the insulin signaling pathway [101]. Drugs that promote IRS2 signaling are expected to promote PDX1 function in β-cells which will support glucose tolerance and cure diabetes.

The progressive loss of β-cell function and the onset of diabetes in $Irs2^{-/-}$ mice might be associated with decreased expression in pancreatic islets of the homeodomain transcription factor PDX1 (also called IDX1 and IPF1) [101]. PDX1 is critical for the development of the pancreas in mice and people, and pancreas agenesis occurs upon the complete disruption of PDX1 [102,103]. Moreover, PDX1 is required in adult humans and mice to promote normal glucose sensing and insulin secretion [104,105]. Genetic defects in the PDX1 gene occur in about 5% of people with Type 2 diabetes. Inactivating mutations are associated with autosomal early-onset diabetes (MODY), whereas missense mutations predispose humans to late-onset Type 2 diabetes [106,107]. The functional association between IRS2 signaling and Pdx1 expression observed in mice creates a molecular link between Type 2 diabetes and the less frequent and less severe autosomal forms of diabetes (MODY) [101]. Dysregulation of PDX1 by genetic or functional mechanisms might be one of the common links between early-onset (MODY) and ordinary Type 2 diabetes (Fig. 6). This hypothesis is supported by the finding that transgenic expression of PDX1 postpones β-cell failure and reduces by years the progression of diabetes in $IRS2^{-/-}$ mice [108]. If insulin resistance in people includes an IRS2 component, then reduced PDX1 function might eventually impair β-cell function as noted in people with MODY, and drugs that promote the IRS2→Pdx1 pathway might provide new strategies to promote β-cell growth and function in both Type 1 and Type 2 diabetes.

Summary

The IRS-protein family, particularly IRS-2, plays a fundamental role in insulin and IL-4, IL-7, and IL-9 signaling. Failure of IRS2 might be central to the development of Type 2 diabetes and its associated complications by dysregulating multiple biological processes, including peripheral insulin sensitivity, insulin secretion, and the immune response. Moreover, understanding the function of IRS-proteins during inflammatory responses might reveal rational approaches to modify the effect of stress on cell function and metabolism. Understanding the differences between IRS1 and IRS2 for growth control might reveal strategies to slow general tissue aging or tumor growth while promoting peripheral insulin action and the survival and regeneration of pancreatic β-cells.

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CHAPTER 73

Eph Receptors

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Introduction

Eph receptors represent the largest known family of receptor tyrosine kinases (RTKs) and are activated by interaction with cell-surface ligands, termed *ephrins*, presented by neighboring cells. The physiological roles of Ephs and ephrins range from early embryonic developmental processes such as cell migration and the establishment of compartment boundaries to processes in the adult brain, such as the regulation of neuronal plasticity. At the cellular level, ephrins guide migrating cells and navigating axonal growth cones by repulsion; that is, they induce, via Eph receptor signaling, the local collapse of cellular processes, thereby redirecting cellular growth. In other processes, ephrins may regulate cellular adhesion and de-adhesion and thereby influence cellular behavior. A peculiarity of this ligand–receptor system is that signals are transduced not only by the Eph receptor (referred to as *forward signaling*), but also by the ephrin ligand (referred to as *reverse signaling*). Components of the signaling pathways downstream of Ephs and ephrins have been characterized and correlated with biological functions [1]. This chapter summarizes current knowledge on Eph and ephrin signaling and makes reference to recent reviews summarizing the relevant biology.

Ephs and Ephrins

Eph receptors are type I transmembrane proteins with amino termini located outside of the cell; they have a single transmembrane-spanning region. The Eph ectodomain contains two fibronectin type III (FnIII) repeats, a cysteine-rich region with homology to EGF-like repeats, and an amino-terminal globular domain that constitutes the primary ligand binding region. Based on sequence similarity and ligand affinity, Eph receptors are subdivided into an A subclass, which contains eight members (EphA1–EphA8) and a B subclass, which contains six members (EphB1–EphB6) [18]. The ephrins also fall into two subclasses (A and B) based on their mode of membrane attachment. The ephrinA ligands (ephrinA1–A5) are tethered to the cell surface via a glycosylphosphatidylinositol (GPI) anchor, whereas ephrinB ligands (ephrinB1–B3) contain a transmembrane domain and a cytoplasmic tail. EphA receptors typically bind most or all ephrinA ligands, and EphB receptors bind to most or all ephrinB ligands, with the exception of the EphA4 receptor, which binds both groups of ephrin ligands. Membrane attachment and clustering appear to be required for ephrins to activate receptors, because only membrane-bound or artificially clustered ligands, but not soluble ligands, can trigger receptor signaling. Because Eph receptors and ephrins are both membrane-bound molecules, it is believed that they mediate direct cell–cell communication rather than long-range interactions. For practical reasons, however, signaling is typically studied using soluble fusion proteins consisting of the Fc portion of human immunoglobulin G (IgG) fused to the ectodomain of either ephrin or Eph (i.e., ephrin-Fc and Eph-Fc, respectively).

Eph Receptor Signaling Via Cytoplasmic Protein Tyrosine Kinases

Upon ligand binding, Eph receptors are thought to dimerize, perhaps oligomerize, and autophosphorylate specific tyrosine residues of the cytoplasmic part of the partner receptor. Somewhat unusual among RTKs, Eph kinase activity is autoinhibited by the unphosphorylated juxtamembrane

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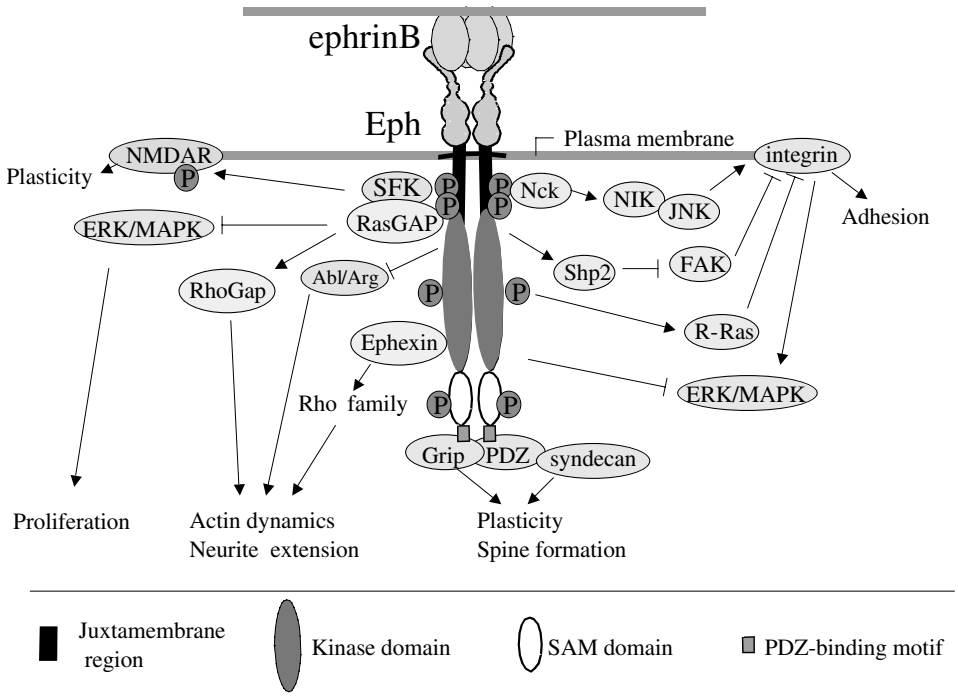


Figure 1 Eph receptor structure and cytoplasmic interactions. The intracellular part of Eph receptors consists of a juxtamembrane region, tyrosine kinase domain, SAM domain (of unknown function), and a PDZ binding site. Upon engagement with ephrins attached to the cell surface, Eph receptors dimerize, perhaps form oligomeric complexes, and autophosphorylate at several tyrosine residues (orange circles). Signaling effectors containing SH2 domains (SFKs, Abl/Arg, RasGAP, Nck) are recruited to these phosphotyrosines. Other effectors bind to the kinase domain (ephexin) or to the PDZ target site (Grip). Again, other effectors may interact with Ephs in an indirect manner (additional interactors are described in references [1], [3], and [18]). Cellular functions mediated by Eph forward signaling include regulation of actin dynamics and suppression of neurite extension, block of proliferation and integrin mediated adhesion, promotion of spine formation, and neuronal long-term plasticity (see text for details).

region, and autophosphorylation of juxtamembrane tyrosine residues not only creates docking sites for phosphotyrosine binding proteins but also releases autoinhibition [2]. Among the first proteins to be identified as Eph effectors were Src family kinases (SFKs), which bind to juxtamembrane phosphotyrosine residues via their SH2 domains (Fig. 1) [3]. Although SFKs mediate mitogenic effects in many cellular contexts, as downstream effectors of Ephs they are more likely to regulate cytoskeletal changes. Activated forms of Src kinases have been implicated in the phosphorylation of various cellular proteins, such as paxillin, Fak, and tensin, which are associated with integrin-harboring focal adhesions and with proteins such as p120 and β-catenin found in cadherin-containing adherens junctions [1].

Recently, the EphB2 receptor was shown to interact and to form clusters with the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors at excitatory synapses in primary neurons [4]. Stimulation with soluble ephrinB-Fc triggered EphB forward signaling through SFKs and caused tyrosine phosphorylation of NMDA receptors. The latter process required SFK activation, as shown by the usage of SFK inhibitors [5]. Functionally, EphB2 protein, but not EphB2 kinase activity, was required for protein-synthesis-dependent, long-lasting changes in neuronal plasticity in the hippocampus [6] [7]. Plasticity defects in ephB2 null mutant mice correlated with defects in performance for learning paradigms requiring an intact hippocampus. Whether or not the interaction of EphB2 with NMDA receptors and the activation of SFKs account entirely for the observed defects remains to be investigated.

The Abelson cytoplasmic tyrosine kinase (Abl) and the Abl-related gene product (Arg) also bind EphB2 and EphA4 receptors via phosphotyrosine-dependent and -independent interactions [8]. Unlike SFKs, Abl and Arg kinase activities are decreased by activated Ephs. Abl kinase is thought to promote neurite growth, whereas Eph forward signaling leads to localized growth-cone collapse and to repulsive guidance of growing axons. Inhibition of Abl kinase may be one of the mechanisms by which Ephs prevent local axonal growth.

Eph Receptor Signaling Via Rho Family GTPases

The small GTP-binding proteins of the Rho GTPase family, including RhoA, Rac, and Cdc42, are important regulators of the actin cytoskeleton and neuronal morphogenesis [9]. Rho GTPases are molecular switches that cycle between

an active GTP-bound state and an inactive GDP-bound state. GTP-bound forms are converted into GDP-bound forms by the action of GTPase-activating proteins (GAPs), whereas guanine nucleotide exchange factors (GEFs) perform the opposite conversion. The Rho-specific GAP, p190RhoGAP, is indirectly recruited to activated EphB2 by binding to p120RasGAP, which binds juxtamembrane phosphotyrosine residues of EphB2 via its SH2 domain [10]. The consequences of p190RhoGAP recruitment to EphB2 are not entirely clear; however, p190RhoGAP appears to be an important link between SFKs and the regulation of neurite outgrowth and axon guidance. Src-mediated phosphorylation of p190RhoGAP is required for binding to p120RasGAP, and mice lacking p190RhoGAP display defects in axon tract formation and fasciculation, reminiscent of the defects seen in mice lacking EphB2 receptors [11].

EphA receptor signaling was also connected to Rho family GTPases, although different *in vitro* assays were employed and direct comparisons are difficult to draw up. Conversely, EphA receptors bind Ephexin (Eph-interacting exchange protein), a novel Rho family GEF required for ephrinA-induced growth-cone collapse of retinal ganglion cells [12,13]. Ephexin appears to have differential effects on Rho GTPases, such that RhoA is activated but Cdc4 and Rac are inhibited, at least *in vitro*. Ephexin-mediated activation of RhoA shifts actin cytoskeleton dynamics to increased contraction and reduced extension, providing an explanation how Ephexin may mediate growth-cone collapse.

Effects on Cell Proliferation

In contrast to other RTKs, Eph receptor forward signaling does not provide a mitogenic stimulus, despite the recruitment of SFKs and the adaptor Grb2 [1]. One possible mechanism may be the recruitment of SLAP, a non-catalytic Src-like adaptor protein that may compete with SFK binding to Ephs and thereby suppress mitogenesis [14,15]. More recently, EphA and EphB forward signaling was shown to suppress MAPK signaling [16,17]. MAPK activation by platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) of different cell lines was reduced by application of ephrinA1 [17]. Moreover, ephrinB1-induced activation of EphB2 in neuronal cells downregulated the levels of GTP-bound Ras, which caused collapse of growth cones and retraction of neurites. Expression of a dominant active form of Ras reversed EphB2-mediated neurite retraction [16].

Eph Receptor Signaling through PDZ-Domain-Containing Proteins

The PDZ binding motif in the carboxy terminus of most Eph receptors has been shown to bind PDZ-domain-containing proteins, including syntenin, Pick1, Grip, and AF6 [18]. For most of these reported interactions, the functional significance of binding is unknown. A recent study has demonstrated that, *in vitro*, the EphB2 carboxy terminus binds the AMPA-type glutamate receptor GluR2 in membranes prepared from rat brain. This interaction is likely to be indirect through the multi-PDZ-domain protein Grip1, which binds EphB2 and GluR2 using different PDZ domains. Interfering with EphB2–PDZ interactions by infusion of EphB2 C-terminal peptide or EphB2 anti-C-terminal antibodies has led to a decrease in tetanus-induced long-term potentiation (LTP) at the mossy fiber–CA3 synapse. Contractor *et al.* [19] speculated that the ephrinB–EphB signaling system may regulate synaptic plasticity by providing a retrograde signal that links postsynaptic induction of LTP to presynaptic changes in neurotransmitter release.

Postsynaptic Eph signaling may also regulate dendritic spine morphogenesis, potentially involving interactions with PDZ domain proteins [20]. The transmembrane cell-surface proteoglycan syndecan-2 induces the formation of morphologically mature dendritic spines in immature hippocampal neurons, in part via regions in its cytoplasmic domain that contain potential tyrosine phosphorylation sites. Activated EphB2 receptors phosphorylate syndecan-2 on tyrosine residues and induce clustering of syndecan-2. Phosphorylated syndecan-2 acquires the ability to recruit cytoplasmic proteins, including PDZ-domain proteins CASK, syntenin, and synbindin, which may also interact with EphB2. It is this complex that is thought to promote the morphological maturation of spines [20].

Eph Receptors and Cell Adhesion

Eph receptors seem to regulate cell adhesion primarily by modulating integrin signaling (Fig. 1). Integrins are receptor molecules embedded in the plasma membrane and consist of two polypeptides: α and β chains. Their ectodomains bind extracellular matrix proteins such as laminin, collagen, and fibronectin. Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that links integrin receptors to intracellular signaling pathways. Activation of endogenous EphA2 in tumor cell lines suppresses integrin-mediated cell adhesion by recruiting the protein tyrosine phosphatase SHP2 and by dephosphorylation of FAK [21]. Similarly, activation of EphB2 inhibits cell adhesion through phosphorylation of R-Ras, which suppresses the ability of R-Ras to support integrin activity [22]. These studies are in contrast to the findings in P19 teratocarinoma cell lines, in which EphB1 signals pro-adhesion to fibrinogen via recruitment of Nck and activation of Nck-interacting Ste20 kinase (NIK) and c-Jun N-terminal kinase (JNK) [23].

Ephrin Reverse Signaling

The concept of ephrin reverse signaling is well established for ephrinB ligands, whose cytoplasmic regions make them look like receptor molecules. GPI-anchored ephrinA

ligands may also signal by interaction with other raft-asso ciated proteins, including Src family kinases [24], or by interaction with an unknown protein that spans the membrane. Evidence for ephrinA reverse signaling was derived from genetic studies in *Caenorhabditis elegans*, whose genome contains four potential GPI-anchored ephrins and one Eph receptor (VAB-1). Different alleles of VAB-1 have been shown to have different effects on cellular organization and have suggested the kinase-independent functions of VAB-1, consistent with reverse signaling by *C. elegans* ephrins [25,26].

EphrinB Reverse Signaling Via Phosphotyrosine

Phosphorylation of conserved tyrosine residues in the cytoplasmic domain of ephrinB ligands is thought to be a critical event in triggering reverse signaling. EphrinB phosphorylation is induced either by stimulation with the soluble ectodomain of Eph receptors or by contact with neighboring cells expressing Eph receptors. Alternatively, ephrinB phosphorylation can be induced by treatment of cells with fibroblast growth factor (FGF) or platelet-derived growth factor (PDGF) via the co-expressed FGF or PDGF receptors. Src family kinases are regulators of ephrinB phosphorylation induced by the EphB ectodomain. SFKs are rapidly recruited into ephrinB-positive membrane patches, and their kinase activity is activated by treatment of cells with the soluble EphB ectodomain (Fig. 2). Most importantly, SFK activity is required for EphB-induced sprouting of endothelial cells that express ephrinB ligands.

Phosphotyrosine-dependent signaling is at least in part mediated by recruitment of the SH2–SH3 adaptor protein Grb4, a relative of Nck (which is implicated in Eph forward signaling). The Grb4 SH2 domain, but not the Nck SH2 domain, binds to phosphorylated ephrinB after engagement with EphB receptors. Grb4 binds, through its SH3 domains, to several polyproline-containing proteins (Abi-1, CAP, axin, and others), all of which are modifiers of the actin cytoskeleton. Consequently, recruitment of Grb4 to ephrinB causes loss of polymerized F-actin structures and disassembly of focal adhesions [27].

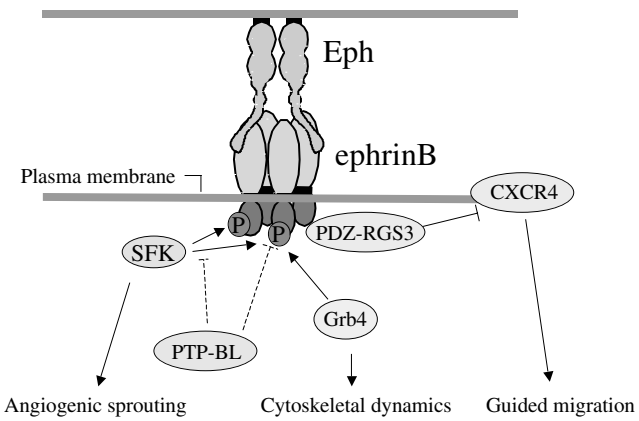


Figure 2 Structure of ephrinB ligand and associated signaling molecules. Upon binding of their cognate Eph receptor on neighboring cells, ephrinB ligands are clustered and Src family kinases (SFKs) are rapidly recruited to ephrinB clusters. SFKs phosphorylate the ephrinB cytoplasmic tail, which leads to angiogenic sprouting through an unknown mechanism. Phospho-ephrinB recruits the Grb4 adaptor, which mediates changes in cytoskeletal organization. The tyrosine-specific phosphatase PTP-BL negatively regulates Src kinase activity and dephosphorylates ephrinB with delayed kinetics (dashed line). PDZ-RGS3, through binding to ephrinB, suppresses CXCR4-induced cell migration (see text for details).

EphrinB Reverse Signaling Via PDZ Domain Interactions

Similar to Eph receptors, ephrinB ligands carry a C-ter minal PDZ binding site, which was shown to interact with a number of PDZ-domain-containing proteins, some of which also interact with Eph receptors, at least *in vitro*; these include syntenin, Pick1, Grip1, Grip2, Fap1, PDZ-RGS3, PTP-BL, and PHIP [18]. For most if these ephrin interactors, it is not known whether they regulate ephrinB localization, clustering, or function. Most notably, PDZ-RGS3 provides a link between cell migration and ephrinB reverse signalling. Coinjection of ephrinB1 and PDZ-RGS3 into *Xenopus* embryos led to de-adhesion of the cells in a PDZ-domain-dependent manner [28]. Furthermore, migration of cerebrellar granule cells by stromal-cell-line-derived factor 1 (SDF-1), a ligand of the G-protein-coupled receptor CXCR4, was inhibited by adding clustered EphB2-Fc receptor bodies. EphrinB reverse signaling may activate PDZ-RGS3, which may regulate the critical GEF activity (GTP exchange factor) required for efficient signaling of CXCR4 [28]. The mechanism by which ephrins stimulate the activity of PDZ-RGS3 is not known, but the interaction between ephrinB and PDZ-RGS3 seems to be constitutive.

The protein tyrosine phosphatase PTP-BL appears to be a negative regulator of ephrinB signaling based on its ability to inactivate SFK activity [29,30] and to dephosphorylate ephrinB [30] (Fig. 2). The action of positive regulators of ephrin phosphorylation, such as SFKs, and of negative regulators, such as PTP-BL, appear to be regulated in time, at least *in vitro*. SFKs are recruited with rapid (5 min) kinetics to ephrin-containing clusters, whereas PTP-BL is recruited slowly (30 min). This may be part of a switch mechanism whereby ephrin reverse signaling shifts from phosphotyrosine-dependent to PDZ-dependent signaling [30].

Summary

Eph receptors and their ephrin ligands play important roles in many developmental and plasticity processes, which are rapidly being elucidated, primarily by mouse genetics. Rapid progress is also being made in the biochemical characterization of cytoplasmic interactors of Ephs and ephrins, mainly in immortalized cell lines. Their functional characterization in cell-based assays, however, turns out to be more

difficult; likewise, the cellular mechanisms underlying ephrin-/Eph-mediated biology is in most cases unclear. Axon guidance is probably mediated by repulsive interactions; however, the cellular mechanism underlying vascular remodeling, and neuronal plasticity is currently unknown.

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CHAPTER 74

Cytokine Receptor Superfamily Signaling

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Cytokine Receptor Superfamily Signaling

A number of cytokines can be functionally grouped by their similarity in structure as well as by the similarity of the receptors that they utilize. One group of 26 cytokines (Table 1) is characterized by a common predicted 4-α-helical bundle structure [1]. This group of cytokines is further characterized by their common utilization of receptors that are members of the cytokine receptor superfamily [2]. The defining structural elements of the receptors include a cytokine binding module that typically contains two fibronectin type III domains with four positionally conserved cysteine residues in the extracellular domain. In the membrane-proximal region of the extracellular domain, most receptors also contain a WSXWS motif of unknown function. The cytoplasmic domains of the cytokine receptors have only very limited similarity, located in the membrane-proximal region and consisting of what has been termed the box 1 and box 2 motifs. Based on structural considerations, the receptors are often further divided into class I and class II receptors, as indicated in Table 1. A single gene (*dom*) in *Drosophila* has functional and structural similarity to the mammalian cytokine receptor superfamily, with the greatest similarity being with the IL-6 subfamily of receptors [3]. This would suggest that the cytokine receptor superfamily has recently evolved to provide expanded opportunities for physiological regulation of cell functions in vertebrates.

As shown in Table 1, a functional cytokine receptor can consist of one or more chains. The nature of the contributions of the individual chains can be quite different depending upon the complex. Frequently a receptor chain contributes only to affinity of cytokine binding. For example, the α-chain of the interleukin-2 (IL-2) receptor, which is the only receptor component listed in Table 1 that is not structurally of the cytokine receptor superfamily, increases the affinity of the cytokine binding complex approximately 10-fold. Conversely, the α-chain of the IL-6 receptor binds the cytokine either as a component of the cell-surface complex or as a soluble extracellular protein. The complex of IL-6 and the IL-6 receptor α-chain in turn has high-affinity binding for the β component of the complex gp130. Similarly, the receptors for IL-3, granulocyte–macrophage colony-stimulating factor (GM-CSF), and IL-5 have a low-affinity, ligand-specific binding chain (α) that associates with a signal transducing chain that is shared (β_c) or, in the case of IL-3 in mice, with a highly related but specific signaling chain (β_{IL3}). Finally, a number of receptors have two chains that are required for signal transduction. For example, in the IL-2 subfamily of receptors both the unique β-chain and the γ_c are required for signal transduction. Similarly, within the type II receptors, the cytoplasmic domains of both chains are required for signal transduction.

In all the receptor complexes, one or more of the receptor chains associates with one or more of the Janus family of protein tyrosine kinases (Jak), as indicated in Table 1 [4–6]. The Jak family of kinases consists of four members (Fig. 1). The family members have a large amino-terminal domain that contains blocks of homology among family members. It is through this region that the Jaks have been shown to interact with receptor chains. The carboxyl domain contains a pseudokinase domain followed by a functional protein tyrosine kinase catalytic domain. The role of the pseudokinase

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Table I

Cytokine receptor	Chains	Associated Janus kinases
Type I receptors		
Single-chain/Jak2		
Erythropoietin (EPO)	1	α (Jak2)
Thrombopoietin (TPO)	1	α (Jak2)
Growth hormone (GH)	1	α (Jak2)
Prolactin (PRL)	1	α (Jak2)
Common β chain		
Interleukin-3	2	α, β_c (Jak2), or β_{IL3} (Jak2)
Granulocyte–macrophage CSF	2	α, β_c (Jak2)
Interleukin-5	2	α, β_c (Jak2)
Common γ or γ-like:		
Interleukin-2	3	α_{IL2} (none), β_{IL2} (Jak1), γ_c (Jak3)
Interleukin-4	2	α_{IL4} (Jak1), γ_c (Jak3)
Interleukin-7	2	α_{IL7} (Jak1), γ_c (Jak3)
Interleukin-9	2	α_{IL9} (Jak1), γ_c (Jak3)
Interleukin-13	2	α_{IL4} (Jak1), α_{IL13} (Tyk2)
Interleukin-15	2	α_{IL15} (Jak1), γ_c (Jak3); α_{IL15} (Jak1), ξ(Jak2)
Interleukin-21	2	α_{IL21} (Jak1), γ_c (Jak3)
Thymic stromal lymphopoietin	2	α_{IL7} (Jak1), γ-like (Jak2)
IL-6 family:		
Granulocyte-CSF	1	α (Jak1, Jak2, Tyk2)
Interleukin-6	2	α, β_{gp130} (Jak1)
Leukemia inhibitor factor	2	α_{LIFR} (Jak1), β_{gp130} (Jak1)
Ciliary neurotrophic factor	3	α_{CNTF}, β_{gp120} (Jak1), β_{LIFR} (Jak1)
Cardiotrophin 1	2	$\alpha_{CT1,}$ β_{gp130} (Jak1), β_{LIFR} (Jak1)
Oncostatin M	2	α_{OSMR}, β_{gp130} (Jak1)
Interleukin-11	2	α_{IL11}, β_{gp130} (Jak1)
Interleukin-12	1	α (Tyk2 or Jak2)
Type II receptors		
Interferon-α,β	2	α (Jak1), β (Tyk2)
Interferon-γ	2	α (Jak1), β (Jak2)
Interleukin-10	2	α (Jak1), β (Jak2)

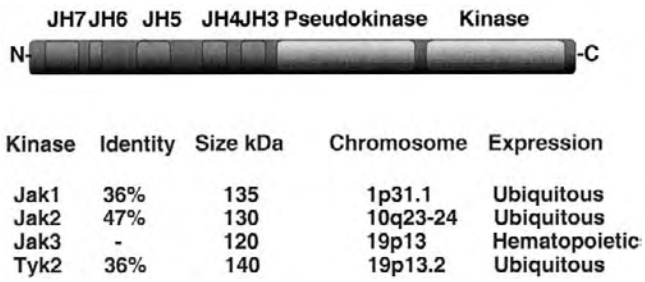


Figure 1 Structure and properties of the Janus family of protein tyrosine kinases.

domain is not known, although some studies have shown that it negatively influences kinase activity and may confer specificity. Unfortunately, no molecular structures have been reported for a Jak. The association of Jaks with receptor chains has been shown to be variable and to exist prior to ligand binding or following ligand binding. Whether the variability is due to technical differences in detecting the association prior to ligand binding or an inherent difference in the nonligated complex is not known.

Irrespective of the number of receptor chains and their individual contributions, the primary function of the complex is to induce the aggregation of the signal transducing component of the complex to activate the associated Jaks. Through the use of cross-linking approaches, ligand induces the aggregation of a number of receptor complexes, resulting in the formation of very large complexes. Recent studies have identified small molecules that can also induce receptor aggregation and thereby mimic ligand binding [7,8]. In one case, it was shown that drug binding occurred at sites distinct from the ligand binding region. Regardless, like ligand binding, drug binding results in the activation of receptor-associated Jaks.

The activation of Jaks involves the transphosphorylation of a specific tyrosine in the activation loop that dramatically increases kinase activity. In addition, there are multiple additional sites of auto- or transphosphorylation, but the potential significance of these additional phosphorylation sites has not been examined in detail. From the structure of the known receptor complexes, it can be deduced that Jak2 is capable of activation in complexes in which Jak2 is the only family member present. However, in the other complexes, it has not been determined whether more than one Jak is required for transphosphorylation between different Jaks. For example, it is known from the phenotypes of Jak-deficient mice that both Jak1 and Jak3 are required for the function of the IL-2 subfamily of cytokines receptors. In addition, evidence has been presented that the Jaks may be required in the receptor complex to form a high-affinity receptor complex [9].

The essential role that the Jaks play in cytokine receptor signaling has been established through the derivation of mice deficient in one or more of the Jaks [4]. For example, a deficiency in Jak3 results in a phenotype of severe combined immunodeficiency (SCID) due to the lack of function of the IL-2 subfamily of cytokine receptors. Genetic deficiencies of Jak3 also occur in children, for whom the deficiency is similar to that associated with a SCID phenotype. Jak2 deficiency is linked with an embryonic lethality caused by the lack of production of sufficient red cells, and Jak1 deficiency is associated with a perinatal lethality and with loss of function of the IL-6 and IL-2 subfamilies of receptors. Finally, Tyk2 deficiency specifically affects the interferon (IFN)-α/β receptor and IL-12. Importantly, in addition to confirming the role of these kinases in cytokine receptor superfamily signaling, analysis of Jak-deficient mice failed to identify an essential role for the kinases in other receptor complexes, in spite of the observation that Jaks have frequently been shown to be inducibly tyrosine phosphorylated

in other receptor systems. In *Drosophila*, a single Jak (hopscotch, *hop*) is critical for signal transduction through the single *Drosophila* cytokine receptor gene (Dome, *dome*) [3]. The identical nature of the phenotypes of mutations of *hop* and *dome* suggest that, as in mammals, the receptor/Jak complex is a dedicated signaling complex.

The cytokine receptor superfamily members activate a variety of signal transduction pathways. One of the most consistently activated pathways is that of induced tyrosine phosphorylation of the transcription factors of the signal transducers and activators of transcription (STATs) family. The details of this family of transcriptions factors are covered elsewhere in this Handbook; however, in general terms, the STATs mediate the specific physiological functions associated with individual cytokines [10]. For example, the function of IFNs to elicit an antiviral response is dependent on STAT1 and, conversely, the primary function of STAT1 is to mediate these responses. Equally striking, STAT4 and STAT6 mediate the unique physiological responses induced by IL-12 or IL-4, respectively. This specificity is dramatically illustrated by the observation that Epo, Tpo, GH, and PRL all induce the activation of STAT5; the physiological functions of GH and PRL are totally dependent upon this activation, but the functions of Epo and Tpo are not.

In addition to specific physiological functions, however, many cytokines have as their primary function the ability to promote the proliferation and survival of cells. The elements that are involved in this response are largely unknown. For example, the primary function of Epo is to expand early erythroid lineage cells to provide sufficient numbers to sustain embryonic development. This capability is not unique to Epo, as the prolactin receptor can mediate the same expansion [11]. Conversely, the cytoplasmic domain of the Epo receptor can fully support the expansion and differentiation of granulocytes when it replaces the cytoplasmic domain of the G-CSF receptor *in vivo* [12]. The ability of the cytoplasmic domain of the Epo receptor to function requires only a small portion of the cytoplasmic domain and specifically does not require receptor tyrosines or the ability to activate a STAT-dependent pathway [13]. The conclusion from these types of studies is that, in these cases, the primary function of the receptor complex may be to activate the Jaks, which then function in much the same manner as the receptor tyrosine kinases by recruiting critical signaling mediators to the kinase.

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CHAPTER 75

Negative Regulation of the JAK/STAT Signaling Pathway

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Introduction

Cytokines are a group of secreted proteins that mediate important cellular processes such as growth, differentiation, and immune defense [1]. They exert their effects by binding to specific receptors expressed on the surface of target cells [2] and trigger intracellular signaling cascades that ultimately result in changes in gene transcription. Despite the diversity of their biological actions, cytokines use a common signal transduction pathway: the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (Fig. 1A). Cytokine receptors typically lack intrinsic tyrosine kinase activity. Instead, they constitutively associate with cytoplasmic JAKs. Cytokine binding induces oligomerization of receptor chains to bring JAKs into close proximity, allowing them to cross-phosphorylate each other and the receptor itself. Downstream signaling molecules such as the STAT proteins are then recruited to the receptor complex. STAT proteins bind via their SH2 domains to specific phospho-tyrosine motifs on the receptor and become phosphorylated by the JAKs. Once phosphorylated, STAT proteins homo- or heterodimerize and enter the nucleus where they activate the transcription of a specific set of genes [3]. The combination of JAKs and STATs utilized by a given cytokine accounts for both the specific and redundant actions of cytokines.

The JAK/STAT pathway activated by cytokines is also tempered to control both the duration and intensity of signaling. There are various levels at which negative regulation can occur, including downregulation of the receptor–ligand complex, dephosphorylation or degradation of signaling intermediates, and production and/or activation of specific suppressor proteins. This review focuses on three classes of proteins: the phosphatases, the protein inhibitors of activated STATs (PIAS), and the suppressors of cytokine signaling (Figs. 1B–D).

The Phosphatases

Cytokine signaling involves a cascade of tyrosine phosphorylation events. Tyrosine phosphorylation of many proteins is rapid but transient. It is therefore not surprising that dephosphorylation of activated signaling molecules by specific phosphatases is an important mechanism for the termination of cytokine signaling [4].

SHP-1

The Src homology domain 2 (SH2)-containing tyrosine phosphatase SHP-1 has been identified as a critical negative regulator of cytokine signaling [5]. SHP-1 is a cytosolic phosphatase that is expressed predominantly in hemopoietic cells, and the importance of SHP-1 is evident in mice harboring a spontaneous mutation in the SHP-1 gene known as *motheaten* mice. *Motheaten* mice, which do not express SHP-1, suffer from a range of hemopoietic abnormalities, including hyperproliferation and abnormal activation of macrophages and granulocytes [6,7].

The multiple hemopoietic defects in *motheaten* mice are likely to be attributed to the ability of SHP-1 to suppress the signaling by various cytokines, including erythropoietin

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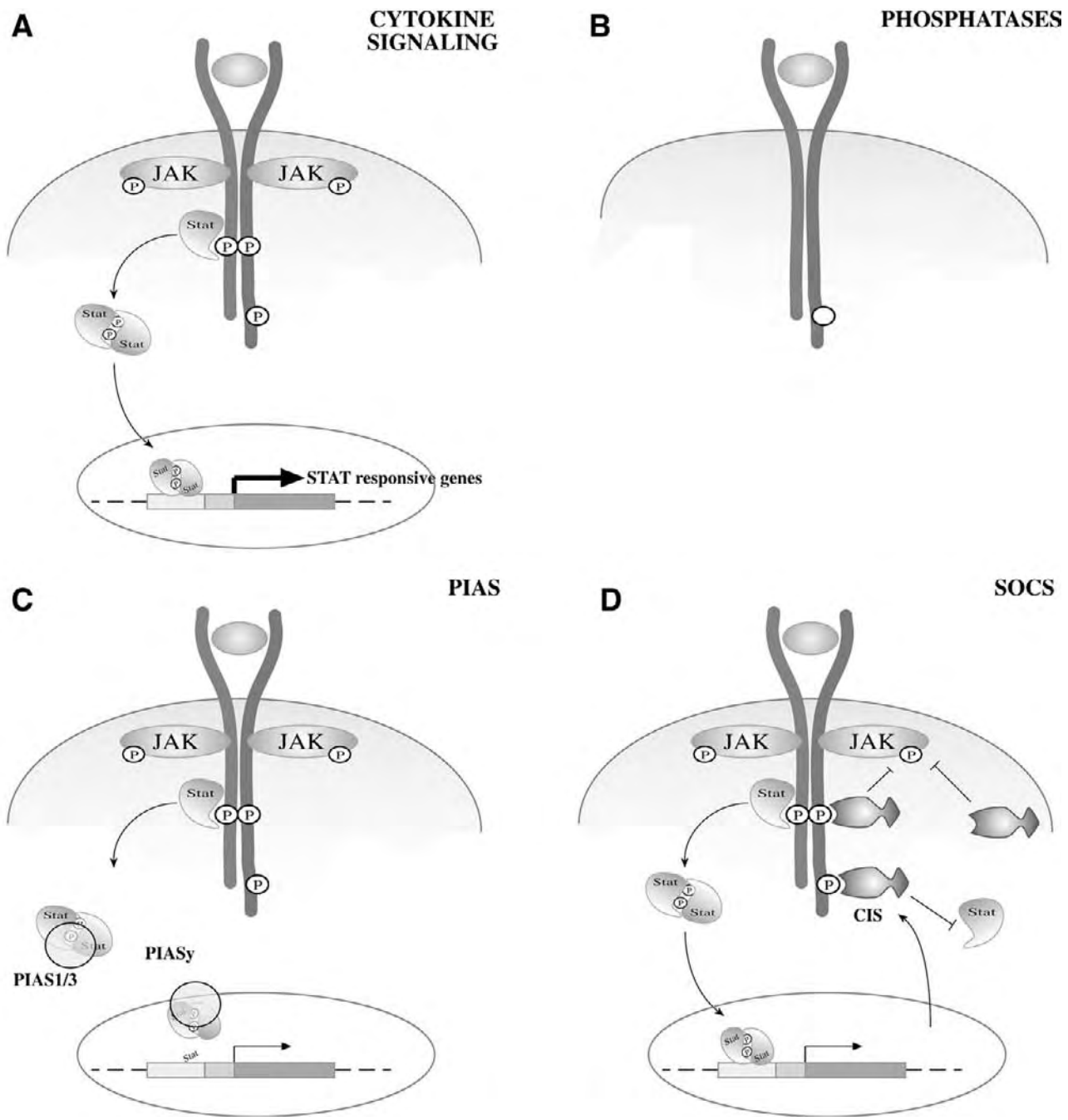


Figure 1 Schematic illustration of mediators and inhibitors of cytokine signaling. (A) Cytokines initiate signaling through binding to multimeric cell surface receptors. Receptor aggregation leads to juxtaposition of JAKs, which cross phosphorylate each other and phosphorylate tyrosines within the receptor cytoplasmic domain. These phospho-tyrosines provide docking sites for cytoplasmic proteins, such as STATs, which are themselves phosphorylated by JAKs. STATs then dimerize, migrate to the nucleus, and regulate the transcription of genes required for mediating the biological response to the cytokine. Phosphatases (B), PIAS proteins (C), and SOCS proteins (D) each attenuate signaling by acting at different levels within the cascade.

(EPO) [8], interleukin-3 (IL-3) [9], IL-4 and IL-13 [10], interferons [11–13], colony-stimulating factor 1 (CSF-1) [14], and Steel factor [15,16].

Among these cytokines, the evidence for regulation of EPO signaling by SHP-1 is most compelling. *In vitro* studies have shown that, upon EPO stimulation, SHP-1 can bind to the activated EPO receptor complex and dephosphorylate the receptor-associated kinase JAK-2. Additionally, cells expressing a mutant EPO receptor that is unable to recruit SHP-1 show prolonged tyrosine phosphorylation of JAK-2 and an enhanced mitogenic response [17,18]. The importance of recruitment of SHP-1 to the phosphorylated receptor cytoplasmic domain in regulation of signaling is highlighted by families in which polycythemia is caused by a mutation that results in truncation of the EPO receptor and an inability to bind SHP-1 [19,20].

CD45

CD45 is a transmembrane phosphatase that was initially identified as being an important regulator of antigen receptor signaling in B and T cells [21–25], with its primary targets being the Src family kinases [26,27] . Recent evidence suggests, however, that CD45 is also a JAK phosphatase and can negatively regulate cytokine signaling [28,29]. CD45 appears to inhibit IL-3-induced proliferation, erythropoietin-induced hematopoiesis, and antiviral responses, both *in vitro* and *in vivo*. *In vitro* studies have shown CD45 can bind and directly dephosphorylate JAKs. Furthermore, IL-3 stimulation of a CD45-deficient, bone-marrow-derived mast cell line induced hyperphosphorylation of JAK-2 and an increased phosphorylation of STAT-3 and STAT-5 [29].

STAT Phosphatases

Phosphorylation of a single tyrosine residue within the C-terminal region of STATs is critical for their activation and function. *In vitro* studies using phosphatase inhibitors have demonstrated the important role of phosphatases in the regulation of STAT phosphorylation [30,31]. Specific regions of STAT proteins have been identified as being critical for their inactivation. The generation of mutant STAT proteins that are constitutively tyrosine phosphorylated revealed that STAT-1 inactivation is dependent on an N-terminal region [13] and contrasts with inactivation of STAT-3 and STAT-5, which rely on a C-terminal region. Phosphatase binding sites are likely to localize to these regions, and consistent with this the N-terminal region of STAT-1 has been shown to bind a phosphatase [13]. The identity of the enzymes responsible for STAT dephosphorylation has been the subject of much study. Recent evidence suggests that PTP-1B [32] and TC-PTP [33] are both STAT phosphatases. The relative importance of each in the physiological dephosphorylation of the various STAT proteins remains to be determined.

PIAS (Protein Inhibitors of Activated STATS)

PIAS Family

The PIAS family is composed of five members: PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy [34]. PIAS proteins are greater than 50% homologous and contain several highly conserved regions, including a putative zinc binding motif and a highly acidic region. PIAS1 and PIAS3 have been shown to negatively regulate cytokine-activated STAT-1 and STAT-3, respectively [34,35]. The specificity of their action has been demonstrated by *in vitro* studies; PIAS1 co-immunoprecipiated with STAT-1 but not STAT-2 or STAT-3, blocked STAT-1 DNA binding in EMSA analysis, and inhibited STAT-1-mediated gene expression in luciferase reporter assays [35]. Similarly, PIAS3 interacts with STAT-3 but not STAT-1 and inhibits STAT-3- but not STAT-1-mediated gene expression [34]. More recently, PIASy has also been shown to inhibit STAT-1 activity [36].

The PIAS–STAT Interaction

A modified yeast two-hybrid system demonstrated that PIAS1 interacts specifically with STAT-1 dimer and cannot interact with STAT-1 monomer [37]. *In vitro* mutational studies have given important insights to the molecular basis of the PIAS1–STAT-1 interaction. In contrast to full length PIAS1, which fails to bind to STAT-1 monomer, the removal of the first 50 amino acid residues of PIAS1 is sufficient to allow the PIAS1–STAT-1 interaction to occur. Therefore, the N-terminal region of PIAS1 serves as a modulatory domain by preventing PIAS1 from interacting with the STAT-1 monomer. In the same study, the C-terminal region of PIAS1 (amino acids 392–541) was defined as being the STAT-1-binding domain [37].

A series of STAT-1 deletion mutants was also generated and used to define the precise region of STAT-1 capable of binding PIAS1. The N-terminal region of STAT-1 (amino acids 1–191) was shown to be the PIAS1-binding domain; however, the PIAS1 fragment used in the assay lacked the N-terminal modulatory region and was therefore capable of interacting with STAT-1 monomer. Consequently, whether this region also represents the physiological PIAS-binding domain in dimeric STAT-1 remains to be confirmed [37].

The *in vivo* PIAS1–STAT-1 interaction requires IFN stimulation. Although it is possible that PIAS1 may be modified by interferon (IFN) stimulation it has been shown that PIAS1 interacts specifically with STAT-1 dimer and cannot interact with phosphorylated or unphosphorylated STAT-1 monomer. This suggests that the critical IFN-stimulated event is the dimerization of STAT-1, which then allows PIAS binding.

The JAK/STAT pathway also operates in *Drosophila* [38,39]. A *Drosophila* PIAS homolog, dPIAS, was found to negatively regulate the STAT homolog, stat92E [40]. Similar to the PIAS1-STAT-1 interaction, the central domain of dPIAS was found to directly interact with stat92E. This observation is important, as it demonstrates the *in vivo* role PIAS family members play in the regulation of cytokine signaling. The generation of mice lacking the various PIAS family members may allow their contribution to the regulation of mammalian cytokine signaling to be assessed.

PIAS: Mechanisms of Action

Different PIAS proteins appear to inhibit STAT activity through distinct mechanisms. PIAS1 and PIAS3 prevent dimeric STAT-1 and STAT-3 binding of DNA [34,35]. In contrast, PIASy inhibits STAT-1-mediated gene activation without blocking the DNA binding ability of STAT-1. A conserved N-terminal LXXLL coregulator motif located in N-terminal region of PIASy is required for the transrepression

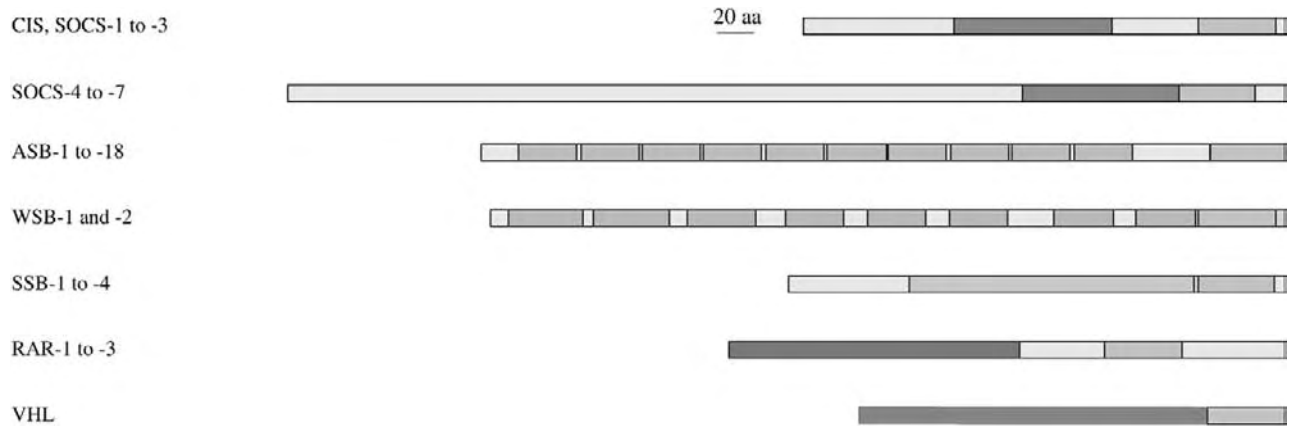


Figure 2 Schematic illustration of SOCS box containing proteins. The various families of SOCS-box-containing proteins are shown. The SOCS box is shown in orange; the SH2 domain of the canonical SOCS proteins (CIS and SOCS-1 through 7), in mauve; the ankyrin repeats of the ASB proteins, in green; the WD40 repeats of WSB1 and 2, in turquoise; the SPRY domain of the SSBs, in grey/blue; and the a domain of the VHL protein, in cerise. Non-conserved regions are shown in grey.

activity of PIASy [36]. It has therefore been proposed that PIASy may act as an adaptor protein to link STAT-1 to a transcriptional corepressor.

PIAS proteins have been shown to exert effects independent of cytokine signaling. PIAS1 shares characteristics of a nuclear scaffold attachment protein; it localizes to nuclei in a speckled pattern and can bind AT-rich, double-stranded DNA. DNA binding depends on a conserved N-terminal sequence (amino acids 11–45) referred to as the scaffold attachment factor (SAF) box [41], SAF-A/B, Acinus, or PIAS (SAP) domain [42]. Additionally, both PIAS1 and PIASxα have been shown to be capable of interacting with steroid receptors, which are ligand-inducible transcription factors [43]. The extent to which the PIAS family acts specifically to modulate cytokine signaling or more widely as regulators of transcription may be clarified by further analysis of *Drospohila* or mice harboring PIAS mutations.

SOCS (Suppressors of Cytokine Signaling) Family

Overview

As with many breakthroughs, three groups simultaneously discovered suppressor of cytokine signaling 1 (SOCS-1), also known as JAK binding protein (JAB) and STAT-inducible STAT inhibitor 1 (SSI-1), using three very different strategies: (1) SOCS-1 was cloned based on its ability to inhibit interleukin-6 (IL-6)-induced macrophage differentiation of the monocytic cell line, M1 [44]; (2) JAB was isolated in a yeast two-hybrid screen for JAK-2 binding proteins [45]; (3) SSI-1 was isolated by an antibody screen for proteins with homology to the SH2 domain of STAT-3 [46].

Database searches using the predicted SOCS-1 amino acid sequence showed it to be related to the previously characterized cytokine-inducible SH2-domain-containing protein (CIS) and identified an additional six SOCS family members (SOCS-2 through SOCS-7) [44–48], each of which contained an SH2 domain and a conserved 40-amino-acid motif, the SOCS box [44]. Further database mining using the highly conserved C-terminal SOCS box revealed that this motif is shared by an additional group of proteins [47–49]. Instead of an SH2 domain, these proteins have a different protein–protein interaction domain (Fig. 2), such as WD40 repeats (WSB proteins), SPRY domains (SSB), ankyrin repeats (ASB), or GTPase (RAR).

SOCS Proteins Are Part of a Negative Feedback Loop

Various *in vitro* studies showed that a range of cytokines can induce SOCS gene and protein expression, and when over-expressed SOCS proteins can inhibit cytokine signaling (Table 1) [50,51]. Taken together, these data suggest that SOCS proteins may act as part of a negative feedback loop to regulate cytokine signaling (Fig. 1D, Table 1). Because many cytokines act via the JAK/STAT pathway, SOCS proteins may also act to mediate cytokine cross-talk. An intriguing feature of certain cytokines is their ability to regulate the response of a cell to subsequent exposure to another cytokine. Although the mechanisms of cross-talk between cytokines are not well understood, recent *in vitro* studies have suggested the involvement of SOCS proteins such that, although SOCS expression can be induced by a specific cytokine, SOCS proteins may have the capacity to regulate multiple cytokine signaling cascades occurring within the same cell. For example, SOCS-1 expression may mediate IFNα-induced inhibition of thrombopoeitin (TPO) signaling [52] and IFNγ-induced inhibition of IL-4 signaling [53]. The evidence for the role of SOCS proteins in mediating cytokine cross-talk remains largely circumstantial and needs to be confirmed *in vivo* using models of specific SOCS protein deficiency.

Table I Specificity of SOCS Protein Production and Action

	Cytokines inducing expression	Cytokine signaling sensitive to inhibition
CIS	IL-2[100], IL-3[63], IL-6[44], IL-9[101], IL-10[102], GM-CSF[63], GH[103], PRL[100], TSLP[104], EGF[105], CNTF[106], leptin[107, 108], EPO[63], TPO[109]	IL-2[100], IL-3[63], GH[110], IGF-I[111], leptin[112], EPO[63]
SOCS-1	IL-2[113], IL-4[46], IL-6[44], IL-9[101], IL-10[102], G-CSF[46], GH[103], PRL[114], TSH[115], SCF[81], insulin[105], LIF[46], CT-1[116], CNTF[106], EPO[45], IFNα/β [52], IFNγ[117, 118]	IL-2[113], IL-3[45], IL-4[119], IL-6[44], IL-7[120], M-CSF[121], GH[103], IGF-I[111], PRL[114], TSLP[104], SCF[81], Flk ligand[81], insulin[122], LIF[46], OSM[44], CT-1[116], EPO[45], TPO[44], IFNα/β[117, 118], IFNγ[117, 118], TNFα[123]
SOCS-2	IL-2[47], IL-6[44], GH[103], PRL[114], insulin[105], CNTF[106]	GH[110], IGF-I[111], LIF[47]
SOCS-3	IL-1[124], IL-2[57], IL-3[125], IL-6[44], IL-9[101], IL-10[102], IL-11[126], IL-22[127], GH[103], PRL[114], TSH[115], EGF[128], insulin[105], PDGF[128], bFGF[129], LIF[130], OSM[125], CT-1[116], CNTF(106), leptin[112], EPO[59], TPO[52], TNFα[131], IFNγ[117, 118]	IL-1[132], IL-2[57], IL-3[57], IL-4[119], IL-6[60], IL-9[101], IL-11[126], GH[103], IGF-I[111], PRL[114], insulin[133], LIF[47], OSM[125], CT-1[116], CNTF[106], leptin[112], EPO[59], IFNα/β[117, 118], IFNγ[117, 118]

Abbreviations: IL, interleukin; GM-CSF, granulocyte–macrophage colony-stimulating factor; GH, growth hormone; PRL, prolactin; TSLP, thymic stromal lymphopoietin; EGF, epidermal growth factor; CNTF, ciliary neurotrophic factor; EPO, erythropoietin; TPO, thrombopoietin; IGF-I, insulin-like growth factor I; G-CSF, granulocyte colony-stimulating factor; TSH, thyrotropin; SCF, stem cell factor; LIF, leukemia inhibitory factor; CT-1, cardiotrophin-1; IFN, interferon; M-CSF, macrophage colony-stimulating factor; OSM, oncostatin M; TNFα, tumor necrosis factor α; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor.

Mechanisms of Action

There is good evidence that SOCS proteins are produced as a result of JAK/STAT signaling and that once produced they can inhibit JAK/STAT signaling. Biochemical analyses over the past few years suggest, as with PIAS proteins, that the different SOCS family members may act in distinct ways: (1) SOCS-1 binds via its SH2 domain to phosphorylated tyrosine residues contained within the activation loop of JAKs and inactivates JAK activity via an N-terminal motif known as the kinase inhibitory region (KIR), which may act as a pseudosubstrate [54–56]; (2) SOCS-3 interacts with phosphorylated tyrosines in the cytoplasmic domain of the various cytokine receptor components and may inhibit JAK activity via its KIR [55–61]; and (3) CIS, like SOCS-3, binds to phosphorylated tyrosine residues within the cytoplasmic domain of receptors and inhibits their signaling by competing with STATs for binding sites [62,63].

Another important component of the ability of SOCS proteins to inhibit cytokine signaling may involve the conserved C-terminal SOCS box. Interaction studies have shown that the SOCS box can interact with a complex of elongins B and C [64,65]. The SOCS box has structural and functional parallels with the F box [49,66,67]. The F box and the SOCS box both couple specific protein–protein interaction domains with generic components of the ubiquitination machinery. The F box complex (SCF) is composed of Skp2, Cullin-1, and an F-box-containing protein, as well as the RING finger protein Rbx/Roc-1 [68]. The SOCS box complex (ECS) is composed of Elongin B/C, Cullin-2 or -5 and a SOCS-box-containing protein, as well as Rbx/Roc-1 [49]. The SCF and ECS complexes are E3 ubiquitin ligases that, with E1 and E2 ubiquitin ligase subunits, mediate poly-ubiquitination of proteins bound to the SOCS-box- or F-box-containing protein. The von Hippel-Lindau (VHL) tumor suppressor protein also contains a SOCS box and has been shown to poly-ubiquitinate the transcription factor hypoxia-inducible factor 1 (HIF-1), leading to its degradation [69–74]. In the case of SOCS-1, there is also good evidence that JAKs are bound by the SH2 domain, are poly-ubiquitinated in a SOCS-1-dependent manner, and are degraded by the proteasome [75–77]. Moreover analysis of mice in which only the DNA encoding the SOCS box has been deleted supports the notion that this motif plays an important physiological role in attenuating signaling. If targeting proteins for proteasomal degradation proves a general mechanism by which SOCS-box-containing proteins act, then it is important to determine which proteins interact with the SH2 domains in other SOCS proteins and the ankyrin repeats, SPRY domains, and WD40 repeats in ASB, SSB, and WSB proteins.

In vitro over-expression studies have also demonstrated that deletion of the SOCS box results in a shorter half-life for SOCS-1 and the VHL protein, leading to the suggestion that a major role for the interaction of the SOCS box with elongins B and C is to stabilize the SOCS protein [65,78]. The reduced half-life of SOCS proteins lacking a SOCS box might be incidental to the primary role of the SOCS box in linking specific substrate recognition with generic components of the ubiquitin ligase complex. Indeed, this increased lability might reflect the degradation of individual proteins that normally form part of a multi-subunit complex, a phenomenon that occurs frequently in the endoplasmic reticulum [79]. It has also been suggested that phosphorylation of SOCS-1 by Pim kinases prolongs the half-life of the SOCS-1 protein [80]. This may provide a mechanism by which the inhibitory effect of SOCS-1 on JAK/STAT activation can be extended.

The regulatory actions of the SOCS proteins may not be restricted to JAK/STAT signaling. In over-expression systems,

SOCS-1 has been shown to interact with KIT and FLT3 receptors, FGF receptor, TEC, VAV, GRB2, and PYK2 [45,81–83]. Similarly, SOCS-2 has been shown to interact with IGF-1 receptor [84], and SOCS-3 has been found to associate with IGF-1 receptor, LCK, the FGF receptor, and PYK2 [85]. The physiological relevance of these *in vitro* observations requires confirmation using primary cells and study of animals lacking one or more of the SOCS genes.

The *In Vivo Role* of SOCS: SOCS Knockout Mice

The apparent promiscuity of SOCS induction and action *in vitro* may be the result of the expression of these proteins in a temporally inappropriate manner and at excessive levels. For this reason, SOCS knockout mice were generated to determine the biologically relevant functions of the SOCS proteins. SOCS-1 knockout mice survive embryogenesis and are born at the expected Mendelian frequency but die neonatally of a severe inflammatory disease that is characterized by monocyte infiltration of several lymphoid organs, abnormal T-cell activation, and fatty degeneration of the liver [86,87]. The SOCS-1 knockout disease is reminiscent of that induced by the administration of IFNγ to neonatal mice [88–90]. Subsequently, IFNγ levels were discovered to be elevated in the serum of SOCS-1 knockout mice [91], and these animals are hyper-responsive to IFNγ [92].

Consistent with the central role that SOCS-1 plays in regulating IFNγ action, administration of neutralizing anti-IFNγ antibodies was shown to delay the disease observed in SOCS-1 deficient mice [93]. Likewise, SOCS-1/IFNγ double-knockout mice survive until adulthood and appear healthy [93–95]. however, SOCS-1/IFNγ double-knockout mice are not completely normal. Inflammatory infiltrates are detectable in several organs, abnormal numbers of activated T cells are present, and the mice die prematurely in their second year of life from a variety of chronic inflammatory diseases [95]. These results suggest that other cytokines may be deregulated in the absence of SOCS-1.

SOCS-2-deficient mice and high growth (hg/hg) mice, which harbor a naturally occurring deletion of the SOCS-2 locus, both exhibit enhanced growth as young adults. This phenotype is typical of an increased response to growth hormone and/or insulin-like growth factor 1 (IGF-1) [96,97]. SOCS-3-deficient mice die embryonically of placental insufficiency [98] and have also been reported to exhibit excessive erythropoiesis, which has been postulated to be caused by an enhanced response to erythropoietin [99].

Concluding Comments

At least three families of proteins act to attenuate cytokine signal transduction: phosphatases, PIAS, and SOCS. These proteins act in fundamentally different ways. Phosphatases and PIAS are present constitutively and are capable of inhibiting signaling in an acute manner. These proteins might be expected to act as buffers to determine the magnitude of an initial response. In contrast, SOCS proteins are, in general, produced as a consequence of signal transduction, some hours after cells are exposed to cytokines. SOCS proteins therefore appear to regulate the duration and perhaps steady-state level of signaling, rather than the magnitude of the initial response.

Despite some progress in understanding how these individual components function, little is currently known of how they act in concert to regulate the intensity and duration of signal transduction in response to acute or chronic cytokine stimulation. Even more tenuous is our grasp of how a cell can integrate signals from several cytokines, each of which stimulates a signaling pathway that contains shared elements (JAKs and STATs) and which is tempered by common negative regulatory proteins (phosphatases, PIAS, and SOCS protein). The reconstruction and reconstitution of such complex situations are key challenges of research in the next decade.

Acknowledgments

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CHAPTER 76

Activation of Oncogenic Protein Kinases

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Introduction

The evolution of a tumor cell requires multiple genetic and epigenetic alterations. Some of these changes result in activation of protooncogenes, while others result in inactivation or loss of tumor suppressor genes [1]. The progressive evolution to malignancy is probably facilitated by the genetic instability that appears to be an intrinsic characteristic of cancer cells [2,3]. The end result is that tumor cells acquire a set of characteristic properties that confer upon them a selective growth advantage [4]. These properties include the ability to proliferate in the presence of inhibitory signals that restrict the growth of normal cells and the ability to proliferate in the absence of signals upon which normal cells are dependent, such as growth factors and attachment to the extracellular matrix. Also critical for tumor growth is the ability to evade processes that normally limit cell proliferation, such as terminal differentiation, programmed cell death (apoptosis), and telomere erosion and replicative senescence. Finally, the growth of solid tumors is limited by the supply of nutrients and room to expand, and in order to continue proliferation solid tumors must elicit a vascular supply (tumor angiogenesis) and then acquire the ability to invade surrounding tissues and migrate to distant sites (metastasis). Tumor progression is thus a Darwinian process in which mutations accumulate and progressively confer upon the evolving cancer cell an ever greater proliferative advantage [4].

All of these changes involve perversions of normal regulatory mechanisms. In normal embryos or adult tissues, complex regulatory mechanisms control cellular proliferation, differentiation, movement, function, and survival. As described elsewhere in this volume, many of these regulatory mechanisms involve protein kinases that function either as receptors transmitting signals across the plasma membrane, or as relays transmitting signals from the plasma membrane to effector proteins in the cytoplasm or within the nucleus. Protein kinases are themselves complex molecular machines that are normally restrained and regulated by autoinhibitory mechanisms or by interactions with *trans*-acting inhibitors [5]. Inactivation of these autoinhibitory constraints, whether by mutational or nonmutational events, results in constitutive activation of kinase activity. Given the central role that protein kinases play in cellular regulation, and the fact that they can be constitutively activated by loss of autoinhibitory regulation, it is (at least in hindsight) no surprise that activation of protein kinases plays a central role in cancer [6]. In this chapter, we review the autoinhibitory mechanisms involved in the regulation of protein kinases and then describe the mechanisms that result in their activation in tumor cells. Finally we discuss the exciting prospect that protein kinase inhibitors will be useful therapeutic agents for the treatment of cancer, an issue that will be discussed in greater detail in the following chapter.

Physiological Regulation of Protein Kinases

As described elsewhere in this volume (see Chapter 67), the catalytic domain of eukaryotic tyrosine and serine/threonine kinases is a conserved structure in which the nucleotide-binding and catalytic sites lie within a cleft between two lobes [7,8] (see Fig. 1). All protein kinases can adopt at least two conformations, an active or "on" state and an inactive or "off" state. Tolstoy wrote in *Anna Karenina* that,

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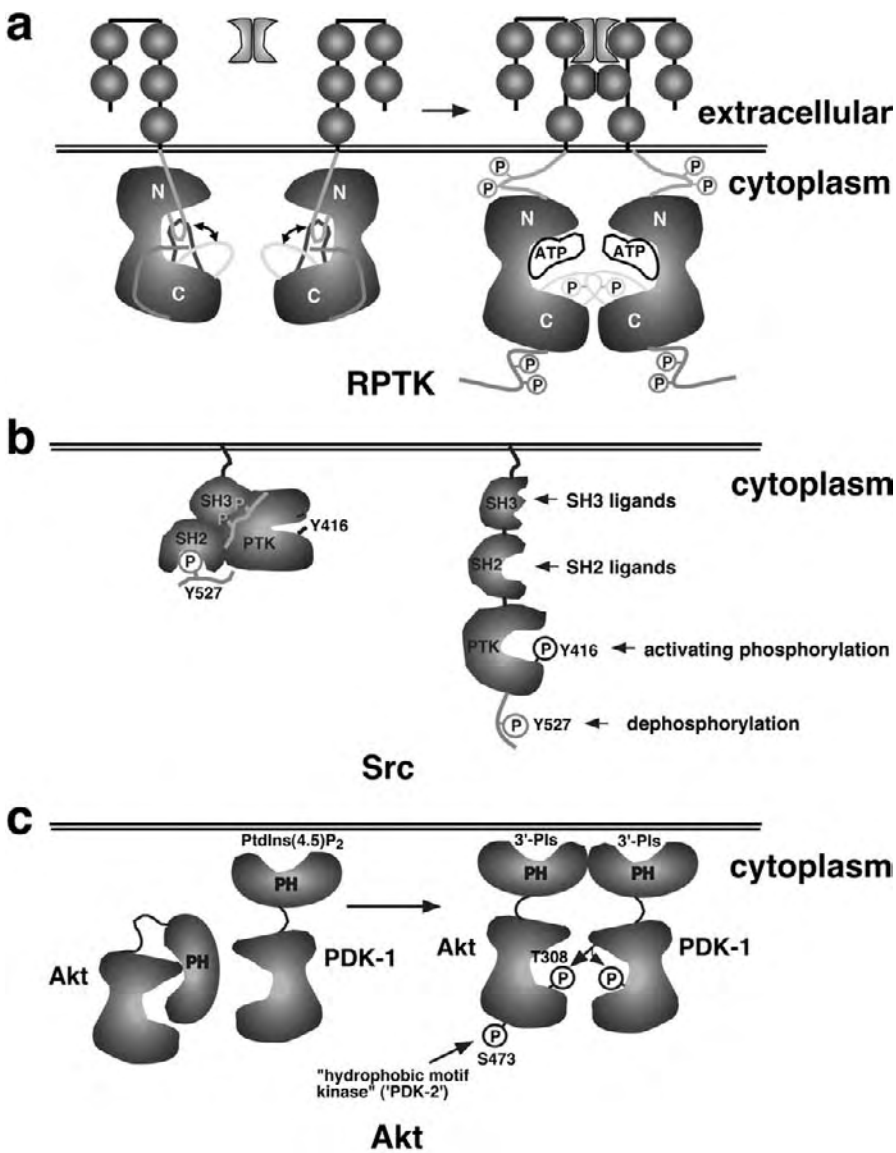


Figure 1 Regulation of protein kinases. (a) Regulation of receptor tyrosine kinases. *Left*, the inactive monomeric state; the activation loop is in equilibrium between conformations that either exclude or allow substrate access. The juxtamembrane and C-terminal regions may also inhibit activity through interactions with the N-lobe or the active site. *Right*, the active dimeric state; ligand-induced dimerization results in tyrosine autophosphorylation and relief from auto-inhibition by the activation loop and juxtamembrane and C-terminal regions. (b) Regulation of c-Src activity. *Left*, the inactive "closed" conformation; phosphotyrosine 527 (chicken c-Src) interacts with the SH2 domain, positioning the SH3 domain to interact with a polyproline type II helix induced in the linker between the SH2 and catalytic domains. The linker region and the SH3 domain in turn interact with the surface of the N-lobe and stabilize the αC helix in an inactive conformation. *Right*, the active "open" conformation; activation results from binding of ligands to the SH2 and/or SH3 domain, dephosphorylation of phosphotyrosine 527, and phosphorylation of Tyr 416 in the activation loop. (c) Regulation of Akt by phosphatidylinositol(PI)-3-kinase. *Left*, the inactive conformation; the N-terminal PH domain of Akt does not interact with membrane lipids and blocks activation by PDK1. *Right*, the PH domain of Akt binds to 3′-phosphoinositides generated by PI 3-kinase. This allows PDK1 to phosphorylate the activation loop of Akt at Thr 308. (From Blume-Jensen, P. and Hunter, T., *Nature*, 411, 355–365, 2001. With permission.)

"Happy families are all alike; every unhappy family is unhappy in its own way." Similarly, the conformations of active protein kinases, which must all catalyze the same reaction, are very similar, but nature has evolved many different ways to inactivate kinase activity [5].

X-ray crystallographic studies on the active and inactive forms of protein kinases have revealed conserved regulatory and catalytic elements. The N-terminal lobe (the "N-lobe" or "small lobe") is primarily a β-sheet structure. However the N-lobe contains an α-helical segment, the αC helix,

which contains a conserved glutamate residue that plays a critical role in regulation. In the active conformation this glutamate residue ion-pairs with and positions a conserved lysine in the N-lobe, that in turn functions to position the α and β phosphates of the bound ATP. The C-terminal lobe (the "C-lobe" or "large-lobe"), which is primarily α-helical, contains the residues that bind substrate and segments critical for catalysis. The catalytic loop at the base of the active site cleft contains an aspartate residue, which (in its nonprotonated form) acts as a catalytic base. At the front of the active site cleft the C-lobe bears a loop termed the *activation loop*. These regulatory elements form an interacting network, so that perturbations of any one element are propagated throughout the structure.

Movements of the activation loop and the αC helix are central elements in the regulation of protein kinases [5]. In the inactive conformation, the activation loop collapses into the active site cleft and inhibits catalytic activity, generally by occluding the access of substrate and/or nucleotide. In the active state, the loop adopts an open extended conformation that permits substrate binding. Activation generally requires phosphorylation of a residue or residues within the activation loop. The phosphorylated residues in the activation loop interact with residues in the C-lobe that position the catalytic aspartate. The phosphorylated residues in the activation loop also interact with the αC helix in the N-lobe, positioning it so that the glutamate residue can ion-pair with the lysine that anchors the α and β phosphates of adenosine triphosphate (ATP). Phosphorylation of the activation loop may be brought about by an intermolecular autophosphorylation. This is the case with the receptor-tyrosine kinases, which are activated by ligand-induced dimerization or oligomerization and/or by a ligand-induced conformational change [9,10] (Fig. 1a); the collapsed conformation of the unphosphorylated activation loop is in equilibrium with the open extended conformation, allowing intermolecular autophosphorylation to occur [11]. Alternatively, phosphorylation of the activation loop may be brought about by an upstream activating kinase, as in the case of members of the MAP kinase family that are activated by activation loop phosphorylation by MAP kinase kinases [12]. Autoinhibition by the activation loop and its relief by phosphorylation represent a conserved mechanism for the regulation of kinase activity.

Superimposed on the regulatory machinery intrinsic to the catalytic domain are a variety of autoinhibitory mechanisms that rely on regions of the molecule outside the catalytic domain itself. In the case of certain receptor tyrosine kinases, such as PDGF-R, c-Kit, CSF1-R, Eph-R, and the insulin receptor, juxtamembrane segments of the receptor can act as negative regulators of catalytic activity by binding to the N-terminal lobe and thus affecting the positioning of the αC helix (Fig. 1a) [13]. This autoinhibition is relieved by phosphorylation of tyrosine residues within the juxtamembrane segment, which results in both dissociation of the juxtamembrane segment from the catalytic domain and the formation of docking sites for SH2-containing signaling molecules. In some receptor tyrosine kinases, the C-terminal segment of the molecule can similarly act as an intrasteric regulator by folding back upon and occluding the active site; again, autophosphorylation of this segment causes dissociation of the inhibitory segment and generation of a phosphotyrosine SH2 docking site (Fig. 1a) [14].

The non-receptor tyrosine kinases provide further examples of autoinhibitory mechanisms that depend on regulatory segments outside the catalytic domains. The mechanism of this inhibition has been characterized in greatest detail for Src and related Src family kinases. These kinases contain SH3 and SH2 domains N-terminal to the catalytic domain and a short regulatory sequence at the C-terminus containing a critical phosphotyrosine residue (Fig. 1b). In the inactive or "closed" state, the SH2 and SH3 domains are located at the back of the kinase domain, positioned there by an interaction between the SH2 domain and the C-terminal phosphotyrosine residue [15,16]. The SH3 domain is thus positioned to interact with the linker between the SH2 domain and the N-lobe of the catalytic domain. The linker region and the SH3 domain in turn interact with the surface of the N-lobe and stabilize the αC helix in an inactive conformation. The interaction between the αC helix and the activation loop stabilizes the activation loop in the "off" conformation that occludes substrate access [17,18]. Activation of Src can thus be achieved by disturbing this network of inhibitory interactions (Fig. 1b). Activation can occur either by dephosphorylation of the C-terminal phosphotyrosine [19] or by interaction of Src with SH2 ligands, such as autophosphorylated receptors [20], or with SH3 ligands, such as PxxP motifs in substrate proteins [21]. Full activation is dependent on phosphorylation of a tyrosine residue in the activation loop.

Intramolecular autoinhibitory mechanisms of this type are not restricted to receptor and non-receptor tyrosine kinases. Many serine/threonine kinases are regulated by active site occlusion by pseudosubstrate sequences [22]. The Raf group of serine/threonine kinases contains two conserved regions (CR1 and CR2) in a regulatory segment N-terminal to the kinase domain; deletion of these regions activates kinase activity [23].

Finally, it should be noted that protein kinases are also regulated by intermolecular interactions with activators or inhibitors. For example, cyclin-dependent kinases are activated by binding of a cyclin molecule. Cyclin binding reorients the αC helix, causes the activation loop to adopt a position accessible to phosphorylation by an activating kinase, and reorients the ATP binding site to allow a productive binding geometry [24,25]. Cyclin-dependent kinases are also regulated by CDK inhibitors such as $p21^{WAF\text{-}1/CIP\text{-}1}$, $p27^{KIP\text{-}1}$, $p16^{INK4a}$, and $p15^{INK4b}$. The INK4 inhibitors bind to both the N- and C-lobe of the cyclin-dependent kinases Cdk4 and Cdk6, distorting the ATP-binding site, reorienting the αC helix, and holding the activation loop in a position that blocks ATP and substrate binding [26,27].

In summary, protein kinases are regulated by a variety of intramolecular autoinhibitory mechanisms. Inhibition by the

nonphosphorylated activation loop within the catalytic domain represents a conserved autoinhibitory mechanism. In addition, kinase activity may be inhibited by segments of the kinase outside the catalytic domain, such as the juxtamembrane region of certain receptor tyrosine kinases, the SH2 and SH3 domains of Src family kinases, and the CR1 and CR2 regions of Raf. Physiological regulators act by modulating these autoinhibitory mechanisms. As we shall now discuss, loss of these autoinhibitory mechanisms is responsible for the activation of oncogenic protein kinases.

Activation of Protein Kinases by Retroviruses

Retroviral oncogenesis, while very rare in humans, is common in avian and mammalian models. The most common mechanism is insertional mutagenesis, in which the provirus integrates into, or adjacent to, a protooncogene [28]. The insertion is believed to result from random integration and is followed by selective growth of cells in which the protooncogene has been activated. Activation of the protooncogene can involve transcriptional promotion or enhancement by the retroviral long terminal repeat and/or structural changes in the product of the protooncogene due to the proviral insertion. The classical example of insertional activation of a protein kinase is the activation of the c-*erbB* gene, encoding the EGF receptor, in line 15_1 chickens infected by an avian leukosis virus [29–32]. In these birds, an erythroleukemia is induced as a result of insertion of the provirus into the first intron of the c-*erbB* gene and splicing of the *gag*-leader into the second exon, resulting in the formation of *gag–erbB* or *gag–env–erbB* fusions that contain the transmembrane and kinase domains of the EGF receptor but lack the ligand binding domain. The resulting insertionally activated EGF receptor can induce erythroleukemia when expressed in a retroviral vector [33]. Activation of transforming capacity appears to involve constitutive dimerization resulting from loss of the ligand binding domain.

A much rarer type of retroviral oncogenesis involves incorporation of part or all of the protooncogene into the retroviral genome, a process referred to as *retroviral transduction*. This appears to occur as a result of multistep recombination between an insertionally activated oncogene and the adjacent provirus. The resulting retrovirus is rapidly transforming and can induce tumors with a very short latency upon injection into a susceptible host. In the case of the line 15_1 chickens mentioned above, the process of retroviral transduction is unusually frequent [29,30]. In approximately 50% of line 15_1 chickens infected with an avian leukosis virus, rapidly transforming erythroleukemia viruses are generated. The high frequency of this event probably results from the fact that the activating proviruses are complete and have not sustained the deletions generally observed in other instances of insertional mutagenesis. The new erythroleukemia viruses encode *gag–erbB* fusions in which the ligand binding domain of the EGF receptor has been deleted and induce a rapid erythroleukemia. In some cases, the erythroleukemia viruses encode an EGF receptor that has also sustained a deletion within the C-terminal segment and/or point mutations within the catalytic domain, and these viruses can induce not only erythroleukemia but also sarcomas [34]. This indicates that additional mutations can induce further activation of transforming capacity.

There are many examples of retrovirally transduced protein kinases, including receptor tyrosine kinases, such as v-Fms and v-Ros, non-receptor tyrosine kinases such as v-Src and v-Abl, and serine/threonine kinases such as v-Raf and v-Mos. The mechanism of activation has been described in greatest detail for the v-Src protein encoded by Rous sarcoma virus. In this protein, the C-terminal 19 amino acids of c-Src have been substituted by 12 amino acids encoded by a sequence downstream from the c-Src gene, deleting the C-terminal phosphotyrosine that anchors the c-Src SH2 domain [35]. In addition, the v-Src gene has sustained activating mutations within the SH3 domain, decreasing the ability of the SH3 domain to interact with the SH2-catalytic domain linker, plus an activating mutation within the catalytic domain. Similarly v-Abl and v-Raf have been activated by deletion or substitution of autoinhibitory segments, the N-terminal "cap" and the SH3 domain in the case of v-Abl [36,37], and the CR1 and CR2 domains in the case of v-Raf [23]. In a few instances where retroviral structural proteins are fused to the activated kinases these retroviral sequences may supply functions necessary for transformation. For example, the myristoylation site in Gag is required for membrane attachment of v-Abl and v-Fgr and for transformation of 3T3 fibroblasts by these kinases [38,39], while additional Gag sequences within v-Abl stabilize the protein in lymphoid cells [40].

Activation of Protein Kinases in Human Cancer

Activation by Chromosomal Translocations

Chromosomal translocations can activate protooncogenes, either by linking the protooncogene to a more powerful or inappropriately regulated promoter or by generating a gene fusion linking the protooncogene product to a heterologous protein. In the latter case, activation can occur either by deletion of an autoinhibitory domain or because the fusion partner provides an activating function, such as dimerization or localization to a novel intracellular compartment. Fusions of receptor or non-receptor tyrosine kinases to heterologous proteins are observed in a variety of cancers and leukemias. Examples include the Tel–PDGFRβ fusion in chronic myelomonocytic leukemia [41], the ZNF198–FGFR1 fusion in certain forms of acute myelogenous leukemia [42], and a variety of fusions involving TrkA, Met, or Ret in papillary thyroid carcinomas [43] (for a complete listing, see Table 1 in Blume-Jensen and Hunter [6]).

The classic example of protein kinase activation by translocation is the generation of Bcr–Abl, which is responsible for the induction of chronic myelogenous leukemia (CML).

In this disease, a reciprocal translocation occurs that results in the fusion of the *abl* protooncogene on chromosome 9 to the breakpoint cluster region (*bcr*) gene on chromosome 22. The resulting fusion protein, Bcr–Abl, is activated by several mechanisms. First, the Bcr coiled-coil domain supplies an oligomerization function [44]. Second, the Bcr sequence retains the Bcr–Abl protein in the cytoplasm, promoting the activation of oncogenic signaling pathways [37] and inhibiting the proapoptotic activity that Abl exerts in the nucleus [45]. Third, the fusion deletes the N-terminal 80 residues of c-Abl, which function as an autoinhibitory "cap" [36]. Finally, the Bcr-sequences contain a tyrosine residue that is subject to phosphorylation by the Abl kinase, generating a phosphotyrosine residue that binds the adaptor Grb2, leading to the activation of Ras [46]. The Bcr–Abl fusion protein is of particular interest because it is a target of the drug Gleevec™ (STI571) (see later discussion).

Activation by Gene Amplification and Overexpression

Because protein kinases can be activated by intermolecular autophosphorylation, an increase in kinase expression can lead to increased activity. Although overexpression can occur without any apparent genetic change, a frequent underlying mechanism is gene amplification. Amplification is often associated with complex chromosomal rearrangements and is manifested by the presence of chromosomal abnormalities, either double-minute chromosomes or homogeneously staining regions. The mechanisms by which these amplified and rearranged segments are generated are not entirely clear and may involve unequal sister chromatid exchange, extrachromosomal amplification and reintegration, localized overreplication, and breakage–fusion–bridge cycles [47]. The process appears to be initiated by DNA double-strand breaks that have escaped repair by the cellular enzymatic repair machinery. In cells that retain p53 function, these events would lead to apoptosis, but in tumor cells deficient in p53 these lesions are not eliminated. In one mouse model system it has been shown that the recombinogenic unrepaired chromosome ends associate into dicentric intermediates, which in turn lead to cycles of breakage–fusion–bridge events, generating amplified and rearranged segments [48].

Amplification of members of the EGFR family, in particular EGFR/ErbB1 and HER2/ErbB2/Neu, is common in mammary carcinomas and other types of cancer, while amplification of both EGFR and PDGFRα occurs in some gliomas and glioblastomas [6,49,50]. Amplification of the *bcr-abl* locus can also occur in response to treatment with Gleevec and is associated with resistance to the drug. Because amplification frequently also results in rearrangements, the products of the amplified genes may also be altered. For example in gliomas there is a common variant form of the amplified EGFR, termed vIII, in which DNA rearrangements and alternative splicing have resulted in the deletion of exons 2 to 7, encoding residues 6 to 276 in the extracellular domain; this yields an EGFR that is ligand independent and constitutively activated and which displays enhanced tumorigenicity [51].

Activation of Protein Kinases by Mutation

The mutations found in tumor cells may arise somatically or can be transmitted as germline mutations that predispose to cancer. It has been argued that the genetic instability characteristic of tumor cells may involve not only an enhanced rate of generation of chromosome abnormalities (translocations, amplifications) but also a mutator phenotype [52]. A subset of tumors are initiated by defects in DNA repair that lead to an increased mutation rate [2]. In addition, many carcinogens appear to act as mutagens; these include both exogenous carcinogens in the diet and endogenous carcinogens such as reactive oxygen species [53].

In the case of receptor tyrosine kinases, mutations can occur within the extracellular domain, the transmembrane domain, the intracellular juxtamembrane region, the catalytic domain, or the C-terminal tail. Deletions or mutations within the extracellular domain can lead to ligand-independent activation, as in the case of the EGFR in gliomas as mentioned above. Another example is provided by the Ret tyrosine kinase, a component of the receptor for neurotrophins of the glial-derived neutrophic factor (GDNF) family: germline Ret mutations in the extracellular domain are the cause of multiple endocrine neoplasia type 2A (MEN2A) [54,55]. These mutations substitute conserved cysteines in the extracellular domain of Ret that are believed to form intramolecular disulfide bonds in the wild-type receptor. These mutations result in an unpaired cysteine, which then forms an activating intermolecular bridge. In rat neuro- and glioblastomas, activating point mutations arise in the transmembrane segment of Neu/ErbB2 (the rodent ortholog of HER2) [56], but similar mutations have not been identified in human cancers. Activating mutations can also occur in the inhibitory juxtamembrane region. Thus, point mutations in the juxtamembrane region of Kit, the stem cell factor receptor, are implicated in gastrointestinal stromal tumors [57,58], while internal duplications in this region of Flt3 occur in acute myelogenous leukemias [59,60]. Mutations within the catalytic domain close to the activation loop can prevent autoinhibition by the activation loop and thus lead to constitutive activity. These types of mutations occur in Ret in multiple endocrine neoplasia type 2B (MEN2B) [55], in Kit in mast cell/myeloid leukemias [61,62] and seminomas [63], and in Met in papillary renal carcinomas [64,65]. The predominant Ret mutation in MEN2B, Met918Thr, is of particular interest because in addition to activating catalytic activity it also alters peptide substrate specificity. Wild-type Ret has the substrate specificity characteristic of receptor tyrosine kinases (which generally have Met at this position), while the mutant Ret has the substrate specificity characteristic of non-receptor tyrosine kinases (which generally have Thr at this position) [54,55,66,67]. Finally, truncations of the C-terminal autoinhibitory segments also occur, as in the case of FGFR2 (K-SAM) in gastric carcinomas [68,69].

Activation of non-receptor tyrosine kinases similarly occurs by mutation in autoinhibitory domains. c-Src is truncated in a fraction of metastatic colon cancers by a point mutation that generates a Stop codon close to the C-terminus, thus deleting the regulatory phosphotyrosine [70]. Mutational activation of Ser/Thr kinases also occurs. For example, the B-Raf protein, a MAP kinase kinase kinase, is mutant in 66% of human melanomas and at a lower frequency in a wide range of human cancers. The most common mutation is a Val to Glu substitution at residue 599 in the activation loop, immediately adjacent to the serine residue that is phosphorylated when B-Raf is activated; the V559E mutation probably activates by mimicking this regulatory phosphorylation [71].

Activation by Mutation of Upstream Regulators

Activation of protein kinases may occur not only as a result of direct mutational effects on the kinases or alterations in their level of expression, but also because of mutations in upstream regulators. This is too large a topic to review here, so two examples must suffice.

Activation of the small GTPase Ras occurs in some 30% of human cancers and results from mutational inactivation of its autoinhibitory GTPase activity. Mutational activation of Ras leads to activation of the Raf/MEK/MAPK cascade. Ras activation also leads to activation of phosphatidylinositol (PI) 3-kinases, which are lipid kinases that generate 3′-phosphoinositides and thereby activate 3′-phosphoinositide-regulated kinases such as PDK1 and Akt (Fig. 1c) [72]. Both Akt and the catalytic subunit of class IA PI 3-kinase are transforming when incorporated into retroviral genomes [73,74]. An increase in 3′-phosphoinositide levels and activation of Akt can also result from mutational inactivation of the tumor suppressor PTEN, a 3′-phosphoinositide phosphatase; PTEN mutations are observed in breast cancers, glioblastomas, and germ cell tumors [75]. Another event leading to activation of Akt is mutation and/or amplification of either the regulatory or the catalytic subunit of PI 3-kinase [76,77].

The cyclin-dependent kinase Cdk4 is an important regulator of the cell cycle, controlling the transition through the G1 restriction point by phosphorylating and inactivating the tumor suppressor Rb. Its activity is dependent on binding to cyclin D1, a protooncogene product overexpressed in many different types of cancer; for example, in a substantial fraction of mantle cell lymphomas expression of cyclin D1 is activated by a translocation involving the cyclin D1 locus (*CCND1*) [78]. Cdk4 is also inhibited by the cyclin-dependent kinase inhibitor INK4a. The locus encoding INK4a (which also encodes p14ARF) is frequently mutated in human cancers. Thus, INK4a is a tumor suppressor that acts by inhibiting the activity of a protein kinase [79,80]. Interestingly, mutations in the *CDK4* gene itself are also observed, particularly in hereditary and sporadic melanomas, although they are much less common than loss of INK4a; these mutants encode a Cdk4 protein that is resistant to inhibition by INK4a [81–83].

Oncogenic Protein Kinases as Targets for Therapy

Because of their prominent role in tumor progression, protein kinases are promising targets for therapy. Many kinase inhibitors are at various stages of development, but two types are of particular interest. One strategy for inhibiting receptor tyrosine kinase function is the use of anti-receptor antibodies that recognize the ectodomain of the receptor, block ligand binding, and induce receptor endocytosis. Herceptin™ (trastuzumab), a humanized version of a mouse monoclonal antibody against HER2, inhibits the growth of breast cancer cells that overexpress HER2 [84] and has been approved for the treatment of breast cancer patients with HER2-positive tumors. This antibody may act by promoting the c-Cbl-dependent polyubiquitination and degradation of the receptor [85]. A chimeric humanized version of a mouse monoclonal antibody against the EGF receptor (Erbitux™, formerly known as IMC-C225 or Cetuximab) has also been reported to give positive results in clinical trials, but these findings have been questioned, and this antibody has not yet received FDA approval.

The other class of drugs under development are small molecule inhibitors that inhibit kinase function. A few inhibitors have been developed that block SH2 domain–phosphotyrosyl ligand binding, thus inhibiting substrate recognition by non-receptor tyrosine kinases. However, most inhibitors developed are those that target the ATP binding site in the catalytic domain. In the case of tyrosine kinases, these inhibitors include a variety of flavone and isoflavone natural products such as quercitin and genistein, quinazolines and pyridopyrimidines, phenylamino-pyri midines, benzylidene malonitriles (tyrphostins), and indoles and oxindoles (reviewed in Al-Obeidi and Lam [86]). Two anti-EGFR inhibitors, OSI-774 (Tarceva™) and ZD1839 (Iressa™), have given positive results in clinical trials.

Of these inhibitors the most spectacularly successful has been the phenylamino-pyrimidine derivative Gleevec™ (or Glivec in the U.K.), otherwise known as STI-571, CGP57148, or Imatinib [87]. This compound was derived from a lead phenylamino-pyrimidine compound by a series of chemical optimization steps that increased activity against tyrosine kinases and increased solubility and bioavailability. The compound efficiently inhibits ($IC_{50} \approx 0.1$ to 0.5 μM) the activity of Bcr–Abl, c-Kit, PDGFR, and the Abl-related kinase Arg but does not inhibit ($IC_{50} > 10$ μM) other tyrosine kinases such as Src, EGFR, insulin and IGF-1 receptors, and Fms. Gleevec inhibits Bcr–Abl by binding with high affinity to, and thus stabilizing the inactive form of the kinase [88]. Its specificity results from the fact that the structure of the inactive form of Abl differs from that of Src and most other tyrosine kinases, in particular in the conformation of the nonphosphorylated activation loop when collapsed into the active site cleft. Gleevec is effective in the treatment of chronic myelogenous leukemia when administered in the chronic phase, increasing progression-free survival. When administered in CML blast crisis, most patients develop resistance, primarily due to amplification of

the *bcr-abl* gene and mutations in the Bcr–Abl kinase domain that render the catalytic activity less sensitive to the inhibitor [89]. Gleevec is also effective in the treatment of c-Kit-positive gastrointestinal stromal tumors, in which as noted earlier, oncogenic mutations in c-*kit* occur in the extracellular domain, the juxtamembrane domain, or the catalytic domain. Gleevec may also prove to be effective in treatment of chronic myeloproliferative disorders with Tel-PDGFRβ fusions, and in targeting PDGF-dependent cells in the tumor stromal microenvironment.

The success of Gleevec as an anticancer drug is particularly encouraging because it suggests that the diversity of inactivation mechanisms in oncogenic protein kinases can be exploited for the development of specific inhibitors. Herceptin and Gleevec provide the first two examples of rationally developed anticancer drugs. It is to be hoped that the next few years will see the development of further successful anticancer drugs that target activated protein kinases.

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CHAPTER 77

Protein Kinase Inhibitors

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Signal Transduction Therapy

The realization that the cancer cell differs from the normal cell in its aberrant signal transduction has given impetus to cancer researchers targeting them for therapy. The altered signal transduction network in cancer cells allows them to utilize their normal environment to their advantage without obeying the network of signals that regulate the normal cell [1]. Cancer cells sprout due to mutations in their growth signaling pathways. These mutations induce stress, which the mutated cells are able to evade due to further mutations that enhance survival signals to overcome the cellular stress. These two sets of mutations enhance the proliferation of tumor cells, which resist apoptotic messages. During its evolution, the cancer cell becomes highly dependent on this abnormal signaling network. Because of the many mutations that accumulate in the cancer cell, it loses a significant portion of its signaling genes and thrives on the few advantageous genes that remain. Unlike normal cells, cancer cells are devoid of the complex signaling networks characteristic of normal cells and are therefore much less robust than normal cells. It depends on fewer, but enhanced, signaling pathways and is deficient in many of the regulatory pathways characteristic of normal cells. Thus, the few enhanced pathways on which the cancer cell thrives are actually its Achilles' heel. Depriving the cancer cell of one or more of these enhanced signaling elements may sensitize it to stress and even induce its demise [2]. Interception of these pathways could inflict a decisive blow to the cancer cell with little harm to its neighboring normal cells, which would retain their robustness. Indeed, this type of reasoning led to the development of tyrosine phosphorylation inhibitors (tyrphostins), as it was recognized early on that protein tyrosine kinases comprise a major fraction of the signaling elements whose activities are enhanced in the cancer cell and on whose activities the survival of the cancer cell highly depends [3–9]. The type of therapy aimed at modifying the signaling pathways of the cancer cell is known as *signal transduction therapy* [1]. Signal transduction therapy primarily targets the aberrant signaling elements within the cancer cell but also consists of anti-angiogenic therapy, which targets the newly dividing endothelial cells lining the fresh blood vessels surrounding the tumor and generated in response to vascular endothelial growth factor (VEGF) secreted by the tumor [10].

Protein Tyrosine Kinase Inhibitors

Although protein kinases have been known since the discovery of protein phosphorylation in the 1950s, no one turned to them as drug targets until protein kinase C (PKC) and tyrosine phosphorylation were discovered over 20 years later. Identifying tyrosine kinase activity as being the hallmark of the oncogenic activity of $pp60^{c\text{-}Src}$ (and dozens of other oncoproteins) prompted researchers to investigate these proteins as novel targets for drugs. Tyrosine phosphorylation inhibitors (tyrphostins) were subsequently developed as a strategy to combat cancer and other proliferative diseases in the late 1980s [3,11,12]. Also discovered were a number of serine/threonine kinases such as cyclin-dependent kinases (Cdks), Erks, Raf, and PKB/Akt, which play a key role in cell proliferation, cell division, and anti-apoptotic signaling. In contrast to most Ser/Thr kinases known to be involved in housekeeping cellular duties, the more recently discovered protein kinases are directly involved with cellular signaling. In the human genome, we currently identify 409 Ser/Thr kinases, 59 receptor protein tyrosine kinases (RPTKs) and 32 non-RPTKs. Most of the Ser/Thr kinases

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Table I Protein Kinase Inhibitors in Clinical Development

Company	Agent	Indication	Target	Status
Genetech/Roche	Herceptin(mAb)	Breast cancer	Her-2	Marketed
Novartis	STI571/Gleevec	Chronic myeloid leukemia (CML)	Bcr-Abl	Marketed
Novartis	STI571/Gleevec	Gastrointestinal stromal tumor (GIST)	C-Kit	Marketed
AstraZeneca	ZD 1839/Iressa	Solid tumors	EGFR, Her-2	Marketed
Pharmacia/Sugen	SU 6668	Solid tumors	VEGFR, PDGFR, FGFR	Phase 3
OSI Pharmaceuticals	Tarceva (OSI 774)	Solid tumors	EGFR	Phase 2
Cephalon/Lundbeck	CEP-1347	Parkinson's disease	Mixed lineage kinase	Phase 1
Cephalon/Lundbeck	CEP-701	Prostate cancer	NGFR	Phase 2
Ludwig Institute for Cancer Research	AG 1478/CDDP	Glioblastoma multiforme	EGFR	Phase 1
Bayer/Onyx	BAY 43-9006	Colon cancer	Raf	Phase 1
Falvopiridol	—	—	Cdk4/1	Phase 1
Eli Lilly	LY 333531	Diabetic retinopathy	Protein kinase C	Phase 3

are housekeeping metabolic enzymes for which only a small fraction is involved in cellular signaling, in contrast to PTKs, which are primarily involved in signaling. The activities of PTKs are associated with enhanced proliferation and strong survival signals, the two most prominent traits of cancer cells.

The development of PTK inhibitors originally known as tyrosine phosphorylation inhibitors (tyrphostins) led to the approach of signal transduction therapy aimed at eradicating cancer cells. Research in this area has demonstrated that one can generate small molecules with a high degree of selectivity against different PTKs, even closely related ones such as endothelial growth factor receptor (EGFR) and Her-2/neu. Table 1 lists PTKs and Ser/Thr kinase inhibitors currently in clinical development. It is interesting to note that, in a number of cases, the PTK inhibitor, even as a single agent, induces apoptosis in the treated cancer cell but has no such effect on normal cells and is well tolerated by the treated animal.

The first striking example is the case of the Jak-2 inhibitor AG 490 (Fig. 1) [13]. This tyrphostin was found to induce apoptosis of recurrent pre-B acute lymphoblastic leukemic (pre-B ALL), eliminating completely the pre-B ALL cells from severe combined immunodeficiency (SCID) mice engrafted with the disease; treatment of the animals began 5 to 9 weeks after disease engraftment. Furthermore, AG 490 was not inhibitory to normal B or T cells when stimulated by various means. This pioneering study validates the hypothesis discussed here [14] and is considered to be an important milestone in signal transduction therapy. The diseased Pre-B ALL cells depend for their survival and growth on the persistently active Jak-2, but normal B cells (and normal T cells) are completely oblivious to the inhibition of Jak-2. Indeed, the inhibition of Jak-2 is sufficient to induce apoptosis in the pre-B ALL cells, whereas its inhibition in normal cells seems to have no effect whatsoever [13]. Similar results have also been obtained with interleukin-6 (IL-6)-dependent multiple myeloma cells, which are also driven by Jak-2. Very recently, a novel class of Jak2/3 kinase inhibitors has been developed but the *in vivo* activity of these inhibitors has not been reported [15].

Chemistry of Tyrosine Kinase Inhibitors

Initially, a large number of natural compounds were found to be rather potent inhibitors of PTKs. Although many showed initial promise, they all were found to be highly promiscuous and toxic in that they hit many cellular targets. The first PTK inhibitors to be synthesized were benzene malononitrile tyrphostins [11,12]. These compounds (Fig. 1) are competitive with the substrate and noncompetitive with ATP. Structure–activity relationship studies generated compounds that are 1000-fold more active against the EGFR kinase as compared to insulin receptor kinase, with no measurable activity against protein kinase A (PKA) and other serine/threonine kinases. As the number of identified tyrphostins grew, a more complex pattern of kinetics of inhibition of the EGFR kinase began to emerge. Tyrphostins competitive against either substrate or ATP were common, but so were compounds competitive with both [17]. Some compounds were found to be partially competitive (mixed competitive); their interactions with the EGFR [17] or PDGFR [18] reduce the binding affinity of ATP and substrate as well as causing a reduction in the catalytic activity of the enzyme [16]. This type of behavior suggests that the inhibitor binds to sites different than the active site and therefore qualifies as an allosteric inhibitor. As tyrphostins became cyclized (Fig. 2), incorporating nitrile nitrogen into the second ring caused most of the compounds to become ATP competitive [19–22].

Since 1994, the main thrust in the development of PTK inhibitors, especially by pharmaceutical companies, has been toward the generation of ATP mimics (ATP-competitive kinase inhibitors) [23]. Most of the inhibitors generated are based on a scaffold structured around two or more

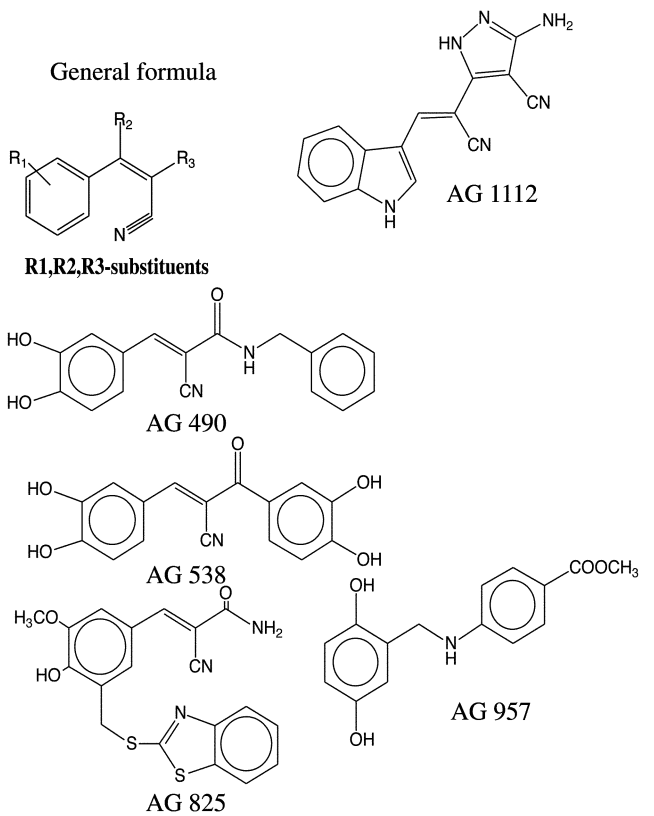


Figure 1 Early Tyrphostins. AG 1112 and AG 957 were prepared as inhibitors of Bcr-Abl; AG 490 is an inhibitor of Jak-2, AG 825 is a Her-2/neu inhibitor, and AG 538 is a potent inhibitor of IGF1-R.

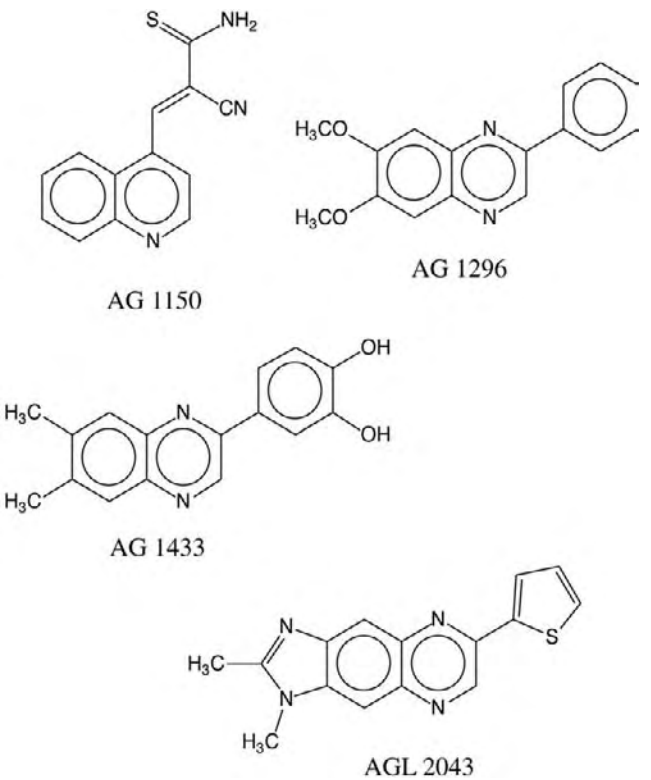


Figure 2 Cyclized tyrphostins. Incorporating the nitrillo(cyano) nitrogen within a second ring generated two-ring tyrphostins as opposed to the one-ring system (see Fig. 1). When a second nitrogen is introduced into the second ring, selective ATP mimics emerge. Interestingly, similar compounds have been identified as PTK inhibitors by random screening rather than by semi-rational design. AG 1150 is rather inactive, whereas AG 1296 and AGL 2043 are potent and selective PDGFR kinase inhibitors.

aromatic rings. These compounds were designed to be ATP mimics, but their kinetic behavior toward the substrate was not always investigated. Because the degree of conservation in the ATP binding site is not absolute, one can obtain a high degree of selectivity among closely related ATP binding domains. In 1993, the Jerusalem group [24] was able to demonstrate that ATP-competitive tyrphostins such as AG 825 (Fig. 1) can discriminate between the kinase domains of Her-2/neu and EGFR by almost two orders of magnitude in affinity, in spite of the almost 80% identity in the kinase domains of the two related PTKs. In 1994, the quinazoline ZD 1839 (Iressa; Fig. 3) was shown to be a potent EGFR kinase inhibitor with excellent bioavailability [25]. Quinazolines were originally identified by Zeneca and were shown to selectively inhibit EGFR at low nanomolar concentrations [25], whereas Her-2/neu is inhibited only at micromolar concentrations. Qunixaloines such as AG 1296 [19,26] or AGL 2043 [27] (Fig. 2) were found to block PDGFR kinase with inhibitory effects on related receptors such as c-Kit and Flt-3 and with 10- to 50-fold less efficacy against the more distantly related receptor VEGFR (unpublished data). The crystal structure of the inactive form of Hck with the Pfizer inhibitor PP1 [28] and of the active form of Lck with PP2 [29] explained why this ATP mimic binds better to the Src family kinase binding domain than does EGFR and much better than to a number of other tyrosine kinases and PKA. It was found that when threonine 338 is substituted for methionine or alanine 402 is substituted for another amino acid, the affinity to PP1 drops markedly [28].

EGFR Family Kinase Inhibitors

The role of EGFR in many cancers was appreciated early on and was one of the first targets for therapy. Indeed, the quinazoline Iressa (ZD 1839) (Fig. 3) [21,25] is in advanced clinical trials for treatment of cancers for which EGFR plays an important role, such as lung cancer and head and neck cancer. Similarly, the quinazoline AG 1478 (Fig. 3) [22] is in clinical development for the treatment of glioblastoma multiforme in which the EGFR and its persistently active Δ (2–7) EGFR are overexpressed [30,31]. This tyrphostin will be used in combination with CDDP, with which it synergizes to induce apoptosis in glioma multiforme cells *in vitro* and *in vivo* [31]. OSI 774 (Fig. 3) is also an effective quinazoline in clinical development [32]. Over the years, we have come to realize that heterodimer combinations of the four members of the Her family play a role in the oncogenic phenotype of many cancers; therefore, attempts are being made to generate inhibitors that inhibit both Her-1 and Her-2. The Glaxo-Welcome Her-1/Her-2 inhibitor GW 2016 (Fig. 4), blocks both receptor tyrosine kinases at 12 n*M* [33], and it is to be expected that more compounds aimed at the Her family will emerge in the near future.

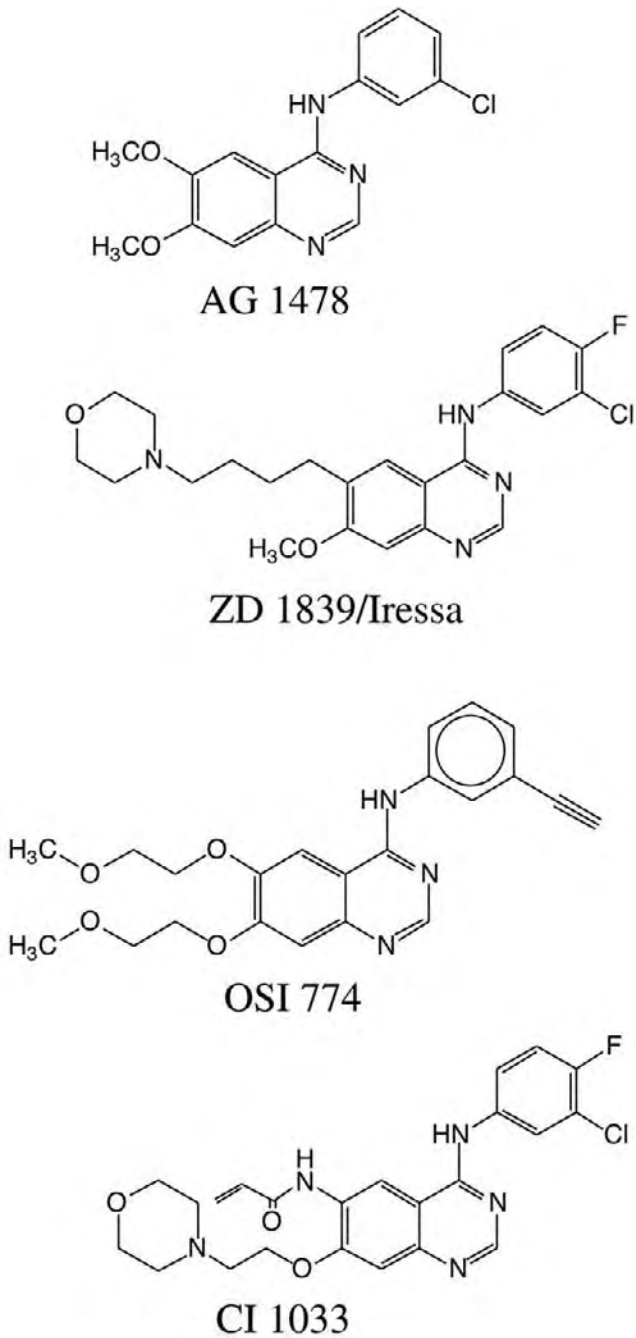


Figure 3 Quinazoline EGFR kinase inhibitors. AG 1478, ZD 1839, and OSI 774 are reversible EGFR kinase inhibitors, whereas CI 1033 is an irreversible inhibitor.

Covalent EGFR Kinase Affinity Labels

The covalent attachment of a selective inhibitor to the EGFR kinase domain (or to any target PTK domain) completely abolishes the catalytic activity of the receptor and is therefore believed to possess better clinical potential. The Parke-Davis compound CI-1033 (Fig. 3) [34] is highly effective *in vivo* as an EGFR kinase inhibitor; the effect of CI-1033 is long lasting and seems to possess higher efficacy than its reversible analogs. The covalent label attaches to cysteine 773, close to the ATP binding domain, and most probably targets the receptor for degradation followed by cell death. Other covalent labels of the EGFR kinase site were also reported to possess strong antitumor activity *in vivo* at relatively low doses.

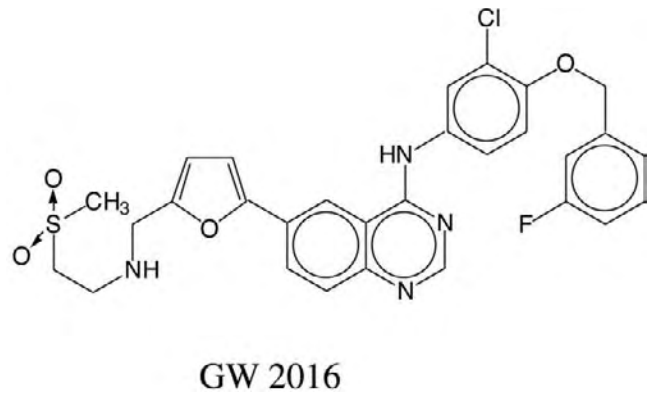


Figure 4 Her-1/2 kinase inhibitor.

From Tyrphostins to Gleevec

In 1993, it was demonstrated that selective Bcr-Abl kinase inhibitors such as tyrphostin AG 1112 (Fig. 1) induce the terminal differentiation of K562 cells [35]. Similarly, another Bcr-Abl-selective kinase inhibitor, AG 957 [36,37], induces the purging of Ph^+ cells and synergizes with anti-Fas receptor antibody to induce their demise [38]. Druker *et al.* [39] followed up on these studies by utilizing CGP 57148, renamed STI 571/Gleevec/Glivec and produced by Novartis (Fig. 5) [40,41]. This highly potent inhibitor inhibits PDGFR kinase as well as its homologous PTK c-Kit but is also a powerful inhibitor of Bcr-Abl kinase. Gleevec was found to induce complete remission in chronic myeloid leukemia (CML) patients which has lasted for nearly 3 years for most patients. It is interesting to note that these patients, who take about 400 to 800 mg daily, suffer only minor side effects and tolerate the drug well. This is a rather surprising, as STI 571 blocks c-Abl, PDGFR, and c-Kit, which play important roles in the function of normal cells. The most likely explanation is that normal cells that utilize c-Abl, PDGFR, or c-Kit can get by even when over 90% of these targets are blocked by utilizing the alternative pathways that all normal cells possess.

Chronic CML cells are highly dependent on Bcr-Abl for their survival and therefore die when the target is blocked, thus validating the principle of enhanced sensitivity of the cancer cell to an inhibitor that targets the element whose signaling is enhanced and on which the cancer cell depends for its survival. The findings on Bcr-Abl are reinforced by the remarkable activity of Gleevec on a subpopulation of gastrointestinal stromal tumor (GIST) patients [42]. The common denominator of the patients who respond to the drug with complete remission or almost complete remission is that their tumors express Kit receptors carrying mutations in exon 11, which converts the receptor to a persistently active kinase. As is true for chronic CML, it seems that the survival of the tumor cells is highly dependent on the signaling of mutated

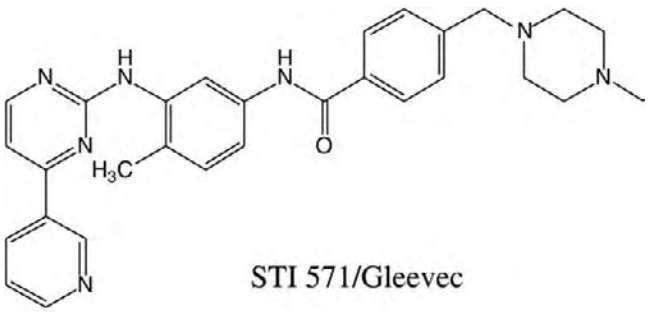


Figure 5 STI 571/Gleevec.

Kit receptor kinase, whereas normal cells can sustain and compensate for c-Kit blockade. Again, as for chronic CML patients, STI 571 has little side effects, probably because normal cells utilize alternative pathways when c-Kit is blocked or can get by even when a high fraction of the normal c-Kit molecules are blocked by utilizing alternative pathways.

ATP Mimics and Substrate Mimics

We have suggested that the optimal PTK inhibitors would be compounds that compete for the substrate binding site within the kinase binding domain. Such agents would be less toxic than ATP mimics because they bind to those domains at the kinase site that are less conserved than the substrate binding domains. Indeed, tyrphostins such as AG 490 (which blocks Jak-2 [13]) and AG 556 (which possesses antiinflammatory properties) have been shown to be highly nontoxic *in vivo* [43–46]. The main problem with these compounds is that they possess hydroxyl groups, which are metabolized relatively quickly, although this characteristic has not eliminated DOPA as an anti-Parkinsonian drug. Recently, we have developed substrate mimics in which the hydroxyls are replaced with *bioisosteres*. Half of the hydroxyl groups in AG 538 [47] were replaced with a type of bioisostere without losing much of the potency against IGF-1R and still retaining the substrate-competitive nature of the compound [48]. The double bond, which is present in many tyrphostins, may be a substrate for the Michael addition, which shortens the half-life of these compounds. Although this is rarely a problem, as most tyrphostins are stable in tissue culture medium for many hours [13,24], higher chemical stability can be achieved by eliminating this double bond. Work in progress indeed suggests that one can probably design substrate-competitive inhibitors devoid of double bonds.

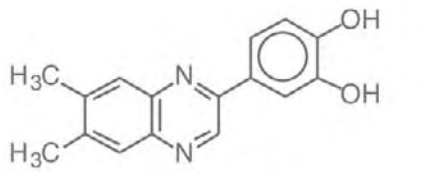
A strong argument for developing substrate-competitive protein kinase inhibitors is that they are likely to offer higher selectivity, as the portion of the kinase site outside the ATP binding domain is less conserved among protein kinases. This in principle should enable one to discover highly selective kinase inhibitors, which even at high doses will exhibit minimal toxicity *in vivo*. All of the kinase inhibitors currently in development (Table 1) are used at high doses, between 20 and 100 mg/kg. These high doses reflect the relatively low efficacy of these compounds *in vivo*, despite the fact that their IC_{50} values for their molecular targets, such as the EGFR, VEGFR-2/Flk-1 etc., are in nanomolar concentrations.

When one examines the efficacy of the ATP-competitive inhibitors in cellular assays, it is observed that these nanomolar compounds act on cells at micromolar concentrations. For example, quinazolines that bind to the EGFR with K_i of a few nanomolars [7,20] inhibit EGFR autophosphorylation in intact cells at micromolar concentrations [22,49]. Similarly, PP1 and PP2 that inhibit Src family kinases with IC_{50} values of 20 to 170 n*M* in cell-free assays [50] block Src activity in cells in the range of 5 to 40 μ*M* [49,51]. It seems, therefore, that the high doses required *in vivo* probably at least partly reflect the competitive relationship between the intracellular millimolar concentrations of ATP and the administered drug. It is noteworthy that drugs such as β-adrenergic blockers are administered at doses that are lower by about 100-fold. In this case, a drug possessing an affinity at nanomolar concentrations must compete with the up to 200-n*M* concentration of the endogenous ligand adrenaline or noradrenaline. Thus, β-blockers can be administered to patients at doses of 1.0 mg/kg or less with great efficacy. The same is true for other receptor-directed drugs for which the endogenous ligand is present at low concentrations within body fluids.

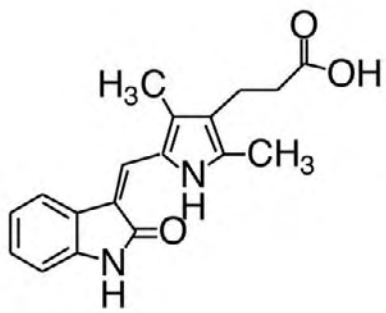
Adenosine triphosphate competitors suffer from another potential problem—the selectivity of newly developed compounds is only tested against a limited number of PTKs and Ser/Thr kinases. The number of PTKs is around 150 and the number of Ser/ kinases ranges from 500 to 600. It has already been observed that the so-called selective Src family kinase inhibitor PP1 is equipotent as a PDGFR kinase inhibitor [52]. Similarly, the Novartis Bcr-Abl kinase inhibitor, STI 571/Gleevec, used to treat CML, is as potent against PDGFR kinase and c-Kit [42]. An inhibitor that competes for both ATP and substrate simultaneously may actually be highly useful. Such tyrphostins have been generated [17], but their toxicity and efficacy *in vivo* have not been evaluated. It has been generally assumed that one should aim to treat patients for short periods in order to diminish side effects, a goal that is particularly applicable to cytotoxic drugs because of their severe side effects. Perhaps not surprisingly, Gleevec has been given to patients for many months with very minor side effects. In fact, the absence of toxicity may allow the prolonged use of protein kinase inhibitors and other signal transduction inhibitors, maximizing their therapeutic effect.

Angiogenesis

Angiogenesis is mediated by the activity of various receptors, primarily VEGFR but also PDGFR and FGFR. Following development of AG 1433 (Fig. 6) [10], Sugen developed SU 6668 (Fig. 6) as a PTK inhibitor for VEGFR, PDGFR, and FGFR simultaneously. This very interesting and promising agent (Table 1) is currently in clinical studies [53].



AG 1433



SU 6668

Figure 6 AG 1433 and SU 6668. AG 1433 was found to be a potent PDFR and EGFR kinase inhibitor. This compound was found to inhibit angiogenesis [10] but was not suitable for development. SU 6668 [53] with a new scaffold was developed and is currently in clinical development (see Table 1).

PTK Inhibitors Synergize with Pro-Apoptotic Agents

It has been observed that transformed cells possess a heightened state of sensitivity to stress/apoptotic signals as compared to their parental nonmalignant cells [2,54]. This sensitized state renders the cancer cells higher sensitivity to stress/apoptotic agents such as *cis*-Platin (CDDP) and pro-apoptotic ligands such as FasL. As the cancer progresses, the potentiated state of sensitivity to stress is covered up by a massive shield of anti-apoptotic signaling networks. Thus, when one applies an anti-apoptotic agent, the potentiated state of the cell is uncovered and the cancer cell becomes hypersensitive to the pro-apoptotic stress agent. This is probably why PTK inhibitors synergize with cytotoxic drugs or pro-apoptotic proteins such as FasL. The first example demonstrating this principle was reported for Her-2/neu-expressing lung cancer cell lines. It was shown that the degree of synergism between a Her-2 kinase inhibitor (AG 825) (Fig. 1) and the cytotoxic agents CDDP, etoposide, or doxorubicin increases with the level of expression of Her-2/neu in a series of isogenic patient-derived NSLC cell lines [55]. It seems that the degree of anti-apoptotic signaling of Her-2 is proportional to the latent potentiated sensitivity of the cancer cell; thus, the degree of synergism is proportional to the level of Her-2/neu signaling. The fact that PTK inhibitors can synergize with pro-apoptotic agents is already being implemented in the clinic. ZD 1839 (Iressa), a potent EGFR kinase inhibitor developed by AstraZeneca, is currently in clinical trials as a single agent and in combination with cytotoxic agents such as Taxol® [56]. Another interesting case is advanced glioblastoma multiforme (GM), where the truncated EGFR Δ (2–7 EGFR) is responsible for its resistance to chemotherapy and radiation therapy. Blockade of the EGFR by the EGFR kinase inhibitor AG 1478 sensitizes the tumor to CDDP and enhances the survival of tumor-bearing nude mice treated with the two agents [30,31]. This combination is in clinical development for the treatment of GM. As mentioned previously, STI 571/Gleevec shows remarkable activity against chronic CML, and patients on STI 571 have been in complete remission for nearly 3 years, but it is much less effective as a single agent in blast crisis CML, where the disease recurs in 80% of the patients within a few months. These patients are likely to be given a combination of STI 571 with pro-apoptotic agents early in the treatment. So far, PTK inhibitors have not been tried in humans in combination with pro-apoptotic proteins or antibodies. In tissue culture, the synergy between PTK inhibitors, cytotoxic agents [55,56], FasL, or anti-Fas receptor antibody [38,57] was established.

PTK Inhibitors for the Treatment of Non-Cancer Diseases

The enhanced activity of PTKs has been related to diseases other than cancer. The enhanced activity of EGFR is the hallmark of psoriasis and papilloma. In both instances, EGFR is overexpressed and the diseased cells produce EGFR-stimulating ligands. This is the reason why EGFR kinase inhibitors inhibit the growth of both psoriatic keratinocytes [58–61] and keratinocytes immortalized with human papillomavirus 16 (HPV-16) [62,63]. It has been suggested that EGFR kinase inhibitors be used as topical agents to treat these conditions. PDGFR is the key player in restenosis following balloon angioplasty; therefore, PDGFR kinase inhibitors have been tested as inhibitors of balloon-induced stenosis. Local application of AG 1295 [64] and AGL 2043 [27] during balloon angioplasty has indeed been shown to be highly effective against the development of stenosis after balloon angioplasty. One can envisage the utilization of PTK inhibitors for other indications, such as pulmonary fibrosis, in which the enhanced activity of PTKs plays an important role in the pathophysiology of the disease.

PTK Inhibitors as Cancer Prevention Agents

Over the past few years, interest has increased in agents that are natural components of foods and have anticancer properties [65]. It has been suggested that males from the Far East suffer less from prostate cancer because they consume food rich in genistein, which is a nonselective PTK inhibitor. Studies suggest that genistein prevents the growth of metastatic cancer [66–68]. Dietary genistein downregulates the expression of the androgen receptor and estrogen receptor-α and -β in the rat prostate, at concentrations comparable to those found in humans on a soy diet [69]. Thus, downregulated sex steroid receptor expression may be responsible for the lower incidence of prostate cancer in

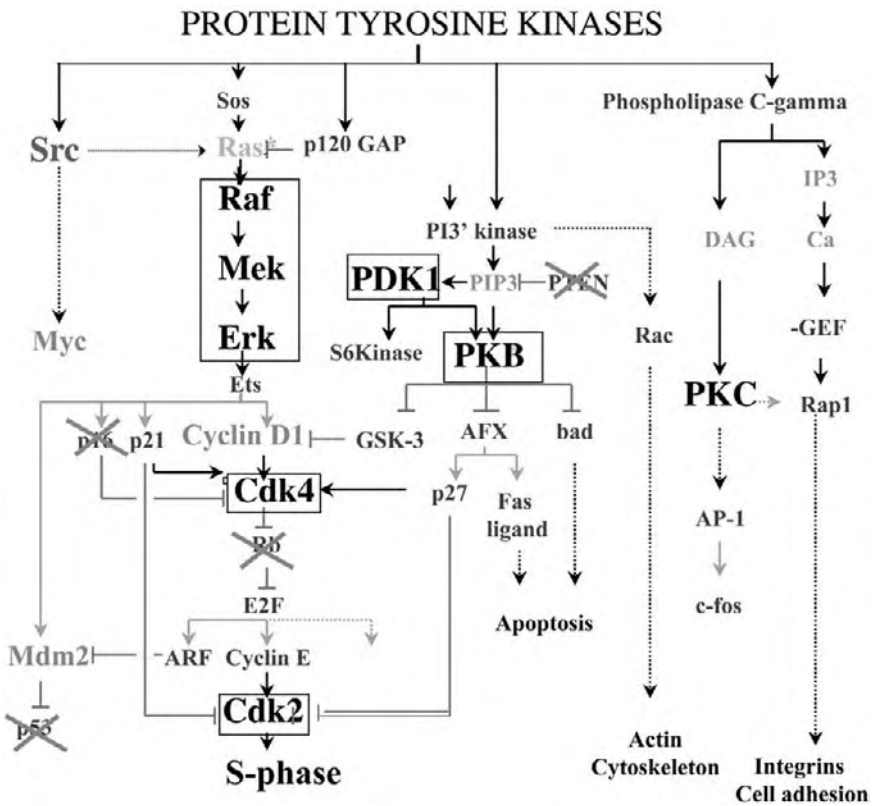


Figure 7 Key signaling Ser/Thr kinases. A small fraction of Ser/Thr kinases are valid targets for drug development; these are indicated by bold type in boxes. Crosses appear on tumor suppressors for which activities are compromised in cancer and therefore augment the activation of various kinases.

populations living on a diet containing high levels of phytoestrogens. Clinical trials are therefore planned for patients with metastatic prostate cancer who will receive genistein to examine whether this agent does, indeed, have any antimetastatic effects. Also, it has been suggested that polyphenols, which are components of wine, possess antineoplastic effects. These effects are largely attributed to their antioxidant properties, but it is possible that the inhibitory effects on PTKs are also responsible.

PTK Inhibitors for Diagnostic Purposes

It is always essential to establish a treatment modality based on the presence of the drug target in the diseased tissue. For example, it is extremely important to know ahead of treatment with an EGFR kinase inhibitor if the tumor does, indeed, overexpress the receptor. This can be achieved by imaging the tumor utilizing an EGFR kinase inhibitor, which is a positron emitter. Recent studies show that a reversible EGFR kinase inhibitor [70] is inferior to an irreversible one [71] for achieving this goal. Positron emission tomography (PET) imaging of the EGFR is extremely important for the determining which patients with non-small-cell lung carcinoma are eligible for treatment with Iressa. Because many of the tumors express either EGFR or Her-2, or both, a dual PET imager based on GW 2016 (Fig. 4) may also be useful.

SER/THR Kinase Inhibitors

Among the few hundred Ser/Thr kinases a handful are involved in transmitting the signals of upstream PTKs, and their activity is essential for cell proliferation and the onset of anti-apoptotic signaling (Fig. 7). Their abnormal enhanced activities are augmented by the deletion of negative regulators such as protein inhibitors of Cdks and the deletion of the tumor suppressor PTEN, the negative regulator of the PI3′ kinase pathway. Thus, the activities of these kinases are enhanced by the synergistic action of the enhanced upstream PTKs combined with the inactivation of downstream negative regulators. As an example, Cdks execute the cell cycle and their activity has been found to be enhanced not only as a result of enhanced upstream signaling but also as a result of the overexpression of cyclin D1 and the deletion of Cdk inhibitors such as p15 and p16. Thus, Cdk2, Cdk1/Cdc2, and Cdk4 have become targets for new antineoplastic agents. Similarly, PI3′ kinase signaling has been found to be enhanced by the deletion of its negative regulation PTEN. This deletion, characteristic of high-grade tumors, potentiates the already strong positive regulation of the PI3′ kinase/PKB/mTor module complex by PTKs. Because of findings such as these, inhibitors of PKB and mTor are in development as antitumor agents.

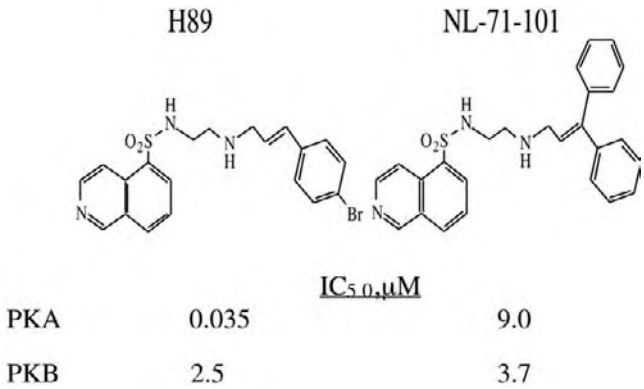


Figure 8 Development of PKB/Akt inhibitors. One of the avenues to develop PKB inhibitors is to reverse the specificity of H-89, a PKA inhibitor. The advantage of this approach is the greater selectivity of the compound against other kinases and the relative simplicity of the experimental protocols.

PKB/Akt Inhibitors

Progress toward generation of PKB inhibitors (Aktstatins) has been recently reported [72]. In this study, the PKA inhibitor H-89 was modified to reduce its affinity to PKA but retaining and even improving its affinity to PKB/Akt (Fig. 8). Attempts are currently being made to widen the gap in selectivity towards PKB/Akt and increase the affinity.

Inhibitors of the Ras Pathway: Raf and Mek Inhibitors

Inhibitors of the Ras–Raf–Mek–Erk pathway have great therapeutic potential, as mutated Ras is the hallmark of many cancers. Mutated Ras occurs in 30% of all human tumors, in 80% of pancreatic cancers, in 50% of colorectal cancers, in 40% of lung cancer, and in 20% of hematopoietic malignancies. So far, no real success has been achieved in developing Ras inhibitors, but promising developments are reported in the development of Raf and Mek inhibitors. A recent study [73] shows that mutations that activate the kinase activity in B-Raf occur in ≈66% of human melanomas, suggesting that Raf kinase inhibitors (such as the recently reported Onyx/Bayer compound BAY 43-9006 [74]) (Fig. 9) may be utilized to treat metastatic melanoma. Mek inhibitors such as PD 184352 [75] are also very promising. PD 184352 (Fig. 9) is a highly potent and selective inhibitor of Mek that is orally active. Tumor growth has been inhibited as much as 80% in mice with colon carcinomas of both mouse and human origin after treatment with this inhibitor [75]. Efficacy was achieved with a wide range of doses with no signs of toxicity and were correlated with a reduction in the levels of mitogen-activated protein kinase in excised tumors. These data indicate that Mek inhibitors represent a promising, noncytotoxic approach to the clinical management of colon cancer. It is highly likely that many more inhibitors of this pathway will emerge in years to come.

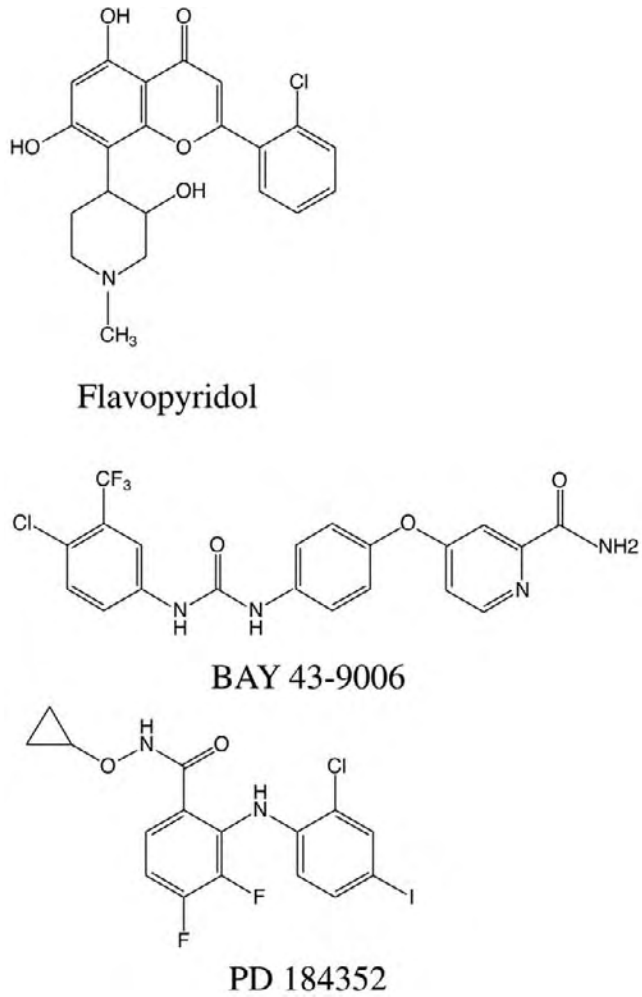


Figure 9 Cdk, Raf, and Mek inhibitors.

Cdk

Inhibitors of Cdks are being developed as anticancer agents [76–81]. Falvopiridol (Fig. 9) inhibits Cdk4/cyclin D and Cdk1/CyclinB1, with an IC_{50} value of 200 n*M* as compared to PKA and PKC, which it inhibits with IC_{50} of 960 μ*M* and 10 μ*M*, respectively [82]. This inhibitor is currently in clinical development (Table 1) [83].

PKC Inhibitors

Although we have been aware of PKC isozymes for a long time, little progress has been made in utilizing PKC inhibitors as therapeutic agents. Some agents are in development as anticancer drugs [77,84–86]. PKC-β inhibitors are also potential agents against vascular dysfunction [87] and are being evaluated as agents against vascular retinopathy, a complication of diabetes.

Rapamycin

Rapamycin, the inhibitor of mTor, is effective as an inhibitor of angiogenesis and therefore is a potential anticancer

drug [88]. Rapamycin is also effective in the inhibition of restenosis when applied on coated stents [89].

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CHAPTER 78

Integrin Signaling: Cell Migration, Proliferation, and Survival

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Introduction

Integrins are a family of heterodimeric, transmembrane receptors that mediate attachment of cells to the surrounding extracellular matrix (ECM) [1]. Different combinations of α and β subunits dictate specificity for the extracellular ligands [2]. The cytoplasmic tail of the β subunit is both necessary and sufficient to mediate the linkage of integrins to the actin cytoskeleton [3]. Although α-subunit cytoplasmic tails bind to cytoskeletal proteins [2], the major functional role of the α subunit is to modulate cytoskeletal interactions by directly interacting with the cytoplasmic tail region of the β subunit. Thus, integrins are ligand-dependent sensors of the ECM environment. As such, they are responsible for directing biochemical signals and cellular forces, which together regulate cell migration, cell growth, and cell survival.

Integrins Nucleate the Formation of Multi-Protein Complexes

A central function of the integrins is to mediate a structural linkage between the dynamic intracellular cytoskeleton and the ECM. More that 50 proteins have been identified either as direct integrin-binding proteins or as proteins that localize to adhesion complexes (e.g., focal adhesions) [4]. The proteins found in adhesion complexes fall into two broad categories: those that serve a structural role to anchor and regulate the actin cytoskeleton and those that are responsible for integrin-mediated signaling and the remodeling of adhesion complexes.

The association of talin, α-actinin and vinculin with integrin receptors serves to illustrate how integrins are linked to actin filaments and the cytoplasm (Fig. 1). Talin is a major structural component of focal adhesions [3]. Talin binds directly to the tails of β1, β2, and β3 integrins. In addition, talin contains binding sites for actin, vinculin, focal adhesion kinase (FAK), and phospholipids. Cells deficient for the expression of talin exhibit significant increases in membrane blebbing, defects in cell adhesion and spreading, and a failure to assemble focal adhesions and stress fibers, underscoring the role of talin in the organization of adhesion structures [5]. α-Actinin is an actin-binding protein that binds the cytoplasmic tails of β1, β2, and β3 integrins [3] as well as several additional focal adhesion proteins, including vinculin and zyxin. Localization of α-actinin to adhesion complexes occurs by a direct interaction with β-integrin cytoplasmic tails. Vinculin is one of the most abundant focal adhesion proteins, although it does not bind integrins directly [3]. Vinculin binds F-actin and the adhesion-associated proteins paxillin and VASP and is recruited to focal adhesions indirectly via an interaction with an integrin cytoplasmic tail binding protein (e.g., talin or α-actinin). Vinculin functions as a molecular bridge to stabilize integrin-F-actin linkages. Vinculin-deficient cells exhibit decreased mechanical stiffness and increased cell motility [3,5].

Integrins also serve to recruit proteins that are directly involved in regulating the formation and turnover of adhesion complexes and the promotion of intracellular signals (Fig. 1). FAK is a focal-adhesion-associated, nonreceptor protein tyrosine kinase. FAK binds *in vitro* to the cytoplasmic tails of β1 and β3 integrins, although to date this interaction

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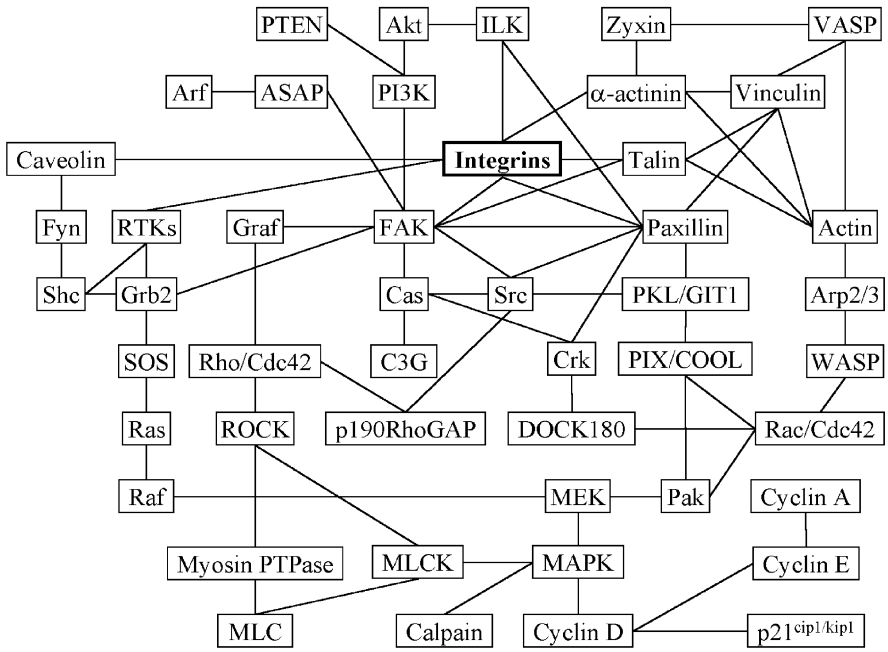


Figure 1 Integrin connections. Schematic diagram of protein interactions initiated by integrin receptors, as described in the text. The following abbreviations are used: ARF (ADP-ribosylation factor), Arp2/3 (actin-related protein 2/3 complex), ASAP (ARF-GAP containing SH3, ankyrin repeats, and PH domain), C3G (Crk SH3-binding GNRP), Cas (Crk-associated substrate), Crk (CT10 regulator of kinase), FAK (focal adhesion kinase), GRAF (GTPase regulator associated with FAK), ILK (integrin-linked kinase), MAPK (mitogen-activated protein kinase), myosin PTPases (myosin phosphatase), p190RhoGAP (p190 Rho GTPase-activating protein), PAK (p21-activated kinase), PI3K (phosphatidylinositol 3-kinase), PIX/COOL (Pak-interacting exchange factor/cloned out of library), PKL/GIT1 (paxillin–kinase linker/G-protein-coupled receptor kinase-interacting protein 1), PTEN (phosphatase and tensin homolog deleted on chromosome ten), ROCK (Rho kinase), RTKs (receptor tyrosine kinases), SOS (Son of Sevenless), VASP (vasodilator-stimulated phosphoprotein), and WASP (Wiskott–Aldrich syndrome protein).

has not been demonstrated *in vivo* [6]. FAK contains an approximately 100-amino-acid domain that is both necessary and sufficient to target FAK to focal adhesions [7]. In addition, FAK also contains sequences that mediate its association with focal adhesion proteins paxillin and talin, as well as to the cytoskeletal adaptor protein Cas and GTPase-activating proteins (GAPs) for Rho (GRAF [8]) and ARF1 (ASAP1 [9]) (Fig. 2). Paxillin is a multidomain protein that not only binds to FAK, but also serves as a scaffold to recruit and organize a number of additional signaling molecules at the sites of adhesion. Paxillin binds Src, Crk, vinculin, actopaxin, and the serine/threonine kinase ILK, as well as the ARF GAPs PKL and GIT1 [10,11]. In addition, paxillin binds directly to the cytoplasmic tails of α4 integrins [12]. Like FAK-deficient cells, paxillin-null cells exhibit defects in cell spreading and cell migration, as well as decreased tyrosine phosphorylation of FAK and Cas [13,14]. Cas is another adaptor protein that binds to both FAK and Src and serves to recruit additional signaling molecules to focal adhesions. Cas associates with the guanine nucleotide exchange factor C3G, protein phosphatases, and adaptor proteins Crk and Nck [15]. Coupling between FAK, Src, and Cas appears to be important for FAK-stimulated cell migration [16].

A number of other proteins and kinases have been classified as integrin binding or integrin-associated proteins, including adaptor proteins and kinases (for example, RACK1, Shc, Grb2, and ILK); growth factor receptors (EGF receptor, ErbB2, PDGF receptor-β, insulin receptor, VEGF receptor); cytoplasmic, chaperone, calcium-binding proteins (calnexin, calreticulin, CIB, endonexin); and membrane-associated proteins (tetraspanins, Ig superfamily proteins, GPI-linked receptors, transmembrane proteins, and ion channels). The functional and structural diversity amongst these integrin-associated proteins underscores the importance of integrins as initiators of many intracellular signaling pathways. How integrins function in a structural versus a signaling role and how such complexes are organized temporally and spatially within the cell remain important and unanswered questions.

Cell Migration: A Paradigm for Studying Integrin Signaling

Cell migration provides an exceptionally relevant model to study integrin signaling. Migration is a complex cellular process that involves the extension of lamellipodia, adhesion

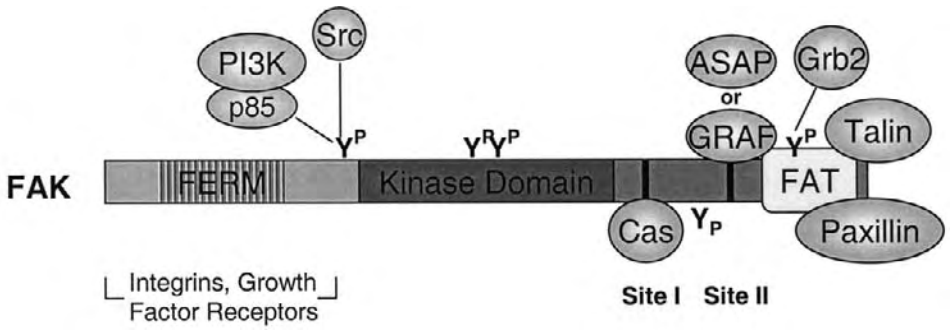


Figure 2 Focal adhesion kinase and its binding partners. FAK consists of a centrally located kinase domain flanked by an amino-terminal FERM domain and a carboxy-terminal region containing proline-rich sequences (site I and site II), as well as the focal adhesion targeting (FAT) domain. FAK autophosphorylation at Y397 in the amino-terminal domain creates a binding site for Src as well as the p85 subunit of PI3K. The carboxy-terminal domain of FAK binds the adaptor protein Cas, the GAP proteins GRAF and ASAP, and focal adhesion proteins talin and paxillin. Phosphorylation of FAK on Y925 creates a binding site for Grb2, potentially leading to the activation of the Ras-MAPK pathway.

at sites within newly formed lamella, organization of force-generating adhesions, contraction and cell-body displacement, and detachment of the cell rear. These events require the coordination of multiple signaling pathways.

Lamellipodia Extension and Formation of New Adhesions

The initial steps in cell migration require the formation of protrusive structures (lamellipodia) at the leading edge of the cell and the stabilization of the protrusion by newly formed adhesion complexes. Cell protrusions are regulated by the activity of surface receptors and Rho family GTPases Cdc42 and Rac [17]. Actin polymerization at the cell front is regulated by Cdc42 and Rac via their interaction with members of the Wiskott–Aldrich syndrome protein (WASP)/Scar1 superfamily [18]. Binding of Cdc42/Rac to WASP/Scar proteins activates the Arp2/3 complex [19], triggering its binding to the sides of preexisting actin filaments and stimulating new filament formation, which results in branched actin networks [20]. The formation of the branched actin network serves to drive the forward extension of the cell membrane, leading to the formation of lamellipodia [20,21].

Formation of new adhesions within lamellipodia involves integrin-induced assembly of FAK/Src complexes and the recruitment of two adaptor proteins, Cas and paxillin. Formation of this signaling complex is likely important to sustain activation of Rac and activation of serine/threonine kinase PAK (p21-activated kinase). FAK/Src-mediated phosphorylation of Cas or paxillin creates binding sites for the adaptor protein Crk. Cas/Crk complexes mediate Rac activation by binding DOCK180 [22,23], the human counterpart of the *Drosophila* and *Caenorhabditis elegans* genes *mbc* and *ced-5*, respectively [24,25]. While paxillin binds Crk following FAK/Src-mediated phosphorylation and signals to Rac, FAK mutants deficient in binding to paxillin efficiently restore migration of FAK null cells to a wild-type level [26]. Thus, in this setting, signaling to Cas appears to be sufficient to mediate adhesion formation.

Paxillin is an important regulator of Cdc42 and Rac through its binding to PKL and the subsequent interaction of PKL with two members of the Cdc42/Rac GEF family PIX/COOL [27–29]. The PIX/COOL family of proteins was originally reported to exhibit GEF activity for Rac and Cdc42 [30], although recently this property has been questioned, raising speculation that PIX/COOL proteins might activate PAK by binding the GTP form of Cdc42/Rac rather than directly activating the GTPases [27]. While the formation of the paxillin/PKL/PIX complex has been reported to activate PAK, recent data indicate that the interaction of paxillin with Rac leads to the downregulation of Rac activity [31], providing a possible mechanism for Rac turnover/downregulation. A suggested pathway (Fig. 1) may be integrin recruitment and activation of FAK/Src, binding and phosphorylation of Cas, and activation of Rac via Cas/Crk/Dock180 complexes. GTP-Rac may then bind to PIX/COOL proteins complexed with paxillin/PKL, resulting in PAK activation and Rac downregulation.

Maturation of Newly Formed Adhesions

Activation of Rho is required for the organization of F-actin into stress fibers and the formation of focal adhesions [32]. Both functions are regulated by the ability of Rho to promote the generation of directional forces, via its regulation of Rho kinase and myosin phosphatase and the subsequent regulation of myosin light chain (MLC) phosphorylation. Rho activation of Rho kinase inhibits myosin phosphatase, thereby maintaining MLCs in a highly phosphorylated (contractile) state. The resultant contractile forces are essential for the organization of actin filaments and adhesion complexes [32].

During cell migration, Cdc42/Rac and Rho signaling are regulated in a reciprocal fashion, leading to the breakdown of stress fibers and focal adhesions (due to the downregulation of Rho) and the commensurate reorganization of cortical actin networks at the leading edge of the cells [33–35]. Plating cells on ECM stimulates a transient decrease in Rho

activity, which is necessary for cell spreading [36,37]. FAK appears to contribute to the transient decrease in Rho activity, as such changes in Rho activity are not observed in cells deficient for FAK expression [38]. The mechanism by which FAK regulates the initial decrease in Rho activity may involve its interaction with the Rho GTPase-activating protein GRAF [8] or its ability to activate Src, which has been shown to phosphorylate p190RhoGAP, resulting in decreased Rho activity upon integrin engagement [36,39]. A subsequent increase in Rho activity is necessary to restore contractile forces, leading to strengthening of attachment sites, stress fiber formation, and generation of the forces necessary for continued cell movement [32].

Detachment and Release of Adhesions

In addition to stabilizing lamellipodia formation at the front of the cell, detachment at the rear of the cell requires sustained contraction and disassembly of integrin complexes. Mitogen-activated protein kinase (MAPK), like Rho kinase, phosphorylates MLCK, stimulating MLC phosphorylation and cell contraction [40]. In addition, phosphorylation of focal-adhesion-localized calpain by active MAPK [41] stimulates calpain-mediated cleavage of adhesion proteins and cell detachment [42,43]. Integrins activate MAPK through three different Ras-dependent pathways. Integrin-mediated activation of FAK and recruitment of Src results in phosphorylation of FAK on Tyr925 [44,45]. Phosphorylation on Tyr925 creates a binding site for Grb2 [44], an SH2/SH3 adaptor protein that links growth factor receptor tyrosine kinases to the Ras/MEK/MAPK pathway through the Ras guanosine diphosphate (GDP)/guanosine triphosphate (GTP) exchange protein SOS (Fig. 1). Integrins also activate SOS through caveolin-1-mediated recruitment of Shc to integrins and subsequent phosphorylation by Fyn [46] (Fig. 1). Finally, integrin engagement results in phosphorylation and activation of the epidermal growth factor (EGF) receptor in the absence of EGF stimulation [47]. Activated EGF receptor recruits Shc to the receptor, where phosphorylation creates a binding site for Grb2/SOS [47] (Fig. 1). Indeed, in this setting, ECM-mediated phosphorylation of Shc and activation of MAPK is blocked by inhibitors of EGF receptor tyrosine kinase activity. Integrin-stimulated migration is inhibited by MAPK inhibitors and stimulated by expression of active MEK [40]. Interestingly, dominant-negative Ras expression has little effect on ECM-stimulated migration [48,49], indicating the existence of Ras-independent mechanisms of MAPK activation.

Several studies show that Rac synergizes with Raf to stimulate MAPK-dependent migration in response to EGF [50]. Rac-dependent activation of PAK stimulates phosphorylation of MEK, resulting in an increased affinity of MEK for Raf [51]. An important consequence of Rac activation may be the enhancement of Raf–MEK interaction, leading to maximum MAPK activity in a setting of only basal Ras and Raf stimulation. This may provide a mechanism by which integrins potentiate signals from growth factor receptors, allowing cells to respond to low levels (gradients) of chemotactic signals in the environment.

Integrin Regulation of Cell Proliferation and Survival: Links to Cancer

The ECM plays a critical role in the altered growth and metastatic behavior of cancer cells. In the case of both normal and cancer cells, these signals contribute to the balance of cell growth and death by regulating the apoptotic machinery of the cell. Under appropriate circumstances, integrin signals regulate G_0-to-G_1 and G_1-to-S progression [52–55], as well as the expression of growth-related gene products associated with these transition states. Transient MAPK activation stimulated by either growth factors or cell adhesion is sufficient to initiate G_0-to-G_1 phase transition and the coincident expression of immediate-early response genes, including c-Fos, c-Myc, and c-Jun [56–59]. Serum stimulation in the absence of adhesion to the ECM abrogates progression through G_1 into S phase by increasing the accumulation of cdk2 inhibitors $p21^{cip1}$ and $p27^{kip1}$ [60]. Cyclin D1 functions to promote G_1 progression by sequestering $p21^{cip1}$ and $p27^{kip1}$ [61]. Cyclin D1 expression requires cell adhesion to mediate sustained MAPK activity initiated by growth factors [62]. Growth factor stimulation of cells held in suspension results in a modest, transient activation of MAPK compared to the robust MAPK stimulation following serum treatment of adherent cells [63]. Maximal, sustained MAPK activation requires FAK and Rho activity [64,65]. Indeed, FAK and Rho have both been implicated in regulating cyclin D1 expression and progression through G_1 [52,65]. Cyclin-D-dependent downregulation of $p21^{cip1}$ and $p27^{kip1}$ is necessary for cyclin E/Cdk2 activity, which induces the expression of cyclin A [60], a key regulator of S-phase progression [66,67]. Therefore, cell-substrate adhesion indirectly promotes cyclin A expression and S-phase progression by stimulating cyclin E/Cdk2 activity. Collectively, these data indicate that the G_0-to-G_1 transition and progression through the G_1 cell cycle is regulated by either serum or adhesion; however, progression through the S phase requires both serum and adhesion.

Cell proliferation is in dynamic balance with cell death. Shifts in this equilibrium, as a result of increasing cell proliferation or decreasing cell death, often result in tumorigenesis. Integrins provide key signals to regulate this balance. Depriving epithelial or endothelial cells of contact with the ECM rapidly induces apoptosis [68,69]. (This specialized form of cell death is referred as *anoikis*). Normal epithelial cells acquire resistance to anoikis upon expression of certain oncogenes [70–72]. A number of studies have implicated integrin signaling to the PI3K (phosphoinositide-3-kinase)–AKT pathway as a central regulator of anoikis [71]. FAK is thought to regulate anoikis by direct activation of PI3K and AKT and perhaps indirectly via interactions with Cas/Crk/DOCK180/Rac [68]. ILK has also been implicated in signaling to AKT, although this pathway is poorly understood.

However, ILK binds to the cytoplasmic tails of β1 and β3 integrin and over-expression of ILK results in activation of AKT [73,74]. Finally, the role of death receptors in anoikis is controversial. In one study, removal of cells from the ECM activated caspases and cell death by a mechanism involving FADD, a death-domain-containing protein [75]. Whether this pathway is important for all forms of anoikis remains to be determined.

In cancer, increasing evidence indicates that integrins synergize with growth factor receptor signals to promote cell proliferation and to stimulate the migration of tumor cells from the primary site and function to promote growth and survival at distant metastatic sites [76]. Growth of tumor cells is influenced by the tissue environment of the tumor (metastasis), and reflects both the upregulation of growth factors and induction of anti-apoptotic signals. Whereas, in most tumors, the exact mechanisms involved in this process are poorly understood, it is important to note that loss of the tumor suppressor gene PTEN leads to the upregulation of AKT and the suppression of anoikis [77]. In addition, many human cancers (including breast, prostate) exhibit upregulation of FAK. Clearly, understanding the complex changes in integrin signaling in cancer cells is a major challenge.

Concluding Remarks

The recognition that integrins are not only adhesive receptors but also "integrators" of growth factor and ECM signaling has had a profound impact on our understanding of cellular processes, including cell migration, differentiation, and cancer. The challenge for the next decade is to understand how different ECM proteins, growth factors, and chemotactic molecules function in coordinating multiple signaling pathways in the context of individual tissues and the organism itself.

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CHAPTER 79

Downstream Signaling Pathways: Modular Interactions

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Introduction

One of the hallmarks of proteins involved in signal transduction is their modularity. Like the proverbial Swiss army knife, signaling proteins often bristle with small, independently folded domains, each of which confers a specific function [1]. Some of these domains have enzymatic activity, catalyzing phosphate transfer, lipid metabolism, or the regulation of GTPases, while others play a purely structural role. The majority of these modular domains, however, mediate specific, high-affinity interactions with proteins or lipids. Indeed, some critical signaling proteins have evolved to the point where they consist entirely of modular interaction domains, serving as adaptors or scaffolds to mediate assembly of protein complexes.

The prominence of modular interaction domains in signaling proteins reflects the critical role played by localization in signaling, particularly in multicellular organisms. It is self-evident that a cell is not a homogeneous aqueous solution, any more than a house is a homogeneous pile of wood, metal, and cement. The specific subcellular localization of a protein within the cell has obvious consequences for its activity; in the case of an enzyme, the local concentration of its activators, inhibitors, and substrates will determine whether the catalytic domain can act on a particular substrate to generate a particular output (i.e., a change in concentration of the modified substrate). Unlike housekeeping processes such as metabolism, which need not occur in a very precise locale in the cell, many signal-induced processes such as assembly of the actin cytoskeleton must be exquisitely regulated in space and time. It is difficult to imagine how such a feat could be performed in a world of solution biochemistry; only in the solid-state world of multifunctional proteins complexes, assembled on surfaces such as membranes, can such spatiotemporal control be achieved [2].

General Properties of Interaction Modules

Modular interaction domains have been introduced in this volume (see Chapter 66), and this chapter considers only the properties specifically relevant to their role in signal transmission. One can divide these domains into two fairly distinct groups based on their mode of interaction. In one group are common folds that have been used over and over to mediate interactions, but for which the specifics of each interaction might vary considerably. These modules include such examples as the ankyrin repeat, WD40 repeat, and LIM domain, among others. Interactions tend to occur over broad surfaces of the folded domain and to be mediated by variable residues that are not conserved among different members of the family [3,4]. For these interaction domains, the common recognizable fold serves as a scaffold upon which variable residues involved in binding are displayed. Because the details of each specific interaction are different, it is therefore difficult or impossible to predict *a priori* what the binding partner for a such a module might be, though closely related members of a family may bind recognizably similar ligands [5].

The second class of modules, and the ones that are most closely associated with signal transduction, are those that

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bind to stereotyped linear peptide ligands. Examples of such domains are the SH2, SH3, WW, PDZ, and PTB domains. In these cases, all of the ligands for a particular class of module have the same general properties: Virtually all SH2 ligands consist of phosphorylated tyrosine and a few proximal amino acids, the great majority of SH3 ligands will have a Pro–X–X–Pro core, and so on. In general, the domain can bind with similar affinity to an intact ligand protein or to a short linear peptide derived from it, indicating that the domain does not recognize an extensive surface of the ligand. Many high-resolution structures exist for such domains, and in all cases (with a few instructive exceptions) highly conserved residues of the domain play a crucial role in binding, and the overall orientation of the peptide ligand is virtually identical for a particular class of domains [6]. This general concept also holds true for lipid binding modules such at the plekstrin homology (PH) domain, where the same binding surface is used to interact with phosphorylated inositol lipids [7].

One important implication of this mode of binding is that discrimination between different ligands cannot be absolute. If all SH3 domains must recognize peptides consisting of a Pro–X–X–Pro core plus a few surrounding residues, there is no great latitude for specificity [8]. Given that there are literally hundreds of SH3 domains, all with very similar conserved residues in the peptide binding groove, it is inevitable that different SH3 domains bind with similar affinity to overlapping sets of ligands. This implies that there cannot be a one-ligand, one-domain correspondence; a particular SH3 domain (or a particular SH3 ligand) is likely to bind a variety of partners in the cell, depending of course on their local concentrations. Thus, in terms of understanding the function of proteins that contain these domains, the news is both good and bad: One can predict with confidence what type of ligand will bind to the domain, but it is very difficult to say which specific ligand might actually be bound at a particular time and place in the cell.

Interactions mediated by binding modules can be either constitutive or induced. Induced interactions are generally regulated by phosphorylation, though other modifications (such as methylation, acetylation, and proline hydroxylation) may also be used. The most venerable example is the SH2 domain, which binds only to peptides containing phosphorylated tyrosine residues. Thus, changes in tyrosine phosphorylation, induced, for example, by activation of mitogen, adhesion, or cytokine receptors, can dramatically alter the subcellular distribution of proteins with SH2 domains. In one sense SH2-containing cytosolic proteins act as sensors, ready to respond at a moment's notice to the creation of phosphorylated binding sites. Other phosphorylation-dependent binding modules have recently been identified, including several whose binding requires serine or threonine phosphorylation [9]. Similarly, a number of modules are now known to bind to specific phosphorylated inositol lipids, thus allowing recruitment of proteins to the membrane in response to changes in phosphoinositide metabolism [10].

Roles in Signaling

From the perspective of the overall logic of signaling mechanisms, overlapping binding specificity means that a particular input is likely to have many different outputs. This is particularly clear for adaptor proteins, which function solely to mediate protein interactions [11]. In the case of SH2/SH3 adaptors, for example, the SH2 domain can recruit it to many different tyrosine-phosphorylated sites in a cell, and the SH3 domains can bring many different effectors along for the ride. This property allows the signaling process to be very sensitive to the potential binding partners available in the cell, their local concentrations, and their state of posttranslational modification—ideal for a system that must integrate many inputs and generate outputs that are precisely controlled in time and space. Such an ability also allows flexibility, in that a limited number of component parts can respond to and generate a diverse array of signals depending on need and context. This is conceptually similar to the ability of an enzyme (e.g., a kinase) to modify many different substrates, thereby radiating an activating signal to many disparate effectors.

It is also important to consider the kinetics of protein interactions mediated by modular domains. Where they have been measured, most of the dissociation constants for such interactions are in the range of 10^{-8} to 10^{-5} *M*, strong enough to be biologically meaningful but hardly so tight as to be functionally irreversible. Assuming reasonably rapid association rates, these modest K_d values imply that the half-times of the interactions are on the order of seconds. Thus, even "constitutive" complexes mediated by these interactions are not at all like the stably associated subunits of a holoenzyme, but are more a dynamic ensemble of interactions that are constantly breaking apart, resorting, and swapping partners. The components of a complex may change rapidly to adjust to an altered landscape of potential partners due to changes in subcellular localization or as other partners are recruited to the vicinity. Despite this overall plasticity, modular binding interactions also have the potential to mediate relatively stable complexes. Many signaling proteins contain multiple modular binding domains and/or binding sites, allowing the formation of multidentate contacts between binding partners. Protein complexes that are held together by multiple interactions are much less likely to dissociate, even if the individual interactions are quite weak.

Although modular protein interaction domains are usually thought of as mediating interactions between two (or more) different proteins, it is now appreciated that there is often a dynamic equilibrium between intra- and intermolecular interactions. In these cases, a relatively weak intramolecular interaction constrains the protein in one conformation; high local concentrations of either an exogenous domain or ligand can out-compete the intramolecular interaction, leading to global changes in the overall conformation of the protein (Fig. 1). The now classic example of this *cis-trans* switch is the Src family of kinases, where the catalytic domain is held inactive by a series of intramolecular

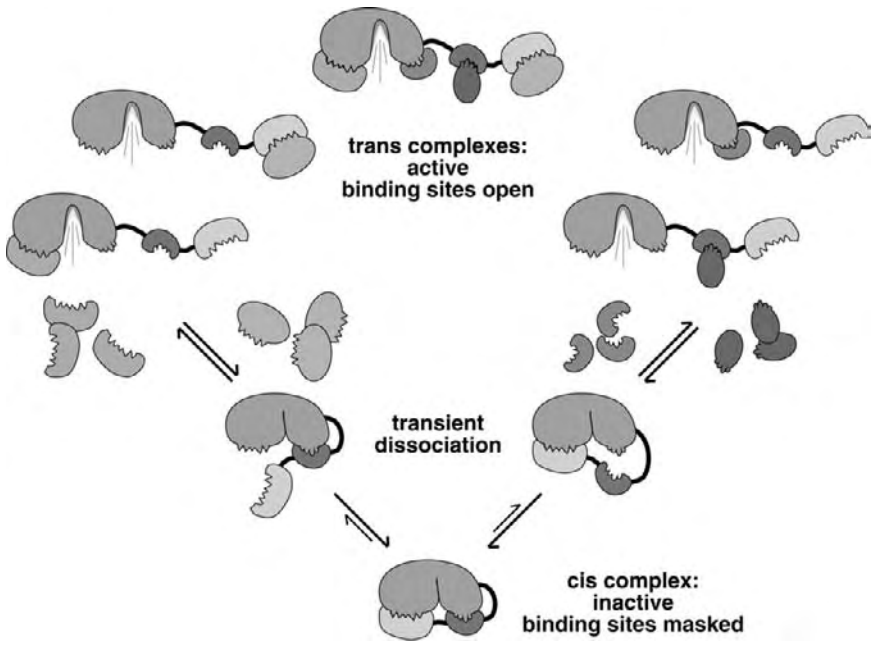


Figure 1 Regulation of protein activity by *cis-trans* binding equilibria. A hypothetical protein (loosely modeled on the Src family of tyrosine kinases) is depicted, consisting of a catalytic domain and two modular protein binding domains. In the absence of high concentrations of binding partners, the protein binding domains interact in *cis* with other regions of the enzyme, inhibiting its catalytic activity and masking binding sites for other proteins (bottom). Occasionally these intramolecular interactions will dissociate, transiently making the binding sites available for interaction with other proteins in *trans* (middle). If high enough concentrations of binding partners are present, binding in *trans* will be favored and the *cis* complex will be disrupted (top). The catalytic domain is now active and multiple binding sites are available to interact with other partners (substrates, scaffold proteins, etc.). Thus, the activity of such a protein will be very sensitive to the local concentration of potential binding partners.

interactions involving its SH2 and SH3 domains [12]. Disruption of these interactions with high-affinity ligands leads to a more open conformation, unleashing the catalytic activity and freeing the SH2 and SH3 domains to bind to other ligands, such as substrates or proteins that can anchor the kinase to a particular subcellular location. This regulatory scheme permits activation of the kinase at very precise sites in the cell or by high local concentrations of a particular substrate which can disrupt the intramolecular interactions and then bind tightly to the activated kinase. A host of other examples could be cited, such as activation of the neutrophil cytosolic oxidase [13], assembly of membrane signaling complexes by MAGUK proteins [14], and regulation of guanine nucleotide exchange factors for small GTPases. Therefore it is important to bear in mind that identification of binding partners may reveal only one part of the role played by modular binding domains in a protein.

Prospects

The fact that signaling pathways are critically dependent on interactions mediated by modular protein domains is fortunate from an experimental perspective, because it gives us a ready means to move up and down signaling pathways by defining interaction partners. Because most interaction domains are easily identified by sequence analysis programs, we know for any protein of interest what domains are present and thus what type of ligands are likely to interact with it. Because the domains fold independently we can assess their role by destroying their activity in the native protein by mutagenesis, or we can express the domain in isolation to fish out its binding partners. Despite these considerable advantages, however, it has proven quite difficult in practice to move from domain to interaction partner to function. In large part this is due to the overlapping binding specificities of modular domains—it is easy to identify partners but frustratingly difficult to assign any functional role to a particular interaction.

In order to truly define the signaling networks in a particular cell, we will ultimately need several types of information. First, we will need to define all of the high-affinity interactions in which a particular domain (or a particular binding site) can engage, preferably with some sense of the relative affinity of each interaction. Genome-wide yeast two-hybrid and phage display projects are well on their way to providing this information for relatively simple organisms [15–17]. Of course many of the possible interactions defined by such projects cannot actually occur in a specific cell, because the two proteins may not be expressed at the same

time or may be localized to different subcellular compartments. Thus, we need to develop tools to identify those interactions that actually occur in a cell of interest, at the time of interest and in the appropriate locale. Co-purification strategies will address some of these issues [18,19], as will simple far-western profiling experiments [20], but more sensitive high-throughput methods are clearly needed. The final piece of the puzzle, and perhaps the most challenging, is to assess the functional significance of a particular interaction. This is remarkably difficult at the moment; we can mutate domains or binding sites but, given that each is likely to interact with multiple partners, we cannot say with any certainty what phenotypic effects are due to a particular pairwise interaction. High-throughput methods to constitutively or inducibly force specific pairs of proteins to interact in the absence of competing interactions must be developed. Once these technical hurdles are overcome, we will be well on our way to truly defining the signaling state of the cell, a complex and dynamic equilibrium of interactions from which arises its precise spatial organization and its ability to integrate and respond appropriately to a diverse array of signals.

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CHAPTER 80

Non-Receptor Protein Tyrosine Kinases in T-Cell Antigen Receptor Function

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Introduction

Engagement of antigen receptors expressed on T and B cells utilize four distinct families of cytoplasmic protein tyrosine kinases (Src, Syk, Tec, and Csk) that are required for the efficient generation of second messengers necessary for lymphocyte function and development. The coordinated activation of these PTKs by the antigen and coreceptors modulate both quantitative and qualitative aspects of signaling that control the biological fate of a given lymphocyte. Loss of any of these cytoplasmic PTKs abrogates antigen receptor function and induces developmental abnormalities of the immune system. Studies over the past 5 years utilizing molecular, structural, and genetic approaches have elucidated multiple mechanisms by which antigen and coreceptors can modulate PTK function. This review captures the basic concepts that have evolved from these studies.

T-Cell Antigen Receptor Structure

Antigen receptors functionally consist of both an antigen binding and a signaling module. In T cells, the predominant T-cell antigen receptor expressed on peripheral T cells is encoded by an antigen-specific and highly divergent disulfide-linked heterodimer consisting of α and β subunits. The antigen-binding module is noncovalently associated with a large signaling complex consisting of one γ, one δ, two ε and two ζ subunits, the latter of which exists predominantly as a disulfide-linked homodimer (Fig. 1) [1]. Each of these signaling subunits contains at least one copy of a signaling motif, the ITAM (for immunoreceptor tyrosine based activation motif); the ζ subunit encodes three ITAM sequence and has a consensus sequence of D/E XXYXXL X_{6-8} YXXL [2,3]. The two tyrosine residues within the ITAM are constitutively phosphorylated in resting T cells [4–6], although the precise stoichiometry of each of basal phosphorylation sites remains unclear. While studies in a T-cell hybridoma had suggested a hierarchy and an ordered sequence of ζ-chain phosphorylations [7], studies in thymocytes and in mice expressing mutant ζ-chain subunits indicate substantial redundancy in ITAM-mediated lymphocyte functions [8,9].

Src PTKs

Two members of the Src PTKs, Lck and Fyn, have been implicated in T-cell antigen receptor function and development. Studies of lymphocytes lacking the Lck and Fyn members of the Src family of PTKs have implicated Lck and, to a lesser degree, Fyn, as being responsible for the phosphorylation of the T-cell receptor (TCR) ITAM sequences [6,10–13]. Fyn was the first cytoplasmic PTK

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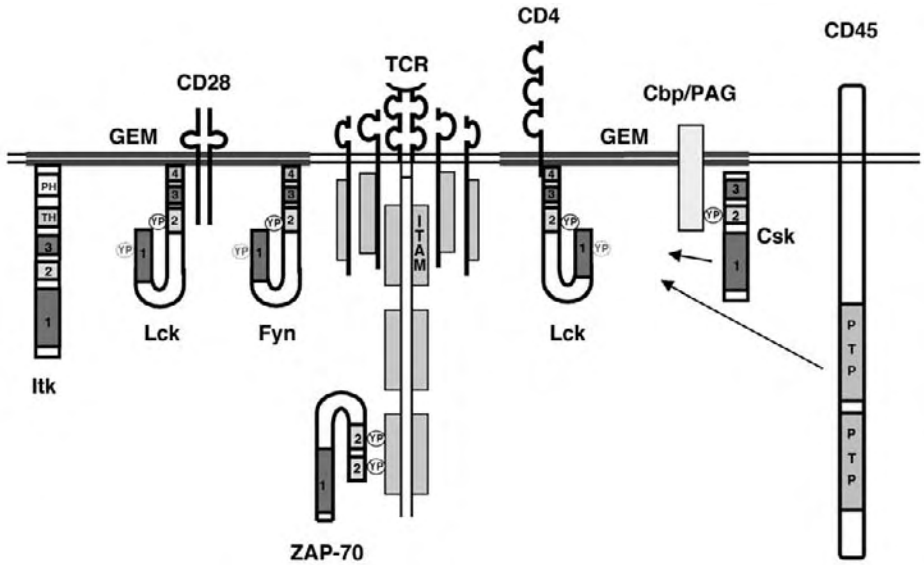


Figure 1 Schematic representation of PTKs in T-cell antigen receptor function. 1 = SH1, 2 = SH2, 3 = SH3, 4 = SH4, PH = pleckstrin homology, TH = Tec homology, YP = phosphorylated tyrosine, PTP = protein tyrosine phosphatase domains, ITAM = immunoreceptor tyrosine-based activation motif.

found associated with the TCR subunits and Lck is noncovalently associated with the CD4 and CD8 coreceptors that colocalize with the antigen receptor upon receptor engagement [14–17]. How the basal phosphorylation of the TCR ITAMs is controlled remains unclear, but undoubtedly represents a net balance of continued phosphorylation, dephosphorylation, and internalization of antigen receptors.

Similar to other Src PTKs, Lck and Fyn contain four structural domains (SH1, SH2, SH3, and SH4) [18,19]. The SH4 domain encodes the amino (N)-terminal 15 amino acids and facilitates the subcellular localization of the kinase through post- and cotranslational modifications that include myristoylation and palmitoylation [20–22]. These modifications preferentially localize Lck and Fyn into subspecialized domains within the plasma membrane referred to as *glycolipid enriched microdomains* (GEMs) [23,24]. These microdomains differ in their cholesterol and phospholipid content which results in differences in the lateral mobility of proteins that reside within GEM and non-GEM fractions. In addition, the association of Lck with the CD4 and CD8 coreceptors that are also enriched within the GEMs further preferentially localizes Lck within these microdomains. T cells that express mutants of Lck that cannot localize into the GEMs are unable to initiate TCR activation events [25,26]. Hence, proper subcellular localization of the Src PTKs, through the SH4 domain, is requisite for TCR functions.

Carboxy (C)-terminal to the SH4 domain are the SH3 and SH2 modules, which bind a variety of signaling proteins to mediate the assembly of signaling complexes as well as to regulate SH1 (kinase) domain enzymatic activity. Determining the structures of the Src family PTKs has provided major insights into their regulation [27,28]. First, a greater understanding of how phosphorylation of the activating autocatalytic and inhibitory C-terminal tyrosine residues regulates kinase activity has been established. Phosphorylation of the C-terminal tyrosine residue (Tyr 505 in Lck and Tyr 527 in Fyn) by Csk (c-Src kinase) downregulates Lck and Fyn activity by promoting an intramolecular interaction with its SH2 domain. This intramolecular binding event favors a "closed, less active" conformation by altering substrate accessibility into the catalytic pocket. Conversely, dephosphorylation of the C-terminal tyrosine residue by the CD45 protein tyrosine phosphatase disengages the inhibitory conformation and permits greater substrate access to the enzymatic core. The SH3 and SH2 domains further contribute to the effect of the C-terminal inhibitory tyrosine by stabilizing the closed conformation with tail phosphorylation [29].

Second, an additional role for the SH3 domain of Src PTKs in regulating Src PTK function has been demonstrated [27]. Binding of a high-affinity SH3-binding peptide or the HIV-Nef viral protein contributes to Hck (another Src family PTK) activation. Recently, SH3-binding peptides derived from the cytoplasmic domain of CD28 have been shown to be capable of activating Lck and Fyn PTKs [30]. Moreover, mutation of the corresponding proline residues within this motif results in loss of CD28-mediated coreceptor functions.

Finally, the mode of lymphocyte activation can also affect the activation status of Lck. While crosslinking of the TCR with anti-CD3 monoclonal antibodies is unable to induce Lck autophosphorylation on Tyr 394, crosslinking of CD4 or CD28 coreceptors alone can induce Lck autophosphorylation [31]. As each of these structural domains contributes to Src PTK activation, receptor and coreceptor engagement likely contributes to maximal Lck functional activity through distinct mechanisms involving relocalization within membrane subdomains as well as direct enzymatic activation.

Csk (c-Src PTK)

Phosphorylation of the negative regulatory C-terminal tyrosine residue of Src PTKs is mediated by a balance between the Csk PTK and the CD45 protein tyrosine phosphatase (PTPase). The Csk PTK is structurally similar to Src PTKs, although they lack the auto- and negative regulatory tyrosine residues within their catalytic cores as well as the SH4 domain responsible for GEM localization [32]. The recent description of the CBP (Csk-binding protein; also known as PAG, for phosphoprotein associated with GEMs) has provided some intriguing insights into Csk regulation. CBP/PAG is a palmitoylated transmembrane adaptor protein that is enriched in GEMs [33,34]. CBP/PAG is phosphorylated on tyrosine residues that mediate its interaction with the SH2 domain of Csk. This interaction mediates the colocalization of Csk to the GEM fraction, which as discussed previously is also enriched in Src PTKs, and facilitates Csk phosphorylation of the negative regulatory tyrosine residue of Src PTKs [35]. In turn, Src PTK enzymatic activity is inhibited. Following receptor engagement, CBP/PAG is thought to be dephosphorylated, resulting in dissociation of Csk from the GEM-localized fraction. Hence, Csk regulation of Src PTKs may regulate the threshold of antigen receptor and provide an inhibitory feedback loop for antigen receptor function [36].

More recently, CBP/PAG has also been implicated in the anchoring of GEMs to the T-cell cytoskeleton. CBP/PAG contains a C-terminal VTRL sequence that mediates its interaction with EBP50 (ezrin/radixin/moesin-ERM binding protein of 50 kDa and also known as NHERF [Na^+/H^+ exchanger regulatory protein]) [37,38]. In light of this cytoskeletal connection, overexpression of CBP/PAG in T cells inhibits GEM mobilization and formation of the immunologic synapse, in addition to inhibiting TCR-mediated signaling functions. How the cytoskeleton interaction may regulate Csk function requires additional investigation.

ZAP-70/Syk PTKs

Tyrosine phosphorylation of the receptor-encoded ITAMs mediates the binding of the ZAP-70 and Syk PTKs to the TCR. This family of cytoplasmic PTKs is unique in that they encode tandem SH2 domains that mediate their interaction with the TCR-encoded ITAMs [39,40]. While studies in transformed T-cell lines initially demonstrated that the TCR-encoded ITAMs undergo tyrosine phosphorylation following TCR crosslinking, subsequent studies of nontransformed T cells indicate that the receptor already contains a substantial amount of constitutively phosphorylated ITAMs [39]. Phosphorylation of both tyrosine residues within the ITAM is required for the high-affinity and functional binding of the ZAP-70/Syk PTKs [41–43]. Solution of the N-terminal domains of these two PTKs demonstrates that they adopt a head-to-tail configuration to bind the dually phosphorylated ITAM [44,45]. Hence, the N-terminal SH2 domain binds the C-terminal phosphorylated tyrosine residue while the C-terminal SH2 domain binds the N-terminal phosphorylated tyrosine residue. Mutation of either tyrosine residue or inactivation of either SH2 domain decreases the activity of the interaction by >100-fold and results in an inactive antigen receptor.

As ZAP-70 is constitutively associated with the TCR, regulation of the ZAP-70 PTK is achieved through transphosphorylation of ZAP-70 by the Src PTKs [46,47]. Phosphorylation of Tyr 493 within the transactivation domain of ZAP-70 results in an upregulation of ZAP-70 enzymatic activity. Intriguingly, the transactivation loop also contains a second tyrosine residue (Tyr 492), which, when subsequently phosphorylated following Tyr 493 phosphorylation, downregulates ZAP-70 enzymatic activity. Hence, sequential tyrosine phosphorylation of the transactivation loop tyrosine residues positively and negatively modulates ZAP-70 enzymatic function.

While ZAP-70 and Syk are structurally homologous, these two PTKs differ in some aspects of their enzymatic activation. Binding of Syk to the doubly phosphorylated ITAM results in an alteration in structural conformation that leads to Syk autophosphorylation and auto-activation [48]. In addition, the intrinsic Syk kinase activity has been estimated to be 10- to 100-fold greater than ZAP-70 [49]. Hence, ZAP-70 appears to be a less active kinase that is subject to greater degrees of enzymatic regulation. In addition, overexpression of Syk in transformed T-cell lines can overcome the requirement for the CD45 PTPase in TCR activation [50,51]. These intrinsic differences between these two structurally homologous PTKs may provide a mechanistic basis for their different roles in cellular and developmental distribution and function.

The *in vivo* functions of ZAP-70 and Syk also differ. During T-cell development, $CD4^-CD8^-$ thymocytes signal through the pre-TCR (consisting of invariant pre-Tα; and β subunits) to mature into $CD4^+CD8^+$ thymocytes. The pre-TCR utilizes either ZAP-70 or Syk to facilitate this transition [52]. In contrast, the αβ-TCR, which facilitates the later transition from $CD4^+CD8^+$ to single positive ($CD4^+$ or $CD8^+$) thymocytes utilizes ZAP-70 and not the Syk PTK [53]. These differences may relate to the differential distribution of the ZAP-70 and Syk kinases during T-cell development as well as to distinct signaling requirements of the pre- and αβ-TCRs [54].

In addition to regulation by tyrosine phosphorylation of the transactivation loop and structural conformation changes induced by binding to a doubly phosphorylated ITAM, the interdomain B region of ZAP-70 and Syk PTKs contains three tyrosine residues that are phosphorylated following receptor activation. Phosphorylation of Tyr 292 in ZAP-70 (and Tyr 319 in Syk) serves as a binding site for the N-terminal SH2-like domain of the Cbl E3 ubiquitin ligase [55–63]. Mutation of these tyrosine residues within interdomain B results in a hyperactive antigen receptor with prolonged phosphorylation of cellular substrates. Conversely, overexpression of c-Cbl results in decreased antigen receptor function but requires the

presence of Tyr 292 in ZAP-70. Hence, the interaction of Cbl with the ZAP-70/Syk PTKs may play an important role in the downregulation of antigen receptor function through a proteosome-dependent mechanism.

While phosphorylation of Tyr 292 within the interdomain B of ZAP-70 serves an inhibitory function in TCR-mediated activation, phosphorylation of two other interdomain B tyrosine residues (Tyrs 315 and 319 of ZAP-70) are required for TCR functions [64–69]. Mutation of either tyrosine residues diminishes, but does not abrogate, activation of phospholipase Cγ (PLCγ)-mediated signaling pathways. Phosphorylation of these residues appears to provide docking sites for the stable assembly of macromolecular signaling complexes that include Lck and PLCγ1. Hence, tyrosine phosphorylation of ZAP-70/Syk is required for enzymatic activation, protein turnover, and the efficient stabilization and phosphorylation of signaling complexes required for lymphocyte function.

Tec PTKs

Two members of the Tec PTKs, Itk/Emt/Tsk and Rlk/Txk, are expressed in T cells and play critical roles in TCR function [70–72]. Studies of mice deficient in either Itk or Rlk demonstrate that these PTKs serve overlapping roles, as these mice exhibit mild T-cell developmental and functional abnormalities [73,74]. Consistent with this functional redundancy, overexpression of Rlk/Txk partially rescues the TCR-mediated signaling defects observed in *itk*$^{-/-}$ T cells [75]. Moreover, mice deficient in both *itk* and *rlk* demonstrate more significant functional defects in coupling to TCR-induced PLCγ-mediated signaling pathways [74]. In turn, these two members of the Tec PTKs are required for T-cell proliferation, differentiation, cytokine production, and immunity against intracellular pathogens [76–79].

The Tec PTK family represents a unique class of nonreceptor tyrosine kinases that is regulated by both Src- and lipid (PI3K) kinases. The structure of the Tec PTKs resembles Src PTKs in that they contain a C-terminal SH1 and N-terminal SH2 and SH3 domains. However, Tec PTKs lack the N-terminal myristoylation signal and the C-terminal inhibitory tyrosine signatures of Src PTKs. In addition, the Tec PTKs have a unique Tec homology (TH) domain, which consists of a Btk homology region, a proline-rich sequence, and an N-terminal pleckstrin homology (PH) domain (except for Rlk/Txk, which has a palmitoylated cysteine string motif). The PH domain and the cysteine string motif facilitate proper subcellular localization of Itk to the GEMs within the plasma membrane [80].

Activation of Itk is first initiated by its localization to GEMs (with the exception of Rlk/Txk, which is constitutively localized) through a PI3K-dependent mechanism [81–83]. TCR engagement activates PI3K to convert PtdIns(4,5)P2 (PIP2) to PtdIns(3,4,5)P3 (PIP3). The direct interaction of PIP3 with the Itk PH domain, localizes Itk with Src PTKs within GEMs, and facilitates Itk transphosphorylation by the Src PTKs [84].

Solution of the NMR structure of the SH3 domain of Itk has also provided further insights into Tec PTK regulation. The SH3 domain of Itk interacts through an intramolecular interaction with a proline motif in the TH domain and has been proposed to hold the kinase in an inactive state [85,86]. In addition to SH3 domain engagement, binding of ligands to SH2 and PRR domains of Itk can coordinate an open conformation and allow for transphosphorylation by Src PTKs [83].

One such mechanism is facilitated through the interaction of Itk interaction with a number of tyrosine phosphorylated adaptor proteins. The LAT adaptor protein is a transmembrane, GEM-localized protein that is phosphorylated by the ZAP-70/Syk PTKs following TCR crosslinking. Tyrosine phosphorylation of LAT facilitates its binding to a number of signaling complexes that include PLCγ1, PI3K, the Grb2/Sos/Ras complex, and the Gads/SLP-76 signaling complex [87]. Tyrosine phosphorylation of SLP-76 facilitates the cooperative binding of the Itk SH2/SH3 domain with the tyrosine phosphorylated SLP-76 molecule [88]. Association of Itk with phosphorylated SLP-76 relieves the intramolecular inhibition of Itk and allows for transphosphorylation of Itk by the Src PTKs. Hence, the dual mechanisms of appropriate subcellular localization mediated by the PH domain of Itk and intermolecular interactions of the Itk SH2/SH3/PRR domain with adaptor proteins regulate Itk activation.

The SH2 domain of Itk also binds and serves as a substrate for the peptidyl-prolyl isomerase cyclophilin A [89]. Nuclear magnetic resonance (NMR) analysis of the Itk SH2 domain has revealed both *cis*- and *trans*-proline conformers in solution. Interestingly, these two conformers bind distinct ligands, with the *cis*-conformer being favored in the presence of the Itk SH3 domain and the *trans*-conformer being favored in the presence of a phosphotyrosine binding peptide. Hence, ligand binding to Itk not only regulates enzymatic function but also may control distinct downstream signaling pathways that result in different biologic fates.

Finally, Itk also plays a role in CD28 coreceptor function [82,90]. The proline-rich motifs within the cytoplasmic domain of CD28 associate with the SH3 domain of Itk and contribute to Tec and Itk enzymatic activation [72,91,92]. While the extracellular domain of CD28 contributes to intercellular adhesion between the antigen presenting and T cells, the cytoplasmic tail of CD28 amplifies PLCγ;1 activation through an Itk dependent mechanism [93]. Together, these data further support the notion that multiple triggering mechanisms contribute to efficient Itk function.

Summary

Structural, biochemical and genetic studies have significantly enhanced our understanding of PTK function in T cell antigen receptor activation. While much has been learned, a precise understanding of the kinetic and spatial arrangements of these mechanisms is still lacking. The application

of more sophisticated techniques of analysis using microscopy and reagents that specifically recognized the activated forms of enzymes and phosphorylated adaptor proteins will undoubtedly further our understanding of this process and how altered forms of signaling may contribute to human disease.

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CHAPTER 81

Cbl: A Physiological PTK Regulator

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Introduction

Protein tyrosine kinases (PTKs) are powerful biochemical switches that require regulatory mechanisms to restrain their potency following activation by physiological stimuli. Indeed, sustained signaling from PTKs promotes tumorigenesis and is involved in the progression of some human cancers. For this reason, members of the *Cbl* protooncogene family are of interest because of their role as negative regulators of PTKs. Three key discoveries led to this classification: first, the identification of a Cbl protein in the nematode *Caenorhabditis elegans* that negatively regulates signaling from a receptor protein tyrosine kinase (RPTK); second, the presence of a unique domain in all Cbl proteins that recognizes phosphorylated tyrosine residues present on activated PTKs; and, third, the ability of all Cbl proteins to recruit ubiquitin conjugating enzymes (E2s) to, and direct polyubiquitylation and degradation of, activated PTKs. Cbl proteins therefore function by specifically targeting activated PTKs and mediating their downregulation, thus providing a mechanism by which signaling processes can be controlled.

Domains of Cbl Proteins

The c-*Cbl* protooncogene was first identified as part of a recombinant mouse retrovirus that induced pre-B-cell lymphomas. Since then, two additional mammalian homologs (Cbl-b and Cbl-3) have been found, as well as orthologs in *Drosophila* (D-Cbl) and *C. elegans* (Sli-1) [1–3]. These proteins share a highly conserved amino-terminal region consisting of three domains—a four-helix bundle, a calcium-binding EF hand domain, and a variant SH2 domain—which together recognize specific phosphotyrosine residues present on activated PTKs (Fig. 1). Because all three domains are required to form this unique phosphotyrosine-binding module, the region is commonly referred to as a tyrosine kinase binding (TKB) domain [4]. Two other regions highly conserved among all Cbl proteins are a zinc-binding C3HC4 RING finger and a short linker sequence connecting the TKB and RING finger domains, which together recruit E2 proteins [5–8]. Sequence homology is less extensive C-terminal to the RING finger; however, with the exception of D-Cbl, all Cbl proteins possess proline-rich regions involved in interactions with Src homology domain 3 (SH3)-containing proteins (Fig. 1). c-Cbl is also a prominent substrate of PTKs and is phosphorylated following the engagement of many cell-surface receptors. Phosphorylation of c-Cbl by PTKs provides docking sites for Src homology domain 2 (SH2)-containing proteins (Fig. 1) and is required for its E3 ligase activity [6,9]. The C termini of c-Cbl and Cbl-b encompass a conserved domain known as a ubiquitin-associated (UBA) domain. The UBA domain interacts with ubiquitin and was identified by sequence homology to regions found in subsets of E2 conjugating enzymes and E3 ligases. The UBA domain in Cbl proteins is of interest, as c-Cbl has been shown to be subject to ubiquitylation [9,10].

Sli-1: A Negative Regulator of RPTKs

Evidence that Cbl proteins have functions regulating PTKs initially came from genetic studies in *C. elegans* [11].

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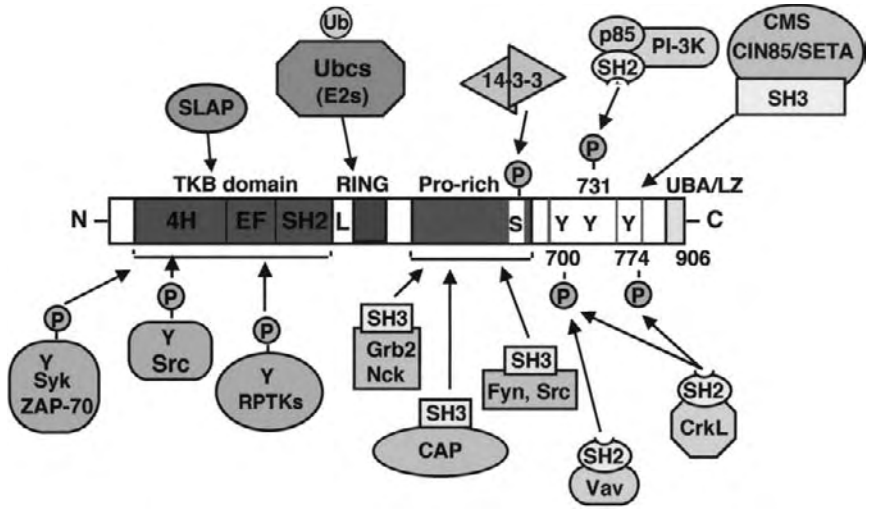


Figure 1 c-Cbl interacts with many signaling proteins. Some of these interactions occur after the activation of PTKs which results in recruitment of c-Cbl to receptor complexes and the targeting of phosphorylated tyrosines on PTKs by the TKB domain. This enables PTKs to be downregulated by c-Cbl-associated proteins such as E2 ubiquitin-conjugating enzymes recruited by the RING finger and linker (L) domains and CIN85, which is recruited by proline and phosphotyrosine motifs and is involved in mediating RPTK internalization. c-Cbl phosphorylation on tyrosine (Y) and serine (S) residues promotes associations with many SH2-containing proteins and 14-3-3 proteins, respectively. Some associations are constitutive, such as those with SH3-containing proteins that bind one or more of the 15 potential SH3-binding proline motifs within the C-terminal half of c-Cbl.

Loss-of-function mutations in *Sli-1* restored signaling for vulval induction and survival through a weakly active epidermal growth factor receptor (EGFR) homolog LET-23, whereas introducing additional copies of *Sli-1* suppressed vulval induction. These experiments indicated that Sli-1 is a negative regulator of LET-23 that functions upstream of LET-60/Ras. Studies in *Drosophila* also suggest that Cbl can negatively regulate RPTK signaling: D-Cbl suppresses the development of R7 photoreceptor cells in flies on a sensitized genetic background [12] and functions as a negative regulator of a dose-sensitive EGFR pathway involved in dorsoventral patterning during oogenesis [13].

PTK Downregulation by Polyubiquitylation

The demonstration of a prominent and inducible association between Cbl proteins and the activated EGFR in mammalian cells clearly demonstrated that genetic studies in *C. elegans* had correctly predicted the site of Cbl action [1–3]. In addition, introducing a corresponding loss-of-function mutation from Sli-1 into c-Cbl blocks interaction of the TKB domain with activated PTKs and abolishes fibroblast transformation by oncogenic forms of Cbl [1–3]. The identification of specific phosphotyrosine residues within the EGFR (pY1045), Met receptor (pY1003), Src (pY416), Syk (pY323), and ZAP-70 (pY292) that are recognized by the TKB domain also supports the proposal that Cbl is involved in their regulation [6,14–16].

A significant breakthrough in identifying a regulatory mechanism that could explain the function of Sli-1 came from observing the profound effect of c-Cbl overexpression on the promotion of PDGF, EGF, and colony-stimulating factor 1 (CSF-1) receptor polyubiquitylation and downregulation [17–19]. Receptor polyubiquitylation was found to be directly mediated by c-Cbl and to be dependent on the integrity of the TKB, linker, and RING finger domains [20,21]. Thus, c-Cbl can promote the polyubiquitylation of activated RPTKs, which targets them for degradation and prevents their recycling from early endosomes back to the cell surface (Fig. 2). *In vitro* reconstituted ubiquitylation assays demonstrated that the c-Cbl RING finger has intrinsic E3 ligase activity and can independently recruit E2s and direct ubiquitin transfer to substrates [5–7]. Furthermore, the structure of c-Cbl bound to an E2 (UbcH7) identified multiple contacts between the RING finger and linker domain of c-Cbl and UbcH7 [8]. These studies defined Cbl proteins as RING-type E3 ubiquitin protein ligases that direct RPTK polyubiquitylation and downregulation. c-Cbl has also been shown to mediate the polyubiquitylation and degradation of active Src, indicating that c-Cbl can also function as an E3 ligase for non-receptor PTKs [22,23]. Interestingly, the E3 ligase activity of Cbl is not solely restricted to PTKs, as Cbl-b overexpression can promote polyubiquitylation of the p85 subunit of PI3-kinase, and c-Cbl polyubiquitylates the T-cell receptor (TCR) ζ chain [24,25].

Cbl-Deficient Mice

The negative regulatory function of Cbl has also been demonstrated in c-Cbl- and Cbl-b-deficient mice, which

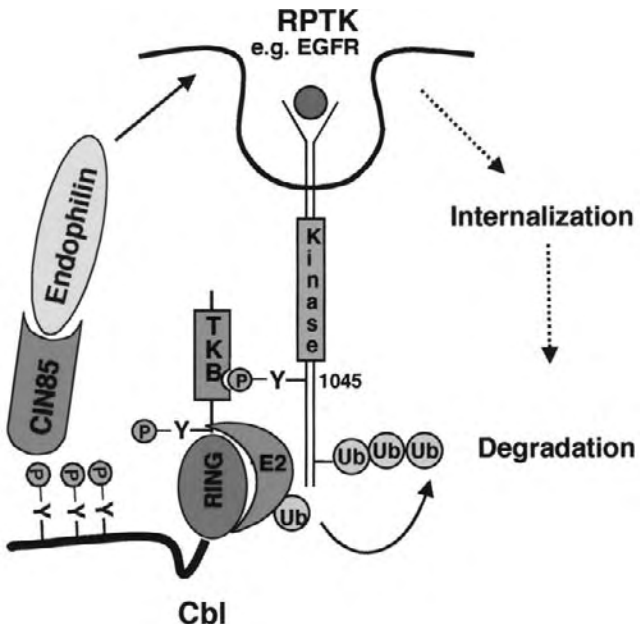


Figure 2 Cbl directs internalization, polyubiquitylation, and degradation of activated RPTKs. Ligand binding induces tyrosine phosphorylation of RPTKs and recruitment of Cbl to the activated receptor (by adaptor proteins not shown). This results in the TKB domain targeting of specific phosphotyrosines on the activated RPTKs (e.g., Y1045 on EGFR). c-Cbl also becomes tyrosine phosphorylated, and this results in the recruitment of a CIN85–endophilin complex that is required for receptor internalization. The E3 ligase function of Cbl catalyzes the transfer of a ubiquitin molecule from the RING-finger-bound E2 to the RPTK. Continued addition of ubiquitin moieties leads to polyubiquitylation, which marks the RPTK for lysosomal or proteosomal degradation. Whether or not the ubiquitin chain assembly that is directed by Cbl involves lysine 48 linkages has not been determined.

show perturbations associated with thymocyte and peripheral T-cell activation, respectively [1,26]. Thymocytes in c-Cbl$^{-/-}$ mice show marked activation of ZAP-70 in response to TCR stimulation, in contrast to wild-type thymocytes, which require costimulation of both the TCR and the coreceptor CD4. The crucial role of CD4 stimulation is to activate Lck, which phosphorylates ZAP-70 to trigger kinase activity. Remarkably, however, in c-Cbl$^{-/-}$ thymocytes ZAP-70 activation can be attained in the absence of Lck activation. The fact that this occurs without hyperactivation of signaling molecules upstream of ZAP-70 suggests that c-Cbl directly regulates ZAP-70, not its activators. The Cbl-b-deficient mouse shows a similar phenomenon, but it occurs in peripheral T cells as opposed to thymocytes. In this case, T-cell proliferation and IL-2 production are uncoupled from a requirement for activation of the coreceptor CD28. Thus, a lowered threshold for TCR signaling is a common theme in both mutant mice. Intriguingly, the uncoupling of a CD28 requirement for T-cell activation in Cbl-b-deficient mice does not involve enhanced ZAP-70 activation but instead results in a significant enhancement in the activation of Vav1. Furthermore, Cbl-b deficiency restores TCR-induced Cdc42 activity and cell proliferation in Vav1$^{-/-}$ mice. Recently, it was shown that the p85 regulatory subunit of PI 3-K, an upstream activator of Vav1, is polyubiquitylated by Cbl-b [24]; however, this does not promote proteolysis of p85 but rather inhibits its recruitment to CD28 and TCRζ, thereby preventing the activation of Vav1 [27]. The Cbl mutant mice have therefore raised interesting questions about the abilities of c-Cbl and Cbl-b to negatively regulate different signaling molecules in distinct T-cell populations.

Future Directions

Recent discoveries have provided a clearer understanding of Cbl function, but key questions remain unanswered. A priority will be to understand whether E3 ligase activity is solely for the purpose of PTK degradation or whether Cbl-directed polyubiquitylation alters the function and fate of PTKs in ways not currently appreciated. Determining the nature of the ubiquitin chain assembly that is directed by Cbl proteins should provide important clues in this area. Furthermore, little is known about the role of Cbl interactions with many other signaling proteins that do not appear to be associated with PTK polyubiquitylation. Indeed, recent findings that loss of E3 activity alone is insufficient to promote transformation [28] and the fact that the RING finger of Sli-1 is partially dispensable for its negative regulation of LET-23 [29] demonstrate that E3 activity is not the sole inhibitory function of Sli-1/Cbl proteins. Clues about how best to approach this aspect of Cbl function may be revealed by further analyses of TKB domain function and a greater reliance on genetically altered organisms using knowledge from the recently resolved structures of the TKB, linker, and RING finger domains. Furthermore, the recent finding that the evolutionary divergent C terminus of Cbl mediates ligand-induced internalization of EGF and Met receptors by recruiting a CIN85/endophilin complex to these receptors is further evidence that Cbl has many adaptations to downregulate PTKs (Fig. 2) [30,31].

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CHAPTER 82

TGFβ Signal Transduction

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Introduction

Transforming growth factor beta (TGFβ) superfamily members are important regulators of many diverse developmental and homeostatic processes, and disruption of their activity has been implicated in a variety of human diseases ranging from cancer to chondrodysplasias and pulmonary hypertension. The superfamily can be divided into functionally distinct subgroupings of ligands; the prototypic TGFβs themselves, the bone morphogenetic proteins (BMPs), and the activins. All members of these TGFβ family subgroups that have been investigated utilize a unique class of signaling receptors that are characterized by the presence of a Ser/Thr kinase domain in their cytoplasmic region. Only 12 transmembrane Ser/Thr kinases have been identified in mammals, and this small family of receptors is further subdivided into two subclasses: the type II and type I receptors. TGFβ family ligands initiate signaling through these receptors by inducing the stable assembly of heterotetrameric complexes of type II and type I receptors. This brings the type II kinase domain, which is constitutively active, into proximity of the type I receptor kinase, thereby allowing the type II receptor to phosphorylate the type I receptor on a Gly/Ser motif called the GS region, which lies on the amino-terminal side of the kinase domain [1]. This phosphorylation event allows the type I receptor to recognize and phosphorylate Smad proteins, which form a key TGFβ signal transduction pathway (Fig. 1).

The Smad Pathway

Smads form a unique class of signaling molecules [2–5]. Eight Smads have been identified in the mammalian genome, and these Smads are subdivided into three distinct classes: the receptor-regulated Smads (R-Smads 1, 2, 3, 5, and 8); the common mediator Smad, of which only one member has been found, Smad 4; and the inhibitory Smads (I-Smads 6 and 7). All Smads are structurally related. They possess an amino-terminal MH1 domain (for MAD homology domain 1) that is poorly conserved in the I-Smads, followed by the linker region and in the carboxy-terminal region a MH2 domain. Each of these domains fulfills critical effector functions in Smad signaling by mediating protein–protein and protein–DNA interactions.

The R-Smads function as direct substrates of the receptor and possess a binding surface on their MH2 domain that accomodates phosphoserine residues separated by single amino acids [6,7]. Thus, phosphorylation of the GS region of the type I receptor likely provides a direct contact point between R-Smads and the receptor that works in conjunction with additional interactions between the MH2 and a specificity loop of the receptor that lies between β-strands 4 and 5 of the kinase domain [8,9]. These interactions are important to mediate matched binding between different type I receptors and specific R-Smads. Consequently, type I receptors that bind BMPs only recognize and phosphorylate R-Smad1, 5, and 8, whereas the TGFβ and activin type I receptors activate R-Smad2 and 3. In addition, an anchor protein called SARA can facilitate activation of R-Smads in the TGFβ/activin pathway by directly binding unphosphorylated Smad2 and recruiting it to cell membranes [10]. SARA has a FYVE domain that binds phosphotidylinositol-3′-phosphate (PI-3′-P). Interestingly, Hrs, another receptor binding protein, also has a FYVE domain and appears to cooperate with SARA in TGFβ signal transduction [11]. Because PI-3′-P and FYVE domain proteins are particularly enriched in the early endosome, these data suggest that trafficking of

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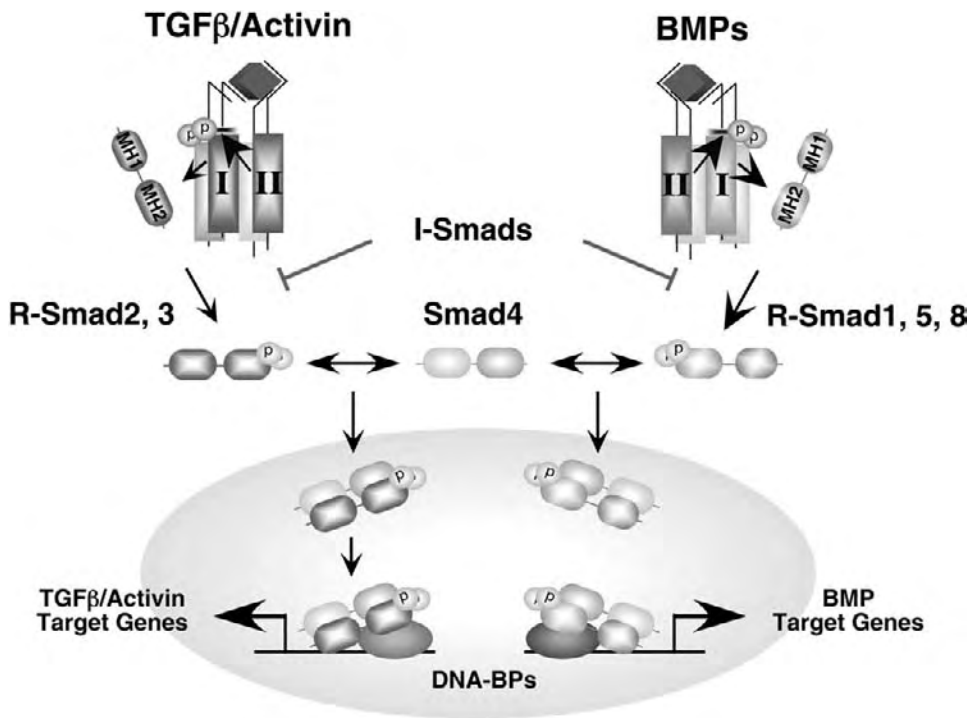


Figure l TGFβ/activin and BMPs signal through distinct receptors and Smads. TGFβ/activin and BMPs bind to different receptor complexes that phosphorylate distinct R-Smads. Phosphorylated R-Smads then bind Smad4 and the complexes accumulate in the nucleus. R-Smad complexes associate with different DNA-binding proteins (DBP-BPs) to regulate distinct target genes and generate diverse biological responses.

the receptors may be important for signaling. Indeed, endocytosis has been noted to be important for initiating TGFβ signal transduction in certain cell systems [12,13]. Another receptor-associated protein that may be involved in this pathway is Disabled-2, which interacts with the TGFβ receptor complex and is important for receptor-dependent activation of Smad2 [14]. This may be particularly important in the visceral endoderm during gastrulation in the mouse [15].

Once bound to the receptor, the type I kinase domain phosphorylates the R-Smad on a carboxy-terminal SSXS motif that is found in all R-Smads [16]. Phosphorylation of R-Smads causes their dissociation from SARA and the receptor and drives homomeric, as well as heteromeric, interactions with the common Smad, Smad4. Interestingly, the same binding surface that likely contacts the phosphorylated GS region also stabilizes the homomeric and heteromeric Smad complexes by binding to the phosphorylated tail of an adjacent R-Smad [6,7]. In addition, phosphorylation drives the R-Smad homomers and R-Smad–Smad4 heteromers to accumulate in the nucleus, where they regulate transcription by interacting directly with DNA and DNA binding partners [2–5]. DNA binding is mediated by the MH1 domain, but in most cases this interaction is not of sufficient affinity and specificity to allow Smad on its own to regulate transcription. Thus, Smad binding to high-affinity DNA binding partners (DNA-BPs) is a critical aspect for recruitment of the Smad complex to specific regulatory elements and functions to facilitate interaction of the MHl domain of Smads with DNA at sites that lie adjacent to the DNA binding partner site. Contact of Smads with DNA in turn plays an important role in stabilizing DNA binding by the ternary Smad–DNA-BP complex. Smads thus bound to regulatory elements can control the transcriptional response by recruiting coactivators such as histone acetyltransferase, MSG, and SMIF or corepressors such as TGIF, SnoN, or histone deacetlyases. In this regard, the role of Smad4 in TGFβ-dependent transcriptional activation seems particularly important, and Smad4 can bind directly to the p300/CBP histone acetyltransferases. Thus, many, but not all, transcriptional responses to TGFβ are lost or blunted in Smad4-deficient cell lines, and, because, Smad4 binds all activated R-Smads, it likely fulfills similar general activation functions in the BMP pathways. Exactly what determines whether Smad complexes recruit coactivators or corepressors is unknown; however, a correlation between the ability of Smad3 to bind DNA via its MHl domain and suppress transcriptional responses of model promoters has been observed [17].

One of the interesting aspects to emerge from this mode of regulation is that many of the Smad DNA-BPs themselves function to mediate trancriptional responses to other cell signaling pathways. For example Smads interact with AP-1, ATF2, vitamin D receptors, and LEF/TCF DNA binding partners which themselves function in MAPK, p38, vitamin D, and WNT signaling pathways, respectively. This leads to an important area of cross-talk between the TGFβ–Smad pathway and other signal transduction pathways. Cross-talk between the Smad pathway can also occur upstream of

transcriptional activation via phosphorylation of Smads in sites other than the carboxy-terminal SSXS motif. Thus, MAP kinases that function downstream of receptor tyrosine kinase pathways can phosphorylate Smads in the linker region and modulate their activity [18], whereas PKC has been shown to phosphorylate Smad3 in the MH1 domain and inhibit DNA binding and activation of Smad3 target elements [19].

In contrast to the R-Smads and Smad4, the two inhibitory Smads, Smad6 and Smad7, function as antagonists of Ser/Thr kinase receptor signal transduction [20–22]. These I-Smads form complexes with the activated type I receptor, prevent access of R-Smads to the receptor, and can mediate ubiquitin-dependent downregulation by recruiting Smurf ubiquitin ligases to the receptor complex [23,24]. Smad6 appears to preferentially target the BMP pathway, whereas Smad7 potently blocks both TGFβ and BMP pathways. Interestingly, expression of the I-Smads is regulated by a variety of signaling pathways that include interferon γ, TNFα, and EGF, as well as TGFβ and BMPs themselves [3]. These latter observations suggests that I-Smads form part of a negative regulatory loop that serves to dampen the activity of ligand-activated Ser/Thr kinase receptor complexes.

Smads and the Ubiquitin–Proteasome System

TGFβ family members are important morphogens that can control distinct cell fate outcomes in response to different concentrations of ligands. By transducing signals directly from the cell-surface receptor to specific gene promoters, the Smad pathway is ideally suited to interpret morphogenetic signals, as the concentration in the nucleus can provide a direct readout of the external concentration of the ligand. Indeed, studies on Smads in *Xenopus* showed that cell fate could be regulated by the concentration of Smad proteins [25]. Smad protein levels are subjected to regulated degradation in the cell by the ubiquitin–proteasome system. In the BMP pathway a C2-WW-HECT domain ubiquitin ligase called Smurf1 (for Smad ubiquitin regulatory factor 1) can block BMP signaling and regulate cell fate by interacting with the R-Smads Smad1 and Smad [26] or MAD in *Drosophila* [27] and target them for ubiquitination and degradation. In certain systems high-level expression of a second Smurf, Smurf2, can also regulate the turnover of R-Smadl [28] or R-Smad2 [29]. In the nucleus phosphorylated R-Smad2 and 3 can be regulated by ubiquitin-mediated degradation [30], mediated at least in part by the ubiquitin ligase Rocl [31], and Smad4 turnover can be regulated by the ubiquitin ligase Jab1 [32]. Moreover, analysis of Smad mutations found in human cancer has shown that some of these mutations inactivate Smads by targeting them for ubiquitin-dependent proteolysis [33]. Thus, Smads are tightly controlled by the ubiquitin-proteasome system.

More than simply targets for ubiquitin-mediated degradation, Smads can also regulate the ubiquitination of associated proteins by functioning as adaptor proteins. The I-Smads play an important role in regulating receptor turnover and do so by forming stable complexes with Smurf1 and Smurf2 and recruiting these C2-WW-HECT domain proteins to the TGFβ and BMP receptor complexes [23,24]. Furthermore, phosphorylated R-Smad2 and 3 can recruit Smurf2 to the nuclear corepressor SnoN, which is a binding partner of Smad2 and Smad3 [34]. This complex potently induces ubiquitin-mediated degradation of SnoN. Interestingly, the anaphase promoting complex ubiquitin ligase also targets SnoN for degradation, and this is enhanced by Smad2- and Smad3-mediated recruitment [35,36]. In addition, Smad3 may mediate degradation of Hef1, which is member of the Cas family of cytoplasmic docking proteins [37]. Thus, Smads may fulfill important roles in regulating protein levels of partner proteins by functioning as adaptors in the ubiquitin–proteasome system.

Smad-Independent Signaling Pathways

Activation of TGFβ family receptors also regulates a number of Smad-independent pathways. One of these involves Daxx, which was found to interact with the carboxy-terminal tail of the TGFβ type II receptor [38]. Dominan-negative Daxx or blocking Daxx expression interferes with TGFβ-dependent apoptosis, and Daxx is important for TGFβ-dependent activation of Jun kinase. As Daxx also mediates similar responses downstream of the Fas receptor, this pathway may reflect a common output of both Fas and TGFβ signaling pathways. Studies on the regulation of S6 kinase have shown that TGFβ can induce protein phosphatase 2A to bind to and dephosphorylate S6 kinase, which, in coooperation with the Smad pathway mediates TGFβ growth arrest in the G1 phase of the cell cycle [39]. As the protein phosphatase 2A regulatory subunit Bα also binds to the receptor [39,40], it suggests that the TGFβ receptor may somehow regulate the activity of PP2A. Whether this pathway plays any role in regulating the phosphorylation status of other substrates in response to TGFβ is unknown.

TGFβ family receptors also can activate numerous other kinases in a cell-type-specific manner. In addition to JNK activation, which is important for induction of fibronectin expression in fibrosarcomas [41], TGFβ has also been reported to stimulate ERK activity in intestinal epithelial cells [42], also, in a variety of cells p38 is activated by TGFβ and BMPs and this may lead to apoptotic responses [43–45]. Furthermore, in epithelial cells derived from the mouse mammary gland, TGFβ can also regulate the Akt/PKB pathway [46]. TAK1, a MAPK kinase kinase that functions in multiple signaling pathways, and its associated activators, TAB 1 and TAB2, have also been implicated to be involved in both TGFβ and BMP pathways [47,48]. Binding of the TAB–TAK complex to Ser/Thr kinase receptor complexes may be mediated by XIAP, which can bind both TAB and the receptor complex [49]. In *Xenopus* systems, this pathway appears to play a role in transducing BMP ventralizing

signals [50]. In addition to kinase cascades, TGFβ also can regulate actin cytoskeletal dynamics by regulating the activity of Rho family GTPases. Thus, Cdc42 and RhoA have been reported to be activated by TGFβ [51,52], with the latter pathway playing a key role in the assembly of stress fibers during TGFβ-induced epithelial-to-mesenchymal transitions. How heteromeric Ser/Thr kinase receptor complexes regulate many of these downstream Smad-independent signaling pathways has to date remained very much a mystery.

Other Receptor Interacting Proteins

A number of other Ser/Thr kinase receptor interacting proteins have been identified through a variety of means [3]. FKBP12 was first identified through yeast two-hybrid screens as a binding partner of multiple type I receptors. FKBP12 binds preferentially to the unphosphorylated GS region of the type I receptors and dissociates upon formation of an active receptor complex. Thus, the protein may function to maintain the type I receptor in an inactive state and prevent spurious activation of the pathway in the absence of ligand. Other proteins that have been shown to associate with receptors include farnesyltransferase-α; TRIP1, which binds the type II receptor; STRAP, which can cooperate with Smad7 to inhibit signaling; TRAP, which also inhibits receptor activity; ARIP, a PDZ and WW domain-containing protein; BRAM, a BMP-receptor-associated molecul; and SNX proteins, which are sorting nexins that might regulate receptor trafficking. Whether these proteins function solely to modulate receptor activation of the Smad pathway or might modulate the activity of the receptor toward Smad-independent pathways is unknown.

Elucidation of the molecular components of the TGFβ superfamily signal transduction pathways has provided important insights into human disease; many human syndromes and illnesses, both hereditary and spontaneous, have been attributed to mutations in this signaling pathway. For instance, mutations in receptors are associated with hereditary hemmorhagic telangiactasia, primary pulmonary hypertension, persistant mullerian duct syndrome, juvenile polyposis syndrome, and colorectal and gastric carcinomas. Mutations in Smads have also been associated with cancers, particularly those of the colon and gastrointestinal tract. Undoubtedly, further elucidation of the molecular mechanisms in this signaling pathway promises to provide new insights into cellular regulation and physiology in health and disease.

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CHAPTER 83

MAP Kinases

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Introduction

The identification in the late 1970s of protein tyrosine kinases (PTKs) as products of oncogenes and growth factor receptors revealed a gaping hole in our understanding of signal transduction. How did such molecules couple to the far more abundant protein serine/threonine kinases that had been known about for several decades? One possibility was that certain serine kinases were themselves phosphorylated on tyrosine. In a search for protein kinases regulated by the insulin receptor-tyrosine kinase, Ray and Sturgill [1] identified a microtubule-associated protein-2 kinase (MAP2 kinase) that, upon treatment of cells with insulin, became phosphorylated on tyrosine (and threonine) and catalytically activated. Many other growth factors were found to activate this enzyme, which led to a refinement of its name to mitogen-activated protein kinase (MAPK). Although MAPKs were soon found not to be the "missing link" for coupling receptor and oncogene tyrosine kinases to intracellular events, their critical role in transducing a variety of signals soon became apparent.

Subsequent molecular cloning of MAPKs revealed additional surprises [2]. First, relatives of the MAPKs were found in other species, most notably yeast [3– 5] With hindsight, the first MAPK to be molecularly cloned [3] was a budding yeast gene termed Fus3 (and a related protein termed Kss1) that encodes a component of the pheromone response pathway. This provided a bonus in that genetic analysis of the pheromone pathway in yeast had revealed several other gene products that regulated the yeast MAPK. Moreover, these components had been ordered and formed a protein kinase cascade [5]. It also became clear that nature had generated multiple variants of MAPKs that played different functions and responded to distinct stimuli. The term *MAPK* is now commonly used to denote an entire class of protein serine kinases that share several key features, including regulation by tyrosine and threonine phosphorylation and organization into a hierarchical cascade of kinases. Generically, the MAPKs are phosphorylated at a threonine–X–tyrosine motif that lies in the T-loop of their kinase domain [6]. Both threonine and tyrosine phosphorylations are catalyzed by a single, dual specificity protein kinase known as MAPK kinase (MAPKK). These enzymes are, in turn, phosphorylated (at serine/threonine residues only) and activated by another class of enzymes, the MAPKK kinases (MAPKKKs) (Fig. 1).

As mentioned, yeast FUS3 was the first MAPK to be genetically identified [3]. Additional genetic analysis in budding yeast identified components for five distinct MAPK pathways. These MAPK pathways are essential for processes such as mating, sporulation, osmoregulation, cell wall integrity, starvation, and filamentous growth [5,7,8]. To date, the sequences of over 100 eukaryotic MAPKs have been reported. In mammals, three major MAPK signaling modules have been described: the original MAPK or extracellular signal-regulating kinase (ERK) and two MAPK cascades that respond to cellular stresses, Jun kinases (SAPK, or c-Jun NH_2-terminal kinase [JNK]) and p38 MAPKs (Fig. 1) [9].

The ERK Module

In mammals, the prototypical MAPKs are encoded by two genes, Erk-1 and -2, generating proteins of 44 and 42 kDa, respectively [2]. Like most of the MAPK proteins, these enzymes are widely expressed and are generally not regulated at the transcriptional level. These enzymes are phosphorylated and activated by MAPK/ERK kinases (MEKs) 1 and 2, which target a threonine and a tyrosine residue within the T-loop of the kinase domain [10,11].

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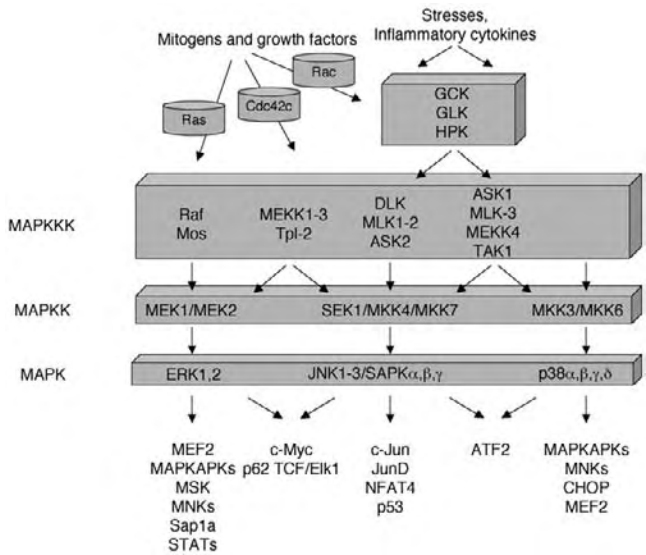


Figure 1 Mammalian MAPK modules. The MAPK module consists of a MAPKKK, MAPKK, and a MAPK. These pathways respond to extracellular signals, including growth factors, hormones, cell stresses, and cytokines. Once activated, MAPKs can phosphorylate a wide variety of proteins, including transcription factors and other kinases. See text for details.

When phosphorylated, this loop swings out, allowing adenosine triphosphate (ATP) and substrate to access the catalytic pocket [6]. MEK activity is similarly dependent upon phosphorylation at two T-loop serine residues by upstream kinases, typically Raf1 but also B-Raf, Mos, or MEKK1 [12].

The availability of phospho-specific antibodies selective for the T-loop phosphorylation sites of ERKs and MEKs has simplified monitoring of the modification of these enzymes following cellular stimulation. Perhaps unexpectedly, such studies have shown that the triad of protein kinases that lead to MAP activation does not function as an amplification circuit, as occurs, for example, in cAMP-dependent activation of glycogen phosphorylase. Instead, the three kinases appear to interact in a one-to-one linear system. So why invest in three kinases when one could do the same job? The answer may lie in the exquisite levels of regulation and sensitivity that are made possible by this arrangement, as well as enhanced specificity. For example, work by Ferrell and colleagues in *Xenopus* has revealed that the MAPK and JNK/SAPK pathways act like switches [13,14]. A stimulus triggers a binary response, on or off with nothing in between. The effect is termed *bistability* and relates to the quantum change required to overcome the activation hurdle (which is opposed by different phosphatases acting on the various MAPK triad components). In yeast, there is another rationale for the three-kinase module. In this case, the same three components can be utilized in different responses, depending on their association with the STE5 scaffolding protein [15]. STE5 acts to insulate the MAPK module by cloistering the kinases together such that they are functionally coupled to a particular stimulus. Loss of STE5 releases the STE11 MAPKKK, which can then couple to a MAPK module activated by hyperosmolarity. Such clustering of signaling molecules is now recognized as a common means to achieve specificity and to counter entropic forces that tend to equalize cellular protein distribution.

Substrates of ERKs include additional protein kinases such as MAPKAP-kinase, signaling molecules such as phospholipases, and transcription factors such as Elk-1/ternary complex factor [9]. These and other ERK targets share an ERK phosphorylation motif minimally comprised of Pro–X–Ser/Thr–Pro, although high-affinity substrates usually harbor additional binding interfaces for the kinase. Online databases such as Scansite [16] are useful tools that use such consensus sequences to predict phosphorylation sites (and the corresponding kinases that modify them) within any protein.

ERK activation is often associated with proliferation (for example, Raf induction is tightly coupled to growth-induced or oncogenic Ras activation), but the consequences of ERK stimulation depends on the signal and cell type. Even in the same type of cell, the kinetics of ERK activation play a defining role in the ultimate response. For example, in PC12 pheochromocytoma cells, slow but sustained ERK activation by nerve growth factor induces neurite outgrowth and differentiation. By contrast, short but sharp activation of the same pathway by epidermal growth factor results in cell proliferation [17]. Thus, the same signaling machinery can deliver different messages within a cell.

Stress-Activated MAPKs, Part 1: SAPK/JNKs

While the ERK family of MAPKs is primarily activated by growth factors and mitogens, the SAPK/JNK and p38 MAPK families are preferentially induced by stress signals, including irradiation, pro-inflammatory cytokines, environmental stress (temperature, osmolarity, pH), oxygen tension, intracellular calcium, and other insults that interfere with cellular integrity.

SAPK/JNKs are encoded by three genes (α, β and γ or JNK2, JNK3, and JNK1, respectively) [18,19]. Differential splicing of the three SAPK/JNK genes generates at least 10 isoforms that encode 54-kDa or 46-kDa proteins. The SAPK/JNKs were originally identified as the major serine/threonine kinases responsible for the phosphorylation of c-Jun, a component of the AP-1 transcription factor [20]. Other SAPK targets include additional Jun proteins (JunB, JunD, and the related activating transcription factor 2 [ATF2]); the ternary complex factor (TCF) subfamily of ETS-domain transcription factors; the tumor suppressor p53; SMAD3; and nuclear factor of activated T cells (NFAT4) [9]. Most characterized SAPK/JNK targets are transcription factors. This is in contrast to ERK and p38 MAPKs, which target proteins throughout the cell. Although the three primary SAPK/JNK proteins exhibit similar substrate specificities, analysis of mice engineered to lack one or more of these genes has revealed differential effects. For example, unlike

the generalized expression of the other two SAP/JNK gene products, SAPKβ/JNK3 is preferentially expressed in neuronal tissue, hinting toward a specialized function. Mice lacking SAPKβ/JNK3 are relatively resistant to kainic acid-induced seizures [21]. Similar results were obtained in mice with a knock-in c-Jun mutation that eliminated the SAPK/JNK phosphorylation sites [22], suggesting that c-Jun is the essential target for SAPKβ/JNK3 in stress-induced neuronal apoptosis. The general consequence of chronic SAPK/JNK signaling is induction of apoptosis, although acute signaling appears protective. In this respect, perhaps this pathway is akin to a long fuse that is lit at the first sign of trouble but if not doused (by subsequent damage control?) it triggers elimination of the cell.

Stress-Activated MAPKs, Part 2: p38 MAPKs

The second family of MAPKs that react to stress is named after the first member to be identified—a lipopolysaccharide (LPS)-inducible kinase activity from mouse peritoneal macrophages [23]. Like the SAPK/JNKs, p38 MAPK activation is often associated with regulation of apoptosis; however, this protein kinase plays a role in skeletal differentiation, inflammatory responses, and the cell cycle.

Mammalian genomes harbor four genes encoding p38 MAPKs that are 60 to 70% identical at the amino acid level: p38α/Mpk2/CSBP, p38β, p38γ/ERK6, and p38δ. p38 MAPKs phosphorylate a number of different proteins, including MAPK-activated protein kinase 2 and the transcriptional regulators myocyte enhancer factor 2 (MEF2), CHOP/GADD153, CREB, and ATF2.

Study of the physiological functions of the p38 MAPKs has benefited from the potent inhibition of the α and β isoforms by pyridinylimidazoles such as SB 203580 which, due to their immunosuppressive effects, are also known as cytokine-suppressing antiinflammatory drugs (CSAIDs) [24].

MAPKKs

As noted above, one of the defining characteristics of the MAPKs is their hierarchical organization within kinase cascades. All of the MAPKs (ERK, SAPK/JNK, and p38 MAPKs) require phosphorylation on threonine and tyrosine for activity. The Erks are specifically phosphorylated by MEK1 and 2 [10]. Two MAPKKs have been identified as upstream activators of the SAPK/JNKs: SEK1 (SAPK/ERK kinase 1, also known as MKK4) [25] and MKK7 (MAPK kinase 7) [26]. Sek1 and MKK7 are not redundant, as mice lacking Sek1 die during embryogenesis of liver malformation, suggesting that these two MAPKKs are coupled to at least partially distinct upstream regulatory proteins [27]. There are also two MAPKKs that phosphorylate and activate the p38 MAPKs: MKK3 and MKK6 [28]; therefore, some six distinct MAPKKs act to selectively induce different MAPKs (see Table 1).

Table I Components of Mammalian Stress-Regulated MAPK Signaling Pathways

MAPKs	
ERK1, 2	Extracellular signal regulated kinases
SAPKα/β/γ	Stress-activated protein kinase (JNK2/3/1, respectively)
p38α/β/γ/δ	p38 MAPK, p38/HOG1, MPK2, Mxi2, CSBP1/2
MAPKKs	
MEK1,2	MAPK/ERK kinases 1, 2
MKK3	MAPK/ERK kinase 3
MKK6	MAPK/ERK kinase 6
SEK1	SAPK/ERK kinase 1 (MKK4)
MKK7	MAPK/ERK kinase 7 (SEK2)
MAPKKKs	
ASK1/2	Apoptosis signal-regulating kinase (ASK1 = MAPKKK5)
DLK	Dual leucine-zipper bearing kinase (MUK, ZPK)
MEKK1–4	MAPK/ERK kinase kinase (MEKK4 = MTK1)
MLK2	Mixed-lineage kinase (MLK2 = MST; MLK3 = SPRK)
Raf	Raf oncoprotein
PAK	p21-activated kinase
TAK1	TGF-activated protein kinase
Tpl2	Tumor progression locus 2 (Cot)
STE20s	
GCK	Germinal center kinase
GCKR	GCK-related
GLK	GCK-like kinase
HGK	HPK/GCK-like kinase
HPK1	Hematopoietic progenitor kinase 1
MST1	Mammalian Ste20-like protein kinase
NESK	NIK-like embryo specific kinase
NIK	Nck-interacting kinase
TAO1/2	One thousand and one amino acid protein kinase 1
Scaffold Proteins	
IB1	Islet-brain 1
JIP1	JNK-interacting protein 1
MP1	MEK partner 1

MAPKKKs

While the MEKs are targeted by only a small number of MAPKKKs (e.g., Raf, Mos, MEKK, Tpl2), over 12 different enzymes act on the stress-activated MAPKs (see Fig. 1 and Table 1). These molecules include the MEKKs that are homologous to yeast STE11. Of note, the mixed lineage

kinase (MLK) group of MAPKKKs contains a Cdc42/Rac1 interaction and binding (CRIB) motif that mediates association with Rho family small GTPases [29]. These molecules are key regulators of the actin cytoskeleton and are implicated in secretion and migratory responses. Expression of activated (GTP-bound) forms of Rac1 and Cdc42 activates the SAPK/JNK and p38 pathways, providing an important link between regulation of the cell shape and control of gene expression by the stress-activated protein kinases [30]. Another MAPKKK, apoptosis signal regulated kinase (ASK1), couples tumor necrosis factor-α (TNF-α) via an adaptor molecule (TRAF2) to activation of SAPK/JNK/p38 MAPK [31,32]. This same MAPKKK also acts as a redox sensor via association with thioredoxin, providing one mechanism for coupling cellular oxidative status to transcription [33,34].

MAPKKKKs

An early indication that there may be a further level of protein kinases in the MAPK cascade, above the MAPKKKs, was discovery of the yeast kinase STE20. Genetic analysis placed this gene as acting upstream of MAPKKK STE11, but physical evidence of direct regulation of STE11 by STE20 has been sparse. Several STE20-related proteins were subsequently identified, such as germinal center kinase (GCK) and GCK-related (GCKR/KHS1) and GCK-like kinase (GLK). The precise mechanisms by which these enzymes interact with the MAPK cascades is not completely understood. For example, although over-expression of these molecules induces SAPK/JNK activity, in some cases mutants that disable the kinase activity or delete the kinase domain entirely retain their ability to activate the MAPKs. In addition to SAPK/JNK induction, some of the MAPKKKs induce NF-κB activation, suggesting that they impact multiple and distinct signaling path-ways [35].

Instead of acting like conventional protein kinases, the MAPKKKs appear to operate more as kinase scaffolds and physically interact with a variety of the MAPKKKs (not necessarily directly phosphorylating them). As mentioned earlier, the pheromone pathway in yeast again led the way in our understanding of scaffolds. In this case, the MAPK FUS3 binds to the scaffold protein STE5 together with the MAPKK STE7 and MAPKKK STE11 [15]. More recently, mammalian scaffolding proteins have been characterized. MEK partner 1 (MP1) interacts with ERK1 and MEK1 [36], whereas the JNK-interacting protein (JIP) family of proteins binds to SAPK/JNK, MKK7, mixed-lineage protein kinases, and the STE20-like protein kinase HPK [37]. As is the case for STE5, the mammalian scaffolds appear to be important for clustering pathway components. The stoichiometry of association, however, is quite low, suggesting that these scaffolds also act to sequester signaling modules within certain subcellular locations, with the majority of the protein kinases remaining untethered and free to diffuse.

Summary

The MAPKs are one of Nature's preferred "solutions" for signaling, and the basic three-kinase module has been replicated for various tasks in all eukaryotes. While the overall structure of the module is remarkably conserved, the inputs and outputs are diverse and unpredictable. Although MAPKs did not turn out to be the "missing link" between tyrosine and serine/threonine kinases, their importance transcends such a simple bridge in acting as adaptable conduits between the environment and the appropriate cellular response. As has already been demonstrated for p38 MAPK, pharmaceutical modulation of the pathways may have therapeutic benefit. Indeed, MEK and Raf inhibitors are undergoing clinical trials for the treatment of certain cancers. As more selective, small-molecule inhibitors are developed, additional indications such as inflammation, arthritis, and autoimmunity may benefit from MAPK medicinal chemistry.

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CHAPTER 84

Cytoskeletal Regulation: Small G-Protein–Kinase Interactions

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Introduction

Work from many laboratories has established that molecular switches of the Rho GTPase family, that including Rho, Rac, and Cdc42, alter the cytoskeleton by recruiting protein effectors. These, in turn, act in cascades that are responsible for altering the three major cytoskeletal networks: the microfilament, intermediate filament, and microtubule systems. The many signals that can impinge on cells to alter their morphology act via a common set of such Rho switches. This chapter discusses three well-studied protein kinases regulated by Rac, Cdc42, and Rho. A comprehensive description of other Rho effectors can be found elsewhere [1]. Lessons learned about the control of these kinases have begun to tell us how cells use the common currency of protein phosphorylation to coordinate complex spacial and temporal signaling events, as is required for processes such as cell movement.

A number of studies have pointed to Rac as playing a critical role in cell migration [2], a property of many cultured cells that can be directly observed and quantified. Recent real-time imaging techniques have allowed visualization of the production of active Rac GTP by growth factors directly to the leading edge [3], confirming that the GTPase is activated in a polarized fashion. Cdc42 drives formation of filopodia [4] and is important for cells to orientate correctly towards a chemotactic gradient [5] or for fibroblasts to align themselves during wound healing [6].

Rho-dependent actin stress fibers are responsible for the contractile forces generated in cultured cells, both within leading-edge structures [7] and between integrin-containing focal-adhesion complexes (FCs), particularly in the retracting tail of migrating cells [8]. Rho, acting through its effector, Rho kinase (ROK), plays a key role in producing these structures by organizing myosin II [9]. In the immune and nervous systems, Rho GTPases function in migration, phagocytosis, neurite outgrowth, and axonal pathfinding. We now have some understanding of how cytosolic protein kinases are coupled to cytoskeletal driven processes (Table 1).

p21-Activated Kinases

The most prominent downstream targets of Rac and Cdc42 in blot overlays are the mammalian p21-activated kinases (PAKs) [10] with homologs Ste20 and Cla4 in *Saccharomyces cerevisiae*; these proteins have been implicated in cell-cycle control, dynamics of the actin cytoskeleton, apoptosis, and transcription (for reviews, see Bagrodia and Cerione [11]). One of the effects of PAK expression is a loss of F-actin; this is thought to result in part from effects on myosin light chain kinase (MLCK; see Fig. 1). However, an opposite effect is thought to result from activation of LIM kinases; cofilin inactivation by LIM kinases facilitates the assembly of actin. LIM kinase 1 is activated by Rac1 [12] and its effector, PAK1/αPAK [13]. It has recently been shown that the related Cdc42 effector PAK4 is a potent *in vivo* activator of LIM kinase 1 [14]. In spite of the close connection between Rho GTPases and focal adhesion complexes, PAKs are the only identified effector (kinase or otherwise) that have been localized to adhesion complexes [15], where PAK is probably locally activated in these fledgling structures [16].

PAKs (α, β, γ isoforms equivalent to PAK1, 3, 2) are 60- to 68-kDa proteins that contain an N-terminal p21 (Cdc42/Rac)

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Table I Protein Kinase Effector Proteins of Rho GTPases

Effector	GTPase selectivity	Protein type	Target	Cytoskeletal effect
MRCK (α,β)	Cdc42,	S/T kinase	MBS	Actin/myosin II Increased focal adhesions
PAK (α,β,γ) (PAK1, 2, 3)	Cdc42, Rac,	S/T kinase	LIM-K MLCK	Actin polymerization Inhibits myosin II
PAK4, 5	Cdc42	S/T kinase	LIM-K	Actin polymerization
PAK6	Cdc42	S/T kinase	Androgen receptor	—
PKN1, 2 (PRK1, 2)	Rac, Rho	S/T kinase	Vimentin (?)	Actin/myosin II IFs disassembly
ROK (α,β) (ROCK, Rho kinase)	Rho	S/T kinase	MBS Vimentin LIM-K	Actin/myosin II Increased focal adhesions IFs disassembly Actin polymerization

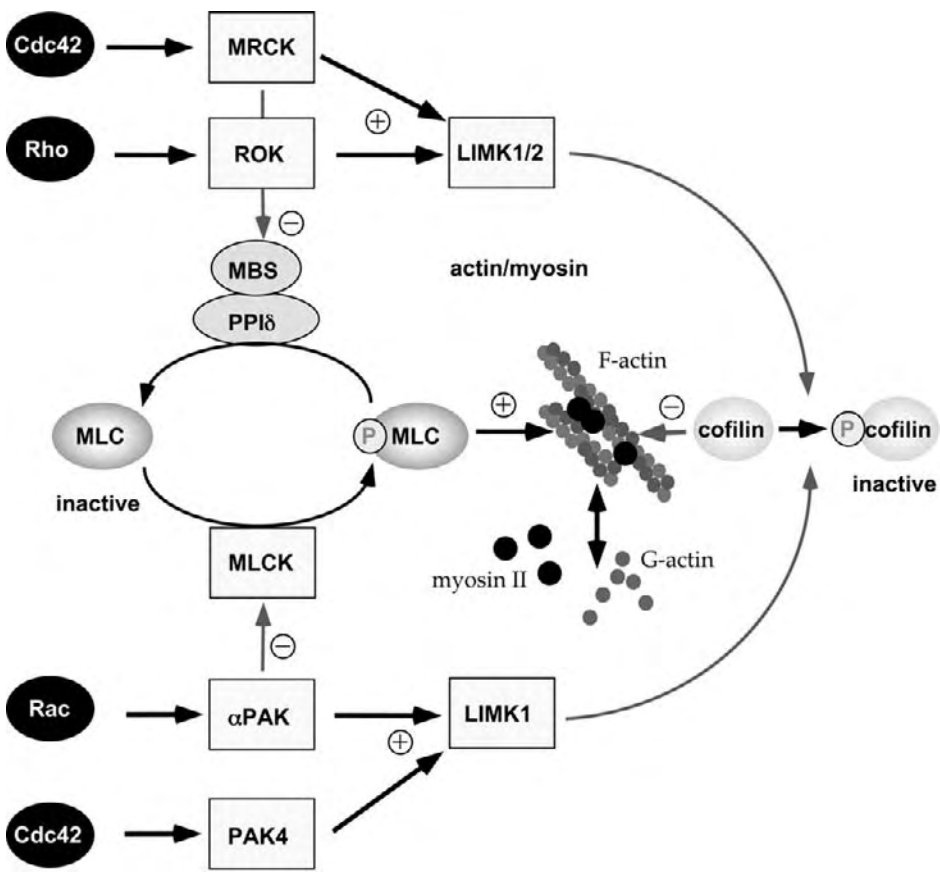


Figure 1 Pathways linking Rho GTPase-associated kinases to actin/myosin II. Rho regulation of actin organization occurs through multiple effectors. The Rho-associated kinase (ROK) and the myotonin-related Cdc42-binding kinase (MRCK) block protein phosphatase delta (PP1δ) activity by phosphorylating the myosin binding subunit (MBS) p85 or p130 subunits. This prevents inactivation of phosphorylated MLC-P assembled into actin stress fibers. Myosin-II-driven assembly of stress fibers favors the formation of focal adhesion complexes. Myosin light chain kinase (MLCK) is a key enzyme for maintaining the myosin heavy chain–light chain complex in an active state but is negatively regulated by Rac via the p21-activated kinase (PAK). It appears that both Cdc42-binding kinases PAK4 and MRCK can act via LIM-kinases (LIMKs) to inactivate cofilin. This stabilizes actin filaments, because cofilin serves to accelerate actin dissociation and may drive the net peripheral actin assembly characteristic of active Cdc42.

binding domain (PBD) and a flanking kinase inhibitory domain (KID) that maintain the C-terminal kinase domain in an inactive state (Fig. 2). The ability of the PBD to bind with high affinity to Cdc42 GTP and Rac GTP forms the basis for biochemical pull-down assays of the active GTPase species. Mutations in the KID lead to constitutive activation of PAK in the absence of GTPase [15]. The binding of Cdc42 GTP or Rac GTP was proposed to allosterically induce activation of the kinase by affecting the KID structure [17]. The structures of an autoinhibited PAK [18] and of complexes between Cdc42 and a CRIB-containing fragment [19] seem to support the model. Cdc42-GppNHp binds with low affinity ($K_d \approx 0.6$ μ*M*) to intact kinase, whereas the affinity to the isolated regulatory fragment is much higher (K_d 18 n*M*), indicating that the difference in binding energy is used for the conformational change leading to activation [20].

PAKs can also be activated by lipids, including sphingosine [21], which may act synergistically with Cdc42 [22]. Proteolytic digestion of αPAK produces a heterodimeric complex consisting of a regulatory fragment (residues 57 to 200) and a catalytic fragment (residues 201 to 491), which is active in the absence of Cdc42 [20]. In the physiological context, this is relevant as PAK2 is caspase activated [23].

It is of particular interest that PAK1 may be autoinhibited in *trans* [24], as illustrated in Fig. 2, a characteristic that could prevent spontaneous N-terminal autophosphorylation of PAK on sites such as Ser144 in the KID segment which is involved in activation. Although Parrini *et al.* [24] propose that Cdc42-binding dissociates PAK1 dimers, different results are seen with PAK/Cdc42 complexes on gel filtration columns [20]. Here, the data suggest that an allosteric control mechanism induces autophosphorylation, which in turn induces the release of the KID and full kinase activation. This finding is contrary to the view that phosphorylation is a late event in the p21-mediated activation mechanism and serves primarily to stabilize the open conformation. The complexity of the PAK activation mechanism requires further analysis.

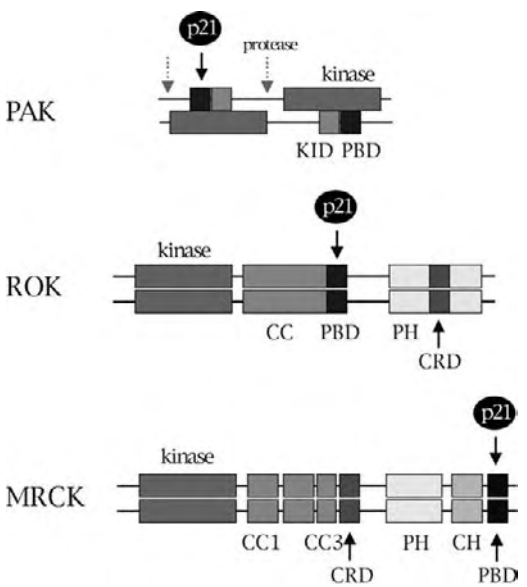


Figure 2 Structural features of Rho GTPase-regulated kinases PAK, MRCK, and ROK. These proteins have in common a p21-binding domain (PBD), which interacts with only the GTP-bound form of the p21, and a catalytic serine/threonine kinase domain. All three kinases exist as dimers in the cell (as illustrated). While PAK is autoinhibited in a head-to-tail fashion, ROK and MRCK are thought to be aligned in parallel. The kinase inhibitory domain (KID) of PAK is a conserved motif that makes numerous contacts with the catalytic domain in the crystal structure [18]. Structures are not available for the other two kinases, which are dimerized via their coiled-coil domains. Abbreviations: PH, pleckstrin homology; CRD, cysteine-rich domain; CC, coiled-coil; CH, citron homology.

Myotonic Dystrophy Kinase-Related Cdc42-Binding Kinase

Myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) is a Cdc42-binding serine/threonine kinase with multiple functional domains that belongs to the myotonic dystrophy family of kinases, which includes ROK and myotonic dystrophy protein kinase (DMPK). These kinases share homology in their N-terminal kinase domains and, to a certain extent, the arrangement of other C-terminal domains. Interestingly, they all contain an N-terminal dimerization domain of approximately 70 amino acids referred to as the leucine-rich domain in DMPK, which is required for kinase activity. MRCKα is implicated in Cdc42-mediated peripheral actin formation and neurite outgrowth in HeLa and PC12 cells, respectively [25]. Its expression is ubiquitous but is highest in the brain. MRCK acts downstream of Cdc42 in actin cytoskeletal reorganization, particularly Cdc42-mediated peripheral actin formation, which may be due to its effects on mysoin II (see Fig. 1). One of the major MRCK targets appears to be the myosin binding subunit of PPIδ [26] which allows inactivation of the phosphatase. In *Drosophila*, mutations of the Gek locus (a *Drosophila* homolog of MRCK) exhibit abnormal F-actin accumulation, a phenotype downstream of *Drosophila* Cdc42 [27]. The involvement of MRCK in the regulation of neurite outgrowth in PC12 cells [28] is consistent with the view that Cdc42, Rac-1, and their effectors are important for neuronal outgrowth [29].

MRCK exists in high-molecular-weight complexes in which three independent coiled-coil domains (numbered CC1 to 3; see Fig. 2) and an N-terminal region preceding the kinase domain are responsible for intermolecular interactions leading to MRCKα multimerization [30]. An N-terminus-mediated dimerization and consequent autophosphorylation regulate MRCKα catalytic activity. The N-terminus-mediated dimerization and the autoregulatory kinase/distal coiled–coil (CC) interaction are two mutually exclusive events that tightly regulate the catalytic state of the kinase. Disruption of this interaction occur when cells are treated with phorbol ester, which can interact directly with a cysteine-rich domain next to the distal CC domain. Diacylglycerol production downstream of phospholipase C therefore causes MRCK activation, but localization of

MRCK is regulated by Cdc42 GTP [30]. The regulation of MRCK probably involves kinase phosphorylation in the activation loop and a hydrophobic motif C-terminal to the kinase domain by autophosphorylation and/or phosphorylation by heterologous kinases.

Rho-Associated Kinase (ROK)

The serine/threonine kinases ROK (also described as Rho-kinase or ROCK) promote maintenance of the phosphorylated state of myosin light chain (P-MLC) by blocking the action of the key phosphatase in the cycle [31]. ROK and MRCK target a single inhibitory threonine phosphorylation site on the p85 [26] or p130 [25] myosin-binding subunit (MBS) of protein phosphatase 1δ. This MBS phosphorylation activates a domain that directly blocks the activity of the phosphatase. Such activity occurs in the context of other Rho effectors—for example, the mammalian Diaphanous (mDia), a formin that can generate F-actin in combination with the action of ROK [32]. Cofilin, which promotes F-actin disassembly, is negatively regulated by LIM kinases and, therefore, by ROK (Fig. 1).

Rho-associated kinase contains an N-terminal serine/threonine kinase domain that is flanked by an extended coiled-coil region and other C-terminal domains such as GTPase binding, pleckstrin homology (PH), and cysteine-rich zinc finger domains (Fig. 2). In ROK, the C terminus contains an unconventional PH domain with an inserted cysteine-rich motif [33]. Besides the formation of stress fibers and focal adhesions, ROK has also been implicated in other Rho-mediated cellular functions, such as the regulation of intermediate filament assembly [34,40], neurite remodeling [35], cytokinesis [36], and transcriptional regulation [37].

An autoinhibitory region for ROK, including the PH domain, has been mapped to the C terminus. Binding of the GTP-bound form of Rho is known to activate ROK; the interaction is believed to disrupt the negative regulatory interaction between the kinase domain and the C-terminal autoinhibitory region to give rise to an active kinase. The compound Y-27632 is widely used as a specific inhibitor of the ROK (ROCK) family of protein kinases. Y-27632 and related Y-30141 compounds inhibit the kinase activity by competing with adenosine triphosphate (ATP) [38]. Their affinities for ROK kinases as determined by K_i values are 20 to 30 times higher than those for two other Rho-binding kinases, citron kinase and protein kinase PKN. Y-27632 abolishes stress fibers in Swiss 3T3 cells at 10 μ*M*, but the G_1-to-S phase transition of the cell cycle and cytokinesis are not affected at this concentration. Activation of RhoA leads to phosphorylation of ERMs (ezrin/radixin/moesin) on a conserved regulatory threonine residue [39]. Activated Cdc42 also drives such phosphorylation of ERMs, but at the tips of filopodia via MRCK[40]. Although ROK has been implicated in this process [41,42], other *in vivo* experiments have shown that phosphorylation of ERMs is not suppressed by the ROK inhibitor Y-27632; rather, overexpression of the lipid kinase effector PIPKα is capable of increasing levels of phosphorylated ERM and concomitant formation of microvilli [43].

Acknowledgments

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CHAPTER 85

Recognition of Phospho-Serine/Threonine Phosphorylated Proteins

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Introduction

The finding that Src homology domain 2 (SH2) and phosphotyrosine binding (PTB) domains could bind to Tyr-phosphorylated motifs on proteins, but not to Ser- or Thr-phosphorylated sequences, suggested that modular domain-mediated regulation of protein–protein complexes might be a feature unique to tyrosine kinase signaling cascades. However, the subsequent discovery of a diverse group of molecules and domains that specifically recognize phosphorylated serine- and threonine-based motifs has dispelled this idea and is leading to a more general appreciation of the role of protein phosphorylation in regulating the reversible assembly of multiprotein complexes [1].

14-3-3 Proteins

The first phosphoserine/threonine-binding molecules that were identified were members of a family of dimeric proteins called 14-3-3 that were first identified as abundant polypeptides of unknown function in brain [2]; they were later identified as activators of tryptophan and tyrosine hydroxylase [3,4] and as inhibitors or activators of PKCs [5]. Mammalian cells contain 7 distinct 14-3-3 gene products (denoted β, γ, ϵ, η, σ, τ, and ζ), while plants and fungi contain between 2 and 15. Several of the mammalian 14-3-3 isotypes are subject to phosphorylation, although the role that phosphorylation plays in 14-3-3 function remains speculative.

The initial observation that 14-3-3-binding might be regulated by ligand phosphorylation emerged from studies of tryptophan hydroxylase [6] and Raf, the upstream activator of the classical mitogen-activated protein (MAP) kinase pathway [7]. Detailed investigation of the 14-3-3 binding sites on Raf [8], together with oriented peptide library screening on all mammalian 14-3-3s [9] led to the identification of two optimal pSer/threonine-containing motifs, RSXpSXP and RXXXpSXP, that are recognized by all 14-3-3 isotypes (pS denotes both pSer and pThr, and X denotes any amino acid, although there are preferences for particular amino acids in different X positions). Over 100 14-3-3 binding proteins have been identified to date, and many, though not all, use phosphorylated sequences that closely match the optimal 14-3-3 consensus motifs for binding.

Comprehensive referenced and tabulated lists of 14-3-3-binding proteins are available within detailed reviews [10,11]. In many cases, the mechanistic role of 14-3-3-binding is not known, though for a smaller subset of ligands detailed studies are beginning to uncover general mechanisms through which 14-3-3 may regulate their function. For some ligands, 14-3-3 proteins can directly regulate their

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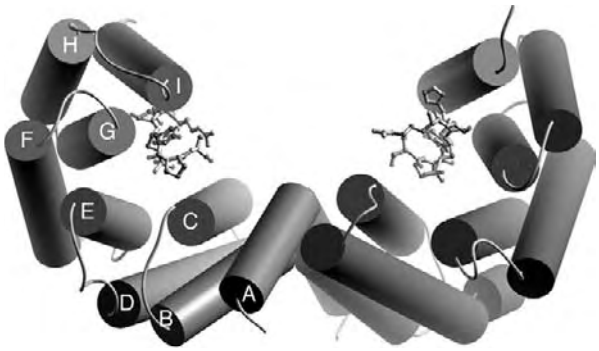


Figure 1 14-3-3/phosphopeptide interactions. Dimerization of two 14-3-3 monomers, each of which is composed of nine α-helices, forms a cleft within which phosphoserine-containing ligands bind (shown in ball-and-stick representation). A single, multiply phosphorylated protein ligand may bind simultaneously to both available sites. Alternatively, two singly phosphorylated proteins can bind, one to each monomer, allowing 14-3-3 to act as a molecular scaffold for the assembly of diverse signaling complexes [9,24]. Reprinted from Yaffe and Smerdon [75], with permission.

catalytic activity, as observed, for example, with Raf, exoenzyme S, and serotonin *N*-acetyltransferase [12–14]. For others, 14-3-3 appears to regulate interactions between its bound protein and other molecules within the cell. For example, growth-factor-mediated phosphorylation of the pro-apoptotic Bcl-2 family protein BAD at Ser-136 and/or Ser-112 facilitates its interaction with 14-3-3 proteins and blocks its association with anti-apoptotic Bcl-2 family members at the mitochondrial membrane [15–17]. Finally, in a number of cases, 14-3-3 proteins appear to play an important role in controlling the subcellular localization of bound ligands such as Cdc25 and Forkhead family transcription factors [18–21].

The X-ray structures of 14-3-3τ and ζ revealed that the molecule is a cup-shaped dimer [22,23] in which each monomeric subunit consists of nine α-helices (Fig. 1). The dimer interface is formed from helices αA, αC, and αD, creating a 35-Å × 35-Å × 20-Å central channel where binding to peptide and protein ligands occurs. Ligand-bound 14-3-3 structures have shown that peptides bind within an amphipathic groove along each edge of the central channel [9,24,25] with the entire phosphopeptide main chain in a highly extended conformation until two residues after the pSer, when there is a sudden sharp change in peptide chain direction required to exit the 14-3-3 binding cleft. More recently, the structure of 14-3-3ζ bound to a *bona fide* protein ligand, serotonin *N*-acetyltransferase, has been solved [26]. In this structure, the 14-3-3 binding portion of the enzyme displays a conformation very similar to that seen in isolated phosphopeptide:14-3-3 complexes, including the extended conformation and sudden alteration in chain direction. Furthermore, 14-3-3-binding at least partially restructures the substrate binding site on serotonin *N*-acetyltransferase, rationalizing some of the 14-3-3 effects on enzyme activity. Additional X-ray structures of 14-3-3-bound complexes are required before a detailed mechanistic understanding of 14-3-3 function emerges.

FHA Domains

Forkhead-associated (FHA) domains are a recently recognized pThr-binding module found in several prokaryotic and eukaryotic proteins including kinases, phosphatases, transcription factors, kinesin-like motors, and regulators of small G proteins. FHA domains are ≈140 amino acids in length and extend significantly beyond the core homology region first identified by sequence profiling [27–33].

Recognition that FHA domains were pThr-binding modules came from findings that the FHA domain of KAPP, a protein phosphatase in *Arabidopsis*, was critical for its interaction with phosphorylated receptor-like kinases [28,34] and that the FHA domains within the *Saccharomyces cerevisiae* cell-cycle checkpoint kinase Rad53p were essential for interaction with the phosphorylated DNA damage control protein Rad9p [35,36]. Durocher *et al.* [36] were the first to demonstrate that FHA domains could bind directly to short pThr-containing peptides in isolation. Data regarding the specificity of different FHA domains for pThr-based sequence motifs comes from peptide library experiments that show sequence-specific binding involving amino acids from the pT−3 to the pT + 3 position [32,37]. Curiously, substitution of pSer (pS) for pThr (pT) completely eliminates phosphopeptide binding, presumably due to a structurally conserved van der Waals interactions with the threonine γ-methyl group or to entropic constraints that are unique to phosphothreonine. Tsai and co-workers found that the C-terminal FHA domain of Rad53 binds to pTyr-containing peptides *in vitro* [29,31] although the *in vivo* relevance of pTyr-dependent signaling mechanisms in budding yeast is not yet clear.

The *in vivo* binding partners for most FHA domain proteins are unknown, though a number of studies and clinical observations involving naturally occurring or engineered mutations or deletions within the FHA domains of key signaling molecules have verified their functional importance. Mutations that impair the ability of the N-terminal FHA domain of the yeast checkpoint kinase Rad53p to bind to phosphopeptides also result in increased sensitivity to DNA damage, whereas mutations within the FHA domain of Chk2, the human homolog of Rad53p, have been implicated in a variant form of the human cancer-prone Li-Fraumeni syndrome [38]. In addition, other mutations in FHA-domain-containing proteins including p95/Nbs1 and Chfr also appear to contribute to human tumor formation [38–41]. Chfr appears to function as a subunit of an E3 ubiquitin ligase, targeting Polo kinase for degradation to establish a cell-cycle checkpoint [42], although the role of the FHA domain in this process is not yet known.

Structures of both FHA domains from Rad53p [29,31,32], together with FHA domains from Chk2 [43] and Chfr [44], have been determined by nuclear magnetic resonance (NMR) spectroscopy or X-ray crystallography (Fig. 2). The FHA domain consists of an 11-stranded β-sandwich, with a topology essentially identical to that of the MH2 domain from Smad tumor suppressor molecules [29,32].

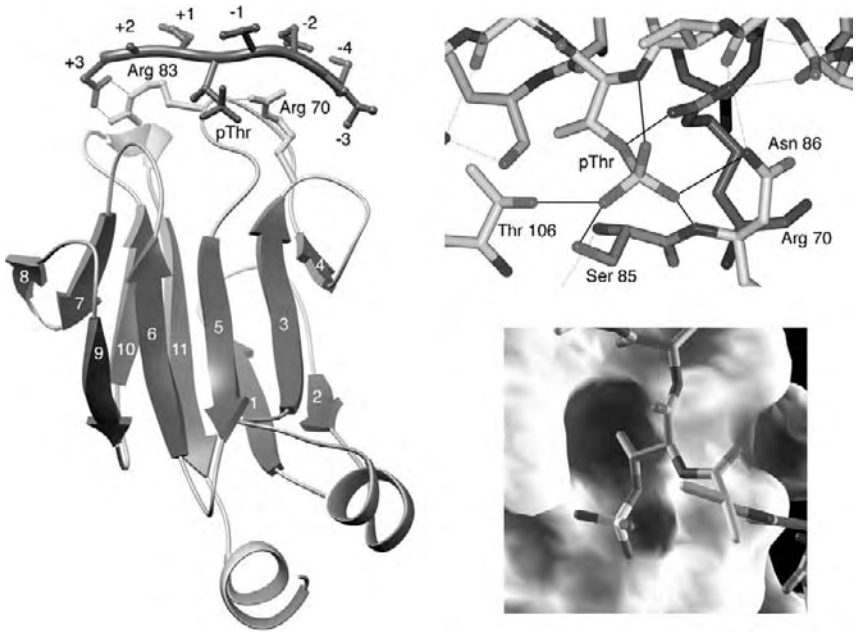


Figure 2 FHA/phosphopeptide interactions. The FHA domain architecture as exemplified by the X-ray structure of Rad53p FHA1 in complex with a Rad9p phosphopeptide (left panel [32]). The peptide binds at one end of the β-sandwich domain in an extended conformation. The highly conserved arginine (Arg70) makes a crucial contact with the phosphate group, while a non-conserved arginine (Arg83) acts to select an Asp at the pT + 3 position (right panel, top). The phosphate group interacts with a constellation of conserved and semi-conserved FHA domain side chains (top panel), while the γ-methyl group of the phosphothreonine binds in a pocket on the domain surface (right panel, bottom), likely explaining the observed preference for pThr over pSer in peptide-selection experiments. Reprinted from Yaffe and Smerdon [75], with permission.

This structural relationship, together with the remarkable similarities in phospho-dependent binding interactions of FHA and MH2 domains, suggests the existence of a superfamily of FHA-like phospho-binding domains [43,45,46]. In all pThr peptide complexes, binding occurs at one end of the domain, through interactions between selected residues in the phosphopeptide and loops connecting the β3/4, β4/5, and β6/7 β-strands. Of the seven most highly conserved residues in the FHA family, three make direct interactions with the peptide (two bind directly to the pThr residue), while the remainder form the structural core or stabilize loop regions of the β-sandwich structure. Interestingly, the Chfr FHA domain structure shows a segment-swapped dimer with the C-terminal half of β7 and β8–10 exchanged between monomers. Whether or not these dimers exist *in vivo* or contribute to Chfr function is not known.

WW Domains

WW domains contain ~40 amino acids with two invariant tryptophan residues (labeled W in single-letter amino acid code, hence the name *WW domain*) that bind to short proline-rich sequences containing PPXY, PPLP, or PPR motifs [47]. A small subclass of WW domains within the proline isomerases Pin1/Ess1 and their homologs, the splicing factor Prp40 and the ubiquitin ligase Rsp5, show specific binding to phosphoserine-proline motifs within mitotic phosphoproteins [48] and the phosphorylated C-terminal domain (CTD) of RNA polymerase II [49–51].

Phospho-specific WW domain function is best understood for the proline isomerase Pin1, a protein that slows progress through mitosis [52] but is also required for mitotic exit [53] and for the DNA replication checkpoint [53]. In addition to its WW domain, Pin1 contains a proline isomerase (rotamase) domain at its C terminus that catalyzes the specific *cis-trans* isomerization of pSer/Thr–Pro bonds [54,55]. Both the WW-domain-mediated pS–P binding and the rotamase-catalyzed pS–P isomerization are necessary for Pin1 biological activity [53,56]. In addition, Pin1 also enhances the dephosphorylation of substrates by protein phosphatases, all of which requires the pSer–Pro bond to be in *trans*. Because the WW domain can only bind to the *trans* geometric isomer, its major role may be to stabilize the *trans*-isomerase product for dephosphorylation [57,58]. WW-domain-facilitated substrate dephosphorylation is likely to be a general mechanism for WW domain function in both cell-cycle progression and regulation of transcriptional elongation.

WW domains fold into three anti-parallel β-strands, forming a single groove that recognizes proline-rich ligands in the context of a type II polyproline helix (Fig. 3). Specificity for different proline-rich motifs is determined largely by residues within the loop regions that connect the β1/β2 and β2/β3 strands, somewhat akin to the mechanism of ligand binding utilized by FHA domains. The structure of the Pin1 WW domain in complex with a Y*p*SPT*p*SPS

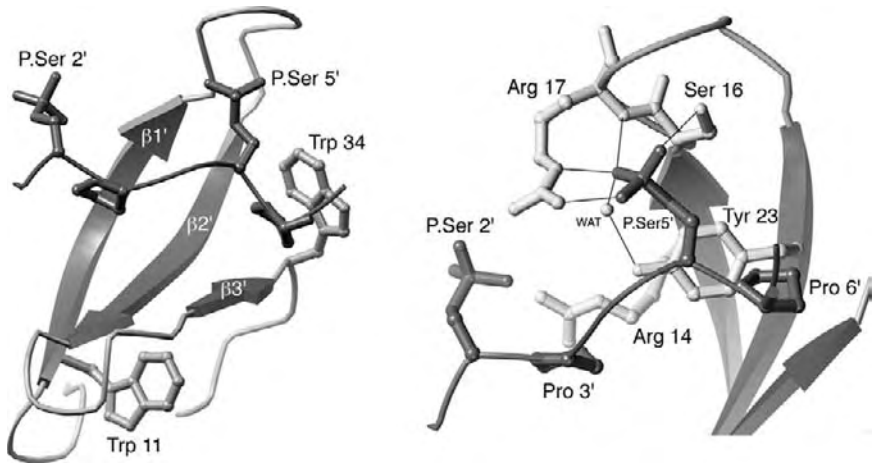


Figure 3 WW/phosphopeptide interactions. WW domains are named after two conserved Trp residues that form the structural core of the three-stranded domain (left panel). The pSer-Pro containing phosphopeptide (purple) derived from RNA polymerase II binds in an extended conformation across the β-sheet (right panel [59]). The oxygen atoms from only one of the two phosphates binds to residues located on the β1–β2 loop. Reprinted from Yaffe and Smerdon [75], with permission.

peptide from the CTD of RNA polymerase II [59] revealed that all of the phosphate contacts were made between the second pSer and two residues of the peptide in the β1/β2 loop (Ser-16 and Arg-17) along with one in the β2 strand (Tyr-23). These findings explain why only a few WW domains are competent to bind phosphorylated sequence motifs, as the majority of WW domains lack an Arg residue within loop 1 [59].

Leucine-Rich Repeats and WD40 Domains

In addition to 14-3-3 proteins, FHA domains, and WW domains, several other modular signaling domains have been shown to bind to their substrates following serine/threonine phosphorylation, most notably leucine-rich regions, and WD40 repeats. These phosphospecific-binding modules are key participants in phosphorylation-dependent ubiquitin conjugation reactions catalyzed by Skp1-Cdc53-F-box protein (SCF) protein complexes that target the ubiquitinated substrates for proteosome-mediated degradation [60–64].

The SCF complexes are E3 ubiquitin ligases that consist of Skp1, Cul1/Cdc53, Roc1, and an F-box-containing protein that confers substrate specificity in a phosphospecific manner [60–64]. The leucine-rich repeats or WD40 domain within the F-box-containing protein appears to directly mediate phosphospecific binding. The yeast F-box protein Cdc4 binds to the phosphorylated form of the yeast Cdk inhibitors Sic1 [62,65,66] and Far1 [66,67], while the mammalian F-box protein Skp2 binds to the phosphorylated form of the Cdk inhibitor p27 [68,69]. In both yeast and mammalian cells, these interactions promote the ubiquitin-mediated destruction of the inhibitor, leading to activation of G_1 cyclin–Cdk complexes. Later in mitosis, an alternative set of F-box proteins is involved in the opposing process of inactivating the cyclin–Cdk complexes through ubiquitin-mediated proteolysis of the cyclin subunit [60–64,70]. Thus, temporally regulated substrate phosphorylation coupled with combinatorial interchange of different F-box proteins with the core SCF complex can result in waves of proteolysis that drive the cell cycle [70]. Similarly, phosphorylation-dependant recognition of IκBα and β-catenin by the WD40 repeat of the F-box protein β-TrCP targets these substrates for ubiquitin-mediated proteolysis to alter patterns of gene expression [71,72].

The WD40 repeat of Cdc4 is sufficient to bind tightly to a singly threonine-phosphorylated peptide from Cyclin E1 containing the consensus motif L/I-L/I/P-pT-P-X where X denotes all amino acids except R and K [66]. However, Sic1, the physiological substrate of Cdc4, does not contain a perfect match to the consensus motif and relies instead on the phosphorylation of multiple suboptimal motifs to provide cooperative binding. Thus, by using a series of weak distributed phospho-dependent motifs to bind Sic1, Cdc4 ensures that a threshold of phosphorylation must be overcome prior to the initiation of DNA replication [66].

Leucine-rich repeats adopt a C-shaped structure built of single α-helix/β-strand repeats [73], while WD40 repeats form β-propeller structures [74]. Currently, no structure of either domain has been found to bind to a phosphopeptide, although site-directed mutagenesis experiments have identified several Arg residues within the propeller blades of Cdc4 likely to be involved in ligand recognition [66].

Concluding Remarks

The identification of several families of pSer/Thr-binding modules has provided fascinating insights into how cellular signaling events are regulated by protein–protein interactions

mediated by Ser/Thr phosphorylation. The structural diversity of these domains suggests that, in general, their phosphobinding functions have evolved through convergent evolution. Importantly, the phosphorylated motifs recognized by different phosphoserine/threonine binding modules differ somewhat from the optimal phosphorylated motifs generated by various protein kinase families which provides specificity in assembly of signaling complexes by requiring a unique overlap motif between the activating kinase and the binding domain and also allows abrupt on-and-off activation states to be created through cooperative binding between multiple weak motifs.

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CHAPTER 86

Role of PDK1 in Activating AGC Protein Kinase

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Introduction

Stimulation of cells with growth factors, survival factors, and hormones leads to recruitment to the plasma membrane of a family of lipid kinases known as class 1 phosphoinositide 3-kinases (PI 3-kinases, [1]). In this location PI 3-kinases phosphorylate the glycerophospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), at the D-3 position of the inositol ring, converting it to PtdIns(3,4,5)P_3, which is then converted to PtdIns(3,4)P_2 through the action of the SH2-containing inositol phosphatases (SHIP1 and SHIP2) or back to PtdIns(4,5)P_2 via the action of the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10).

PtdIns(3,4,5)P_3 and perhaps PtdIns(3,4)P_2 play key roles in regulating many physiological processes, including controlling cell apoptosis and proliferation, most of the known physiological responses to insulin, and cell differentiation and cytoskeletal organization [2]. PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 exert their cellular effects by interacting with proteins that possess a certain type of pleckstrin homology domain (PH domain). A number of types of PH domain containing proteins that interact with PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 have now been identified. These include the serine/threonine protein kinases protein kinase B (PKB; also known as Akt) [3], tyrosine kinases of the Tec family [4,5], numerous adaptor molecules such as the Grb2-associated protein (GAB1 [6]), the dual adaptor of phosphotyrosine and 3-phosphoinositides (DAPP1 [7–10]), and the tandem PH-domain-containing proteins (TAPP1 and TAPP2 [11]), as well as guanosine triphosphate (GTP)/guanosine diphosphate (GDP) exchange [12–14] and GTPase-activating proteins [15,16] for the ARF/Rho/Rac family of GTP binding proteins (Fig 1). This chapter focuses on research aimed at understanding the mechanism by which PtdIns(3,4,5)P_3 regulates one branch of its downstream signaling pathways, namely enabling PDK1 to phosphorylate and activate a group of serine/threonine protein kinases that belong to the AGC subfamily of protein kinases. These include isoforms of PKB [3,17], p70 ribosomal S6 kinase (S6K) [18,19], serum- and glucocorticoid-induced protein kinase (SGK) [20], p90 ribosomal S6 kinase (RSK) [21], and protein kinase C (PKC) isoforms [22]. Once these diverse AGC kinase members are activated, they phosphorylate and change the activity and function of key regulatory proteins that control processes such as cell proliferation and survival as well as cellular responses to insulin [2,3,23].

Mechanism of Activation of PKB

The three isoforms of PKB (PKBα, PKBβ, and PKBγ) possess high sequence identity and are widely expressed in human tissues [17]. Stimulation of cells with agonists that activate PI 3-kinase induce a large activation of PKB isoforms within a few minutes. The activation of PKB is downstream of PI 3-kinase, as inhibitors of PI 3-kinase such as wortmannin or LY294002, or the over-expression of a dominant-negative regulatory subunit of PI 3-kinase inhibit the activation of PKB in cells by virtually all agonists tested [24–26]. Over-expression of a constitutively active mutant of PI 3-kinase induces PKB activation in unstimulated cells [27],

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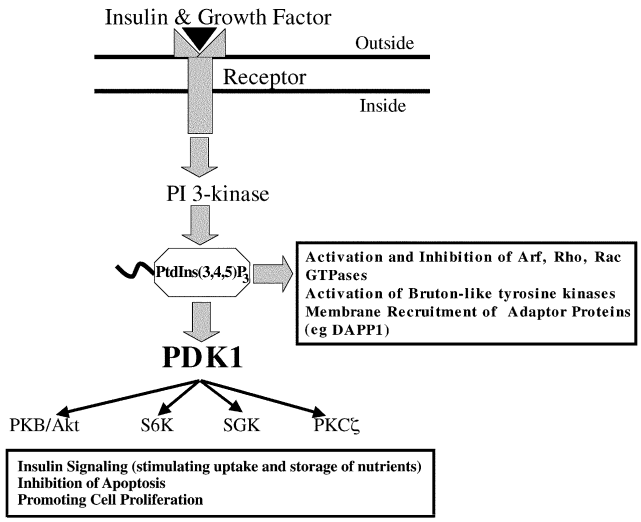


Figure 1 Overview of the PI 3-kinase signaling pathway. Insulin and growth factors induce the activation of PI 3-kinase and generation of PtdIns(3,4,5)P_3. In addition to leading to the activation of PKB/Akt, S6K, SGK, and atypical PKC isoforms such as PKCζ, PtdIns(3,4,5)P_3 also recruits a number of other proteins (outlined in the text) to the plasma membrane to trigger the activation of non-PDK1/AGC-kinase-dependent signaling pathways. Key challenges for future experiments are not only to define the specific cellular roles of the individual AGC kinase but also to understand the function and importance of other branches of signaling pathways activated by PI 3-kinase.

as does deletion of the PTEN phosphatase which also results in increased cellular levels of PtdIns(3,4,5)P_3 [28–32].

All PKB isoforms possess an N-terminal pleckstrin homology (PH domain) that interacts with PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 followed by a kinase catalytic domain and then a C-terminal tail. Stimulation of cells with agonists that activate PI 3-kinase induces the translocation of PKB to the plasma membrane, where PtdIns(3,4,5)P_3 as well as PtdIns(3,4)P_2 are located and, consistent with this, translocation of PKB is prevented by inhibitors of PI 3-kinase or by the deletion of the PH domain of PKB [33–35]. These findings strongly indicate that PKB interacts with PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 *in vivo*. The binding of PKB to PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 does not activate the enzyme but instead recruits PKB to the plasma membrane where it becomes phosphorylated at two residues at this location, namely Thr308 and Ser473. Inhibitors of PI 3-kinase and dominant-negative PI 3-kinase prevent phosphorylation of PKB at both residues following stimulation of cells with insulin and growth factors [17]. Thr308 is located in the T-loop (also known as *activation loop*) between subdomains VII and VIII of the kinase catalytic domain, situated at the same position as the activating phosphorylation sites found in many other protein kinases. As discussed later, Ser473 is located outside of the catalytic domain in a motif that is present in most AGC kinases and which has been termed the *hydrophobic motif*. The phosphorylation of PKBα at both Thr308 or Ser473 is likely to be required to activate PKBα maximally, as mutation of Thr308 to Ala abolishes PKBα activation, whereas mutation of Ser473 to Ala reduces the activation of PKBα by approximately 85%. The mutation of both Thr308 and Ser473 to Asp (to mimic the effect of phosphorylation by introducing a negative charge) increases PKBα activity substantially in unstimulated cells, and this mutant cannot be further activated by insulin [3]. Attachment of a membrane-targeting domain to PKBα results in it becoming highly active in unstimulated cells and induces a maximal phosphorylation of Thr308 and Ser473 [33,36]. These observations indicate that recruitment of PKB to the membrane of unstimulated cells is sufficient to induce the phosphorylation of PKBα at Thr308 and Ser473. Furthermore, there must be sufficient basal levels of PtdIns(3,4,5)P_3/PtdIns(3,4)P_2, T308 kinase, and Ser473 kinase located at the membrane to stimulate phosphorylation and activation of membrane-targeted PKB. PKBβ and PKBγ are activated by phosphorylation of the equivalent residues in their T-loops and hydrophobic motifs [37,38].

PKB Is Activated by PDK1

A protein kinase was purified [39,40] and subsequently cloned [41,42] that phosphorylated PKBα at Thr308 only in the presence of lipid vesicles containing PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2. Because of these properties it was named 3-phosphoinositide-dependent protein kinase 1 (PDK1) and is composed of an N-terminal catalytic domain and a C-terminal PH domain which, like that of PKB, interacts with PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 [42,43]. The activation of PKB by PDK1 is stereospecific for the physiological D-enantiomers of these lipids, and neither PtdIns(4,5)P_2 nor any inositol phospholipid other than PtdIns(3,4)P_2 can replace PtdIns(3,4,5)P_3 in the PDK1-catalyzed activation of PKB [39,42].

Although co-localization of PKB and PDK1 at the plasma membrane through their mutual interaction with 3-phosphoinositides is likely to be important for PDK1 to phosphorylate PKB, the binding of PKB to PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 is also postulated to induce a conformational change in PKB, exposing Thr308 for phosphorylation by PDK1. This conclusion is supported by the observation that in the absence of 3-phosphoinositides, PDK1 is unable to phosphorylate wild-type PKB under conditions where it is able to efficiently phosphorylate a mutant form of PKB that lacks its PH domain, termed ΔPH-PKB [40,41]. Consistent with this, a PKB mutant in which a conserved Arg residue in the PH domain is mutated to abolish the ability of PKB to bind PtdIns(3,4,5)P_3 cannot be phosphorylated by PDK1 in the presence of lipid vesicles containing PtdIns(3,4,5)P_3 [40]. Moreover, artificially promoting the interaction of PDK1 with wild-type PKB and ΔPH-PKB by the attachment of a high-affinity PDK1 interaction motif to these enzymes is sufficient to induce maximal phosphorylation of Thr308 in ΔPH-PKB but not in wild-type PKB in unstimulated cells [44].

More recently, the three-dimensional structure of the isolated PH domain of PKB complexed with the head group of

PtdIns(3,4,5)P_3 has been solved [45]. Interestingly, the structure of the PH domain of PKB complexed to the inositol head group of PtdIns(3,4,5)P_3 revealed that the 3- and the 4-phosphate groups form numerous interactions with specific basic amino acids in the PKB PH domain, but in contrast the 5-phosphate group does not make any significant interaction with the protein backbone and is solvent exposed, thus providing the first structural explanation of why PKB interacts with both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 with similar affinity [45].

The interaction of PDK1 with PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 is thought to be the primary determinant in enabling PDK1 and PKB to colocalize at membranes and permitting PDK1 to phosphorylate PKB efficiently. These conclusions are supported by the finding that the rate of activation of PKBα by PDK1 *in vitro*, in the presence of lipid vesicles containing PtdIns(3,4,5)P_3, is lowered considerably if the PH domain of PDK1 is deleted. Furthermore, the mutant of PKB that lacks its PH domain is also a very poor substrate for PDK1, compared to wild-type PKB, as it is unable to interact with lipid vesicles containing PtdIns(3,4,5)P_3.

Activation of Other Kinases by PDK1

The finding that the T-loop residues of PKB are very similar to those found on other AGC kinases suggested that PDK1 might phosphorylate and activate these members [46,47]. An alignment of the T-loop sequences of insulin and growth-factor-stimulated AGC kinases is shown in Fig. 2. It was found that the AGC kinases activated downstream of PI 3-kinase (namely, S6K1 [48,49], SGK isoforms [50–52], and atypical PKC isoforms [53,54]) were phosphorylated specifically at their T-loop residue by PDK1 *in vitro* or following the over-expression of PDK1 in cells. Moreover, AGC kinases that were not activated in a PI 3-kinase-dependent manner in cells—such as the p90 ribosomal S6K (p90RSK) isoforms [55,56], conventional and related PKC isoforms [57–60], PKA [61], and the non-AGC Ste20 family member PAK1 [62]—were also proposed to be physiological substrates for PDK1, as they could all be phosphorylated by PDK1 at their T-loop residue *in vitro* or following over-expression of PDK1 in cells.

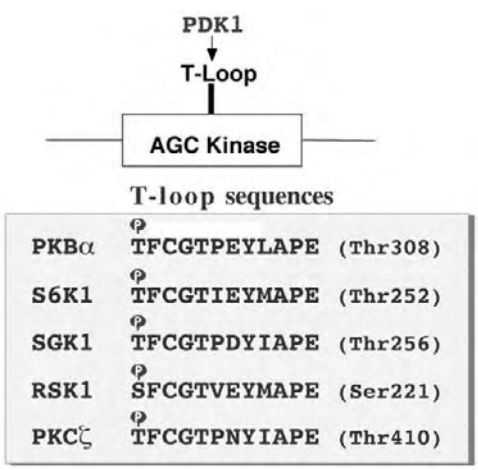


Figure 2 Alignment of the amino acid sequences surrounding the T-loop of insulin and growth-factor-stimulated AGC kinases.

Genetic evidence for the central role that PDK1 plays in mediating the activation of these AGC kinases was obtained from the finding that in PDK1$^{-/-}$ ES cells, isoforms of PKB, S6K, and RSK could not be activated by agonists that switch on these enzymes in wild-type cells [63]. In ES cells lacking PDK1, the intracellular levels of endogenously expressed PKCα, PKCβI, PKCγ, PKCδ, PKCε, and PRK1 are also vastly reduced compared to wild-type ES cells [64], consistent with the notion that PDK1 phosphorylation of these enzymes plays an essential role in post-translational stabilization of these kinases [65,66]. The levels of PKCζ were only moderately reduced in the PDK1$^{-/-}$ ES cells and PKCζ in these cells is not phosphorylated at its T-loop residue [64], providing genetic evidence that PKCζ is a physiological substrate for PDK1. In contrast, PKA was active and phosphorylated at its T-loop in PDK1$^{-/-}$ ES cells, to the same extent as in wild-type ES cells [63], thus arguing that PDK1 is not rate limiting for the phosphorylation of PKA in ES cells. It is possible that PKA phosphorylates itself at its T-loop residue *in vivo*, as it has been shown to possess the intrinsic ability to phosphorylate its own T-loop when expressed in bacteria. Thus far, we have no genetic data in PDK1-deficient cells as to whether or not PAK1 is active, but it should be noted that PAK1 can also phosphorylate itself at its T-loop in the presence of Cdc42-GTP or Rac-GTP, stimulating its own activation in the absence of PDK1 [67].

Phenotype of PDK1 PKB- and S6K-Deficient Mice and Model Organisms

PDK1$^{-/-}$ mouse embryos die at day E9.5, displaying multiple abnormalities that include a lack of somites, forebrain, and neural-crest-derived tissues, although the development of the hind- and midbrain proceeds relatively normally [68]. Other eukaryotic organisms also possess homologs of PDK1 that activate homologs of PKB and S6K in these species [69]. As in mice, knocking out PDK1 homologs in yeast [70–72], *Caenorhabditis elegans* [73], and *Drosophila* [74,75] results in nonviable organisms, confirming that PDK1 plays a key role in regulating normal development and survival of these organisms. Elegant genetic analysis of the PI 3-kinase/PDK1/AGC kinase pathway in *Drosophila* has demonstrated that this pathway plays a key role in regulating both cell size and number [76,77]. For example, the over-expression of dPI 3-kinase [78,79] or inactivation of the PtdIns(3,4,5)P_3 3-phosphatase dPTEN [80–82] results in an increase in both the cell number as well as the cell size of *Drosophila*. Moreover, loss-of-function mutants of Chico, the fly homolog of insulin receptor substrate adaptor protein [83], dPI 3-kinase, or over-expression

of dPTEN results in a decrease in cell size and number. More recently, a partial loss-of-function mutation in dPDK1 was shown to cause a 15% reduction in fly body weight and a 7% reduction in cell number [74]. Loss of function mutants of dS6K1 [84] or dPKB reduce *Drosophila* cell size without affecting cell number [82,85]. PKB and S6K have also been knocked out in mice, but these studies are complicated by the presence of two isoforms of S6K (S6K1 and S6K2) and three isoforms of PKB (PKBα, PKBβ, and PKBγ) encoded for by distinct genes, in contrast to *Drosophila*, which have one isoform of these enzymes. Mice lacking S6K1 were viable, but adult mice were 15% smaller and possessed 10 to 20% reduced organ masses [86]. It was subsequently shown that S6K1 knockout mice possessed a reduced pancreatic islet β-cell size but the size of other cells types investigated was apparently unaffected [87]. Mice lacking PKBα were also reported to be 20% smaller than wild-type animals, but it was not determined whether the lack of PKBα resulted in a reduction of cell size or cell number [88,89]. In contrast, deletion of PKBβ caused insulin resistance without affecting mouse size [90].

PDK1 hypomorphic mutant mice that express only ≈10% of the normal level of PDK1 in all tissues have been generated [68]. These mice are viable and fertile, and despite the reduced levels of PDK1, injection of these mice with insulin induces the normal activation of PKB, S6K, and RSK in insulin-responsive tissues. Nevertheless, these mice have a marked phenotype, being 40 to 50% smaller than control animals. The volumes of the kidney, pancreas, spleen, and adrenal gland of the PDK1 hypomorphic mice are reduced proportionately. Furthermore, the volume of adrenal gland zona fasciculata cells is 45% lower than control cells, whereas the total cell number and the volume of the nucleus remains unchanged. Cultured embryonic fibroblasts from the PDK1 hypomorphic mice are also 35% smaller than control cells but proliferate at the same rate. Embryonic endoderm cells completely lacking PDK1 from E7.5 embryos were 60% smaller than wild-type cells [68]. These results establish that, as in *Drosophila*, PDK1 plays a key role regulating cell size in mammals. However, the finding that AGC kinases tested are still activated normally in the PDK1 hypomorphic mice may suggest that PDK1 regulates cell size by a pathway that is independent of PKB, S6K, and RSK, although this hypothesis requires further investigation. In this regard, Tian *et al.* [91] have recently reported that PDK1 can interact via its noncatalytic N terminus with the PI 3-kinase-regulated Ral GTP exchange factor, leading to its activation. The Ral GTPase has not been implicated in regulating cell size, but it will be important to investigate whether activation of Ral GTPases is defective in PDK1 hypomorphic or knockout cell lines or mice tissues.

Hydrophobic Motif of AGC Kinases

All insulin and growth-factor-activated AGC kinases, in order to become maximally activated, require phosphorylation of a residue located in a region of homology to the hydrophobic motif of PKBα that encompasses Ser473. This is located ≈160 amino acids C-terminal to the T-loop residue lying outside the catalytic regions of these enzymes. This hydrophobic motif is characterized by a conserved motif: Phe–Xaa–Xaa–Phe–Ser/Thr–Tyr/Phe (where Xaa is any amino acid and the Ser/Thr residue is equivalent to Ser473 of PKB). Atypical PKC isoforms (PKCζ, PKCλ, PKCτ) and the related PKC isoforms (PRK1 and PRK2), instead of possessing a Ser/Thr residue in their hydrophobic motifs, have an acidic residue. PKA, in contrast, possesses only the Phe–Xaa–Xaa–Phe moiety of the hydrophobic motif, as the PKA amino acid sequence terminates at this position [92]. PDK1 is the only AGC kinase member that does not appear to possess an obvious hydrophobic motif [92], and the implications of this are discussed below. A major outstanding challenge is to characterize the mechanism by which PKB and other AGC kinases are phosphorylated at their hydrophobic motifs. In spite of considerable effort to discover the kinases responsible for the phosphorylation of AGC kinase members, no convincing evidence has thus far been obtained. The extensive literature and considerable controversy in this area have been extensively reviewed [93]. The only exception is for RSK and conventional PKC isoforms. For RSK, the phosphorylation of the C-terminal non-AGC kinase domain of this enzyme by ERK1/ERK2 triggers this domain to phosphorylate the N-terminal AGC kinase domain at its hydrophobic motif [21]. In the case of conventional PKC isoforms, there is good evidence that these enzymes can autophosphorylate themselves at their hydrophobic motifs following phosphorylation of their T-loops by PDK1 [22].

Mechanism of Regulation of PDK1 Activity

An important question is to determine the mechanism by which the ability of PDK1 activity to phosphorylate its AGC kinase substrates is regulated by extracellular agonists. When isolated from unstimulated or cells stimulated with insulin or growth factors, PDK1 possesses the same activity toward PKB or S6K1 [41,49,94]. Furthermore, although PDK1 is phosphorylated at 5 serine residues in 293 cells, insulin or insulin-like growth factor 1 (IGF1) did not induce any change in the phosphorylation state of PDK1 [95]. Only one of these phosphorylation sites (namely, Ser241) was essential for PDK1 activity. Ser241 is located in the T-loop of PDK1, and, because PDK1 expressed in bacteria is stoichiometrically phosphorylated at Ser241, it is likely that PDK1 can phosphorylate itself at this residue [95]. Although PDK1 becomes phosphorylated on tyrosine residues following stimulation of cells with peroxovanadate (a tyrosine phosphatase inhibitor) or over-expression with a Src-family tyrosine kinase [96–98], no tyrosine phosphorylation of PDK1 has been detected following stimulation of cells with insulin [95,96].

Taken together, these observations suggest that PDK1 might not be activated directly by insulin/growth factors.

Instead, one possibility that might explain how PDK1 could phosphorylate a number of AGC kinases in a regulated manner is that PDK1, instead of being activated by an agonist, is constitutively active in cells and that it is the substrates that are converted into forms that can interact with PDK1 and thus become phosphorylated at their T-loops. In the case of PKB as discussed above, it is the interaction of PKB with PtdIns(3,4,5)P_3 that converts it into a substrate for PDK1. In the case of other AGC kinases that are activated downstream of PI 3-kinase, such as S6K, SGK, and PKC isoforms, which do not possess a PH domain and thus do not interact with PtdIns(3,4,5)P_3 and whose phosphorylation by PDK1 *in vitro* is not enhanced by PtdIns(3,4,5)P_3, it is not obvious how PtdIns(3,4,5)P_3 can regulate the phosphorylation of these enzymes *in vivo*. Recent studies indicate that a conserved motif located C-terminal to the catalytic domains of isoforms of most AGC kinases (the hydrophobic motif of S6K1,or SGK1 [44]) and atypical (PKCζ) and related PKC (PRK2) isoforms [57] can interact with a hydrophobic pocket in the kinase domain of PDK1 (the PIF pocket) [92]. Evidence indicates that this results in a docking interaction, which is required for the efficient T-loop phosphorylation of AGC kinases that do not interact with PtdIns(3,4,5)P_3/PtdIns(3,4)P_2. These experiments indicate that the interaction of S6K and SGK with PDK1 is significantly enhanced if these enzymes are phosphorylated at their hydrophobic motifs in a manner equivalent to that of the Ser473 phosphorylation site of PKB [44]. It is therefore possible that PtdIns(3,4,5)P_3 does not activate PDK1 but instead induces phosphorylation of S6K and SGK isoforms at their hydrophobic motifs, thereby converting these enzymes into forms that can interact with PDK1 and hence become activated. Consistent with this notion, the expression of mutant forms of S6K1 and SGK1 in which the hydrophobic motif phosphorylation site is altered to Glu to mimic phosphorylation is constitutively phosphorylated at their T-loop residues in unstimulated cells [50,99,100]. It is currently not clear how PtdIns(3,4,5)$_3$ could stimulate the phosphorylation of the hydrophobic motif, but it is possible that it could either activate the hydrophobic motif kinases or inhibit the hydrophobic motif phosphatases.

Frodin *et al.* [101] demonstrated that phosphorylation of the hydrophobic motif of p90RSK (which is induced following phosphorylation of p90RSK by ERK1/ERK2 [21]) strongly promotes its interaction with PDK1, therefore enhancing the ability of PDK1 to phosphorylate p90RSK at its T-loop motif. Thus, the phosphorylation of p90RSK by ERK1/ERK2 converts RSK into a form that can interact with and be activated by PDK1. Thus, the mechanism by which PDK1 recognizes isoforms of RSK is analogous to that by which it recognizes SGK/S6K, the only difference being the mechanism regulating phosphorylation of the hydrophobic motifs of these enzymes. The model of how isoforms of PKB, S6K, SGK, and RSK are activated by PDK1 is summarized in Fig. 3.

Related PKC isoforms (PRK1 and PRK2) and atypical PKC isoforms (PKCζ and PKCτ) possess a hydrophobic motif in which the residue equivalent to Ser473 is Asp or Glu, and these enzymes can in principle interact with PDK1 as soon as they are expressed in a cell [57]. However, it is possible that the interaction of related PKC isoforms and atypical PKC isoforms with PDK1 could be regulated through the interaction of these enzymes with other molecules. For example, the interaction of PRK2 with Rho-GTP [60] or PKCζ with hPar3 and hPar6 [102] might induce a conformational change in these enzymes that controls their interaction with PDK1.

PDK1 would be expected to activate PKB at the plasma membrane and its other non-3-phosphoinositide binding substrates in the cytosol. Consistent with this finding, PDK1 has been found to be localized in mainly the cytosol and plasma membrane of both stimulated and unstimulated cells [43,94]. It is controversial as to whether or not PDK1 translocates to the plasma membrane of cells in response to agonists that activate PI 3-kinase. Three reports [43,94,96] indicate that a small proportion of PDK1 is associated with the membrane of unstimulated cells, and they do not report any further translocation of PDK1 to membranes in response to agonists that activate PI 3-kinase and PKB. However, other groups have reported that PDK1 translocates to cellular membranes in response to agonists that activate PI 3-kinase [103,104]. Indeed, as mentioned earlier, there is evidence that at least some PDK1 is likely to be located at cell membranes of unstimulated cells as the expression of a membrane-targeted PKB construct in such cells is active and fully phosphorylated at Thr308 [33,36].

Structure of the PDK1 Catalytic Domain

Further insight into the mechanism by which PDK1 interacts with its AGC kinase substrates has been obtained recently from the high-resolution crystal structure of the human PDK1 catalytic domain. The structure defines the location of the PIF pocket on the small lobe of the catalytic domain—a marked hydrophobic pocket in the small lobe of the kinase domain [105] that corresponds to the region of the catalytic domain predicted from previous modeling and mutational analysis to form the PIF pocket [92]. Interestingly, mutation of several of the hydrophobic amino acids that make up the surface of this pocket abolish or significantly inhibit the ability of PDK1 to interact and activate S6K1 and SGK1 [44], indicating that this hydrophobic pocket does indeed represent the PIF pocket. As phosphorylation of the hydrophobic motif of S6K1 and SGK1 promotes the binding of S6K1 and SGK1 with PDK1, this suggests that a phosphate-interacting site is located near the PIF pocket. Interestingly, close to the PIF pocket in the PDK1 crystal structure, an ordered sulfate ion was interacting with four surrounding side chains (Lys76, Arg131, Thr148, and Gln150). Mutation of Lys76, Arg131, or Q150 to Ala reduces or abolishes the ability of PDK1 to interact with a phospho-peptide that encompasses the phosphorylated residues of the hydrophobic motif of S6K1, thereby

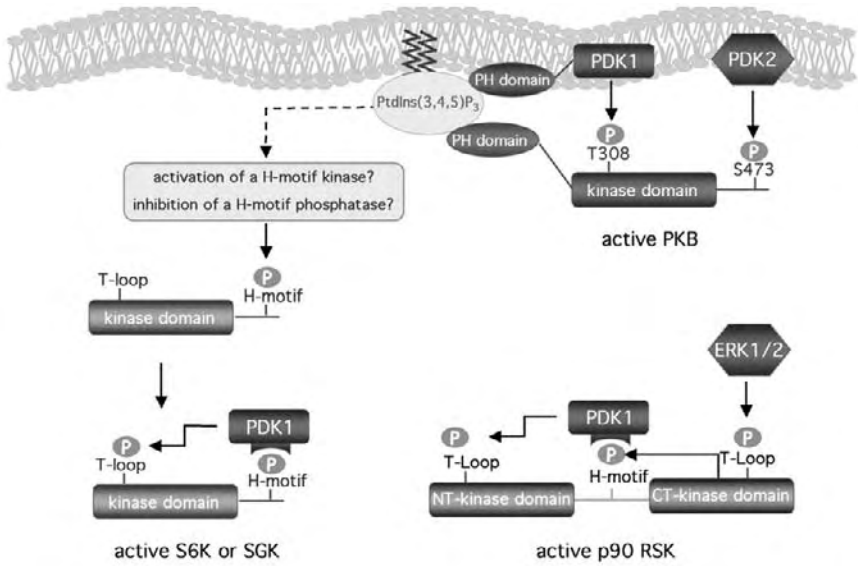


Figure 3 The mechanism by which phosphorylation of PKB, S6K SGK, and RSK by PDK1 is regulated. It should be noted that in this model of how PKB, S6K, SGK, and RSK are phosphorylated at their T-loop, PDK1 activity is not directly activated by insulin or growth factors, consistent with the experimental observation that PDK1 is constitutively active in cells. Instead, it is the substrates of PDK1 that are converted into forms that can be phosphorylated. In the case of PKB, it is the interaction of PKB with PtdIns(3,4,5)P_3 at the plasma membrane that colocalizes PDK1 and PKB and also induces a conformational change in PKB that converts it into a substrate for PDK1. In the case of S6K and SGK, which do not possess PH domains and cannot interact with PtdIns(3,4,5)P_3, this is achieved by the phosphorylation of these enzymes at their hydrophobic motif (H-motif) by an unknown mechanism, which thereby generates a docking site for PDK1. RSK isoforms possess two catalytic domains: an N-terminal AGC-kinase-like kinase domain and a C-terminal non-AGC kinase domain. The activation of RSK isoforms is initiated by the phosphorylation of these enzymes by the ERK1/ERK2 classical MAP kinases, which phosphorylate the T-loop of the C-terminal kinase domain. This activates the C-terminal kinase domain, which then phosphorylates the hydrophobic motif of the N-terminal AGC kinase. This creates a binding site for PDK1 to interact with RSK isoforms, leading to the phosphorylation of the T-loop of the N-terminal kinase domain and activating it. Phosphorylation of all RSK substrates characterized thus far is mediated by the N-terminal kinase domain; however, it is possible that the C-terminal domain of this enzyme will phosphorylate distinct substrates that have not as yet been identified.

suggesting that this region of PDK1 does indeed represent a phosphate docking site [105]. The only other AGC kinase for which the structure is known (namely, PKA) also possesses a hydrophobic pocket at a region of the kinase catalytic domain equivalent to that of PKA which is occupied by the four C-terminal residues of PKA(FXXF) and resembles the first part of the hydrophobic motif phosphorylation site of S6K and SGK (FXXFS/TY) in which the Ser/Thr is the phosphorylated residue [92]. Occupancy of this pocket of PKA by the FXXF residues is likely to be essential to maintaining PKA in an active and stable conformation, as mutation of either Phe residue drastically reduces PKA activity toward a peptide substrate, as well as reducing PKA stability [106,107]. In contrast to the PIF-pocket in the PDK1 structure, PKA does not possess a phosphate docking site located next to the hydrophobic FXXF binding pocket. Sequence alignments of the catalytic domains of AGC kinases, including PDK1, indicate that all AGC kinases possess a PIF pocket, and kinases such as isoforms of RSK, PKB, S6K, and SGK possess a phosphate docking site next to this pocket. The role of these pockets of the AGC kinases is probably to interact with their own hydrophobic motifs, and this interaction may account for the ability of these kinases to be activated following the phosphorylation of their hydrophobic motif. However, unlike other AGC kinases, PDK1 does not possess a hydrophobic motif C-terminal to its catalytic domain and therefore utilizes its empty PIF/phosphate binding pocket to latch onto its substrates that are phosphorylated at their hydrophobic motifs, thereby enabling PDK1 to phosphorylate these enzymes at their T-loop residue and activate them.

Concluding Remarks

Elucidation of the mechanism by which PKB was activated by PDK1 in cells provided the first example of how the second messenger PtdIns(3,4,5)P_3 could activate downstream signaling processes. However, there remain many major unsolved questions for future research to address. A major challenge will be to clarify the mechanism by which PtdIns(3,4,5)P_3 induces the phosphorylation of the

hydrophobic motif of PKB and other AGC kinases members, which is a key trigger for the activation of these enzymes. The results discussed in this chapter also provide a framework within which drugs could be developed to inhibit the PDK1/AGC kinase pathway to treat forms of cancers in which this pathway may be constitutively activated. Indeed, it is now estimated that PTEN is mutated in up to 30% of all human tumors, resulting in elevated PtdIns(3,4,5)P_3 levels and hence PKB and S6K activity which are likely to contribute to the proliferation and survival of these tumors [108]. It could be envisaged that a PDK1 inhibitor would be effective at reducing the PKB and S6K activities that contribute to growth and survival of these tumors.

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CHAPTER 87

Regulation of Cell Growth and Proliferation in Metazoans by mTOR and the p70 S6 Kinase

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Introduction

TOR, a ubiquitous 290-kDa polypeptide, is the founding member of the PI-3-kinase-related protein (Ser/Thr) kinase (PIKK) family. The TOR proteins were discovered by investigating the mechanism of action of the drug rapamycin, a natural product of a strain of *Streptomyces hygroscopicas* collected on Easter Island (Rapa Nui). The agent was initially investigated because of its substantial antifungal activity and subsequently was found to have potent immunosuppressant and antitumor activity. Rapamycin is structurally related to another potent macrolide immunosuppressant agent, FK506 [1]. These two agents in fact share the same cellular receptor, an abundant (□□) basic 12-kDa polypeptide named FK506 binding protein-12 (FKBP-12), one of a family of peptidylprolylisomerases. Despite their structural similarity, common receptor, and shared ability to inhibit prolylisomerase activity, FK506 and rapamycin exhibit entirely distinct cellular actions. FK506 potently inhibits T-cell receptor (TCR) activation of interleukin-2 (IL-2) and other cytokine gene expression, in part by preventing the calcium-dependent dephosphorylation and nuclear entry of the transcription factor NFAT. Rapamycin, in contrast, has no effect on TCR signaling, but strongly inhibits T-cell proliferation by interrupting IL-2R signaling. In addition, the two agents are mutually antagonistic [2–3]. These features pointed to the likelihood that the active pharmacophore was the drug– FKBP complex rather than the drug itself, an idea first verified for FK506 by the demonstration that FK506 in complex with FKBP-12, but neither component alone, binds directly and inhibits the protein phosphatase (2B), Calcineurin.

Progress on the molecular basis of rapamycin action was first achieved in *Saccharomyces cerevisiae*; elimination of both alleles encoding the *S. cerevisiae* homolog of FKBP-12, although having no effect on viability, rendered yeast completely resistant to growth inhibition by rapamycin. A genomic library prepared from *S. cerevisiae* selected for rapamycin resistance enabled the isolation of a gene that conferred a dominant form of rapamycin resistance; this gene encoded a mutant form of TOR2, one of two yeast TOR genes. The TOR2 mutation, Ser1972 Arg or Asn, abolishes the ability of TOR to bind the FKBP–rapamycin complex [4,5]. Mammalian homologs (called variously FRAP [6], RAFT [7], RAPT [8], and mTOR [9]) were independently isolated soon thereafter, through a variety of approaches.

Functions of TOR

The two TOR proteins of *S. cerevisiae* serve in a nutrient sensing pathway; a decrease in *Sc*TOR activity, whether by exposure to low-quality sources of N or C, by treatment with the specific inhibitor rapamycin, or by gene deletion, results in a 90% inhibition in overall mRNA translation [10],

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cell-cycle arrest, and activation of autophagy [11] and the N-and-C discrimination programs of gene expression [12–14], which reorient the metabolism to maximize glutamine synthesis and energy yield from suboptimal N and C sources (Table 1).

TOR activity is also the dominant regulator of overall mRNA translation in the primitive metazoan *Caenorhabditis elegans* [15]; in higher metazoans, such as *Drosophila*, and in vertebrates, the control of translation by TOR is narrowed to specific classes of mRNAs and shared with the class 1 PtdIns(3′)OH kinase [16]. Together, these two signaling elements control the facultative component of protein synthesis necessary for insulin-/mitogen-induced cell growth by exerting joint control over the activity of the p70 S6 kinases; the phosphorylation state of eIF-4E inhibitory proteins, 4E-BPs, which control the efficiency of eIF-4F-mediated translation; and other translational and metabolic targets [17–21].

The p70 S6 kinases are responsible for the phosphorylation of the 40S ribosomal subunit protein S6, a rapid and ubiquitous response to all growth-promoting stimuli. The ability of rapamycin to rapidly cause the dephosphorylation and deactivation of p70 S6K in all cultured mammalian cells while having no effect on the activity of mitogen-activated protein kinase (MAPK) or ribosomal S6 kinase (RSK) [3,4] immediately suggested a role for the target of rapamycin in the regulation of mRNA translation in mammalian cells. The deletion of the gene encoding p70 S6 kinase in *Drosophila* (and, to a lesser extent, S6K1 in mice) has established that the p70 S6 kinase is a critical determinant of cell growth [22]. Nevertheless, the function of S6 phosphorylation and its role in translational regulation remain unknown; although several other plausible *in vivo* substrates for the p70 S6 kinases have been identified (CREM [23], CBP80 [24], EF2 kinase [25], GSK3 [26], and BAD [27]), the molecular targets through which p70 S6 kinase controls cell growth and the specific role of the p70 S6 kinase in translational regulation are not known [28].

Table I TOR Outputs

A. Binding partners
1. Raptor
2. ? TSC1/2
B. Signaling intermediates
1. Protein Kinases
p70 S6Kinases
40S-S6, EF-2 kinase, CBP80
CREMτ, BAD, GSK3, others
nPKCs δ and ε
? Others
2. Protein phosphatases
PP2A
? α4, PP6, PP4
3. Transcription factors
STAT3
? p53, others
C. Translational targets
eIF-4 BPs (eIF-4E)
eIF-4G
cMyc, IGF2, ODC, others
D. Cell-cycle regulators
p27 KIP
Cyclin D1
E. Autophagy
F. Misc
IRS1,
Bcl 2

The discovery of the eIF-4E binding proteins (4E-BPs) and the finding that 4E-BPs, like p70 S6K, are rapidly dephosphorylated in response to rapamycin, provided a more secure example of TOR regulation of translation [16]. The 4E-BPs are small (11-kDa) acidic proteins that bind to eIF-4E, the mRNA cap-binding protein, in a manner competitive with the scaffold protein eIF-4G; the latter protein also binds the RNA helicase, eIF-4A, which, together with eIF-4B (to create the eIF-4F complex), functions to unwind secondary structure in mRNA 5′UT segments, enabling efficient scanning of mRNAs by the 43S pre-initiation complex. By blocking the binding of eIF-4E–mRNA complexes to the eIF-4F assembly, the 4E-BPs interfere most strongly with the translation of mRNAs whose 5′UT segments contain substantial secondary structures. The 4E-BPs were first detected as a family of heat- and acid-soluble polypeptides whose phosphorylation was greatly stimulated by insulin (PHAS-I) [29]. The discovery of their association with eIF-4E [30,31] was followed by the demonstration that their binding to eIF-4E is inhibited by a PI-3-kinase-stimulated, rapamycin-sensitive, multisite 4E-BP phosphorylation [32,33]. The ability of rapamycin to inhibit the phosphorylation of p70 S6K and 4E-BP is clearly due to the binding of a rapamycin–FKBP12 complex to mTOR and inhibition of the mTOR kinase activity, inasmuch as expression of recombinant, rapamycin-resistant, mutant mTOR proteins (Ser2035Thr or Ile) can rescue both p70 S6K and 4E-BP [34–36] from rapamycin-induced dephosphorylation, but only if the mTOR kinase domain is intact.

Thus, the ability of TOR to control at least some components of mRNA translation has persisted during metazoan evolution. With regard to the specific mRNAs whose translational initiation is regulated by mTOR, one set includes those with extensive secondary structure in their 5′UT regions, such as ornithine decarboxylase, c-Myc, IGF-2 promoter 2, and others [16]. A second class includes mRNAs characterized by a 5′ polypyrimidine tract (5′TOP), a run of 5 to 14 consecutive pyrimidines immediately at the transcriptional start site [37]. This motif, found only in metazoan mRNAs, occurs in all mRNAs encoding ribosomal polypeptides, as well as in those encoding other components of the translational apparatus (e.g., EF1α, EF2, PABP). The translation of these mRNAs is especially sensitive to the presence of insulin or mitogens and is inhibited by rapamycin and

inhibitors of the class1 PtdIns(3′)OH kinases. Considerable evidence indicates that the regulation by TOR of these translational targets [17–20], together with other anabolic actions (e.g., diminished degradation of nutrient transporters [21] or stimulation of glycogen synthesis [26]) and anticatabolic actions (e.g., inhibition of autophagy [38,39]), underlies the stimulated cell growth induced by growth factors, especially the component mediated by PI-3 kinase (PI-3K).

Cell growth (i.e., accumulation of mass) and cell proliferation, while closely related, are to some degree mechanistically separable processes [40]. Cell growth requires global protein synthesis, whereas cell proliferation depends on the timely expression and/or activation of a small group of cell-cycle regulatory proteins, as well as their timely degradation/removal [41]. In organisms where TOR controls a major portion of overall protein synthesis, control of cell-cycle progression probably follows *pari pasu*. In mammalian cells, the ability of rapamycin to interrupt cell-cycle progression both *in vivo* and in cell culture varies dramatically despite the unfailing inhibition of p70 S6 kinase and 4E-BP phosphorylation. The ability of mTOR to control cell-cycle transit is best correlated with its ability to regulate the level of the cyclin D and $p27^{KIP}$ polypeptides. Thus, in NIH3T3 cells, rapamycin causes a reduction in cyclin D1 polypeptide, with a consequent decrease in cyclin D1/Cdk4 kinase activity, reduced Rb phosphorylation, and a slowing of progress into S phase [42]. Multiple mechanisms appear to underlie the rapamycin inhibition of the accumulation of cyclin D1 (e.g., inhibition of cyclin D1 gene transcription, destabilization of the mRNA, and accelerated degradation of the cyclin D1 polypeptide through a proteosomal pathway).

In T lymphocytes, as in many other cells, phosphorylation of Rb sufficient to enable progression into the S phase requires the sequential combined actions of cyclin D/Cdk4 and cyclin E/Cdk2 [41]. Activation of cyclin E/Cdk2 in response to IL-2 or TCR stimulation is due to an increase in the expression of Cdk2 and cyclin E polypeptides as well as to a decrease in the level of the general Cdk inhibitor protein $p27^{KIP}$ [41,43]. The content of p27 is regulated at the level of transcription [44], translation [45], and polypeptide degradation [46]. The availability of p27 may also be regulated by the abundance of cyclin D/Cdk4; it has been suggested that the mitogen-induced increase in the abundance of cyclin D/Cdk4 complexes creates a reservoir for binding of p27, further facilitating the activation of Cdk2/cyclinE [47]. IL-2 induces the proteasome-dependent degradation of p27 [48]; this requires the phosphorylation of p27 (Thr187), which is catalyzed by active cyclin E/Cdk2 itself as well as other, as yet unidentified, kinases, and the subsequent ubiquitination of p27, which is mediated by the E3 ubiquitin ligase SCF. PI-3K, through PKB, upregulates the expression of Skp2, the substrate-targeting subunit of SCF, and thereby promotes p27 degradation [49]. Rapamycin blocks the IL-2 induced degradation of p27, and this effect is crucial to the inhibition of cell-cycle progression in T cells [48]. In addition, the ability of rapamycin to block cyclin D1 expression may further contribute to maintaining p27 at levels sufficient to prevent cyclin E/Cdk2 activation. Thus, T cells and MEFs from p27 knockout mice exhibit substantial resistance to the inhibition of proliferation by rapamycin [50]. Similarly, several cell lines selected for continued growth in the presence of rapamycin exhibit low levels of p27 that are unresponsive to further suppression by serum. In summary, it appears that a significant component of the ability of rapamycin to inhibit cell cycle progression in T cells, a critical pharmacologic target in immunosuppression, is attributable to the inhibition of p27 degradation. Rapamycin also inhibits p27 degradation in vascular smooth muscle cells, which appear to be particularly sensitive. Rapamycin inhibits not only proliferation but also vascular smooth muscle cell migration. As a consequence, rapamycin-impregnated vascular stents have shown considerable efficacy in inhibiting intimal hyperplasia and restenosis after percutaneous transluminal catheter (balloon) angioplasty (PTCA) [51].

Early studies of rapamycin pharmacology demonstrated a potent ability of the drug to inhibit the growth of a variety of human tumor cell lines, and recent work has reinvigorated the application of rapamycin derivatives as antitumor agents [53]. Aoki *et al.* [54] observed that chick embryo fibroblasts (CEFs) transformed with oncogenic versions of PI-3K or PBK/Akt are rendered extremely sensitive to growth inhibition by rapamycin, whereas rapamycin was without substantial effect on CEFs transformed with a variety of tyrosine kinase oncogenes, v-*crk*, v-*mos*, v-*jun*, or v-*fos*. Moreover, human tumors with spontaneous overactivity of the PI-3K/Akt pathway (e.g., those exhibiting PTEN loss of function) are very common and exhibit great sensitivity to growth inhibition by rapamycin or the rapamycin derivative, CCI779 [55]. A similar sensitivity to rapamycin is evident in tumors arising in $Pten^{+/-}$ mice [56]. Overexpression of the GLI transcription factor, a proproliferative element in the Sonic Hedgehog pathway [57], also confers rapamycin sensitivity, whereas transformation of these same cells by Ras or Myc does not. GLI expression is upregulated in the embryonal type of rhabdomyosarcoma, a childhood tumor that is frequently rapamycin sensitive.

In summary, the potent and highly specific mTOR inhibitors related to rapamycin have found clinical application in three circumstances: (1) as effective immunosuppressants through their ability to halt T-cell proliferation; (2) as antitumor agents, because the growth of human tumors characterized by overactivity of PtdIns(3′)OH kinase pathways is strongly and selectively inhibited by rapamycin derivatives; and (3) as inhibitors of vascular stent occlusion, through their ability to inhibit the proliferation and migration of vascular smooth muscle cells.

Signaling from TOR

In addition to the kinase catalytic domain, all TORs contain three other functionally relevant, structurally conserved segments (Fig. 1). Amino-terminal to the catalytic domain is a conserved segment of about 500 amino acids, termed

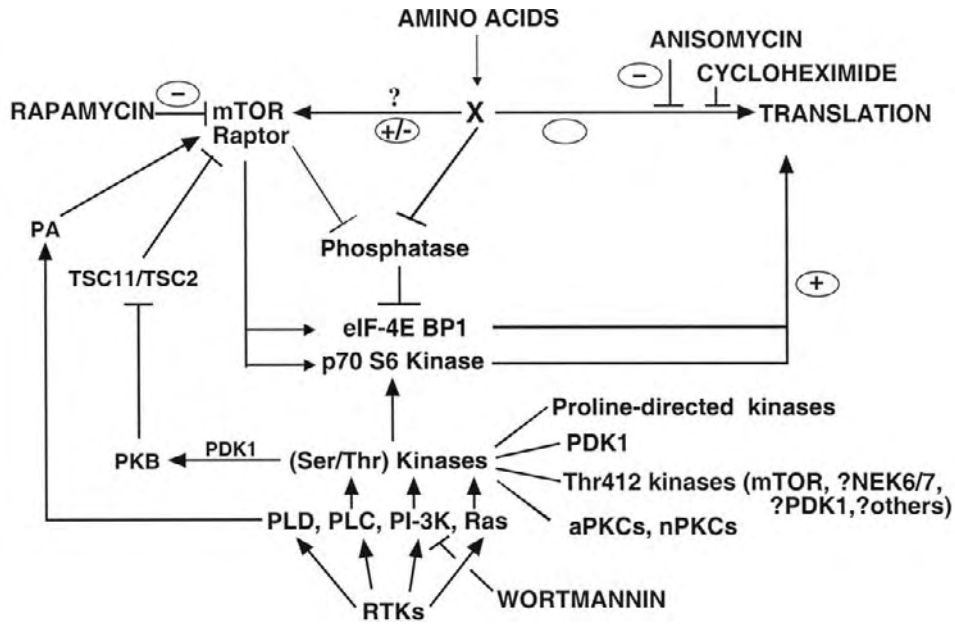


Figure 1

FAT (because it is found only in the FRAP/TOR, ATM, and TRRAP polypeptides). The FAT motif is always found in conjunction with a short motif at the polypeptide carboxy terminus, thus the name FATC. Mutations or deletions in these segments abolish mTOR kinase activity [58]. Situated between the FAT domain and the catalytic domain is a conserved segment that mediates the binding of the FKBP-12-rapamycin complex (the FRB domain). A crystal structure of the minimal binding domain expressed as a prokaryotic, recombinant 11-kDa fragment reveals four tight α-helices in a structure for which the N and C termini lie close together, suggestive of an independently folding module [59]. Within this segment, Ser 2035 (equivalent to Ser 1972 in yeast TOR2) is indispensable for binding the rapamycin/FKBP12 complex; replacement of Ser2035 by any amino acid other than alanine abolishes binding completely and, as in yeast TOR2, renders mTOR resistant to inhibition by rapamycin *in vivo*. At least several of the substitutions at Ser2035 (e.g., to Thr or Ile) do not appear to substantially interfere with mTOR kinase activity *in vitro* or *in vivo*, in that expression of these mTOR Ser 2035 mutants can rescue coexpressed p70 S6K [43,35] or 4E-BP [35,36] from rapamycin-induced dephosphorylation. Despite the conservation of overall amino acid sequence in the FRB domain, as well as of the universal presence (thus far) of a Ser at the residue equivalent to Ser1972 in yeast TOR2, not all TORs are sensitive to inhibition by rapamycin [60,61]. Nevertheless, the importance of this region in TOR signaling is reinforced by the finding that although *Schizosaccharomyces pombe* TOR does not bind FKBP-12/rapamycin complex, mutation of the serine in the FRB domain of *S. pombe* TOR homologous to ScTOR Ser 1972 inactivates *S. pombe* TOR function *in vivo* [61]. A fourth recognizable TOR domain is comprised by the multiple HEAT (Huntingtin, eIF3, PP2A-A subunit, TOR) repeats, a motif of 37 to 43 amino acids, each of which (as first defined for the scaffold (A) subunit of PP2A) contains an antiparallel pair of α-helices; the HEAT hairpin modules assemble in a linear, repetitive fashion to form an elongated stack of double helices [62]. The intrarepeat turns form a continuous ridge, presumably a protein interaction surface. mTOR has 20 HEAT motifs, which occupy nearly all of mTOR AA71-1147. The HEAT domains of yeast TOR have been reported to mediate membrane attachment [63]. The bulk of mTOR in mammalian cells is also particulate, although a precise localization is not available; a portion of mTOR polypeptides appears to be nuclear [64], and the nuclear entry may be critical for certain signaling functions (e.g., in transcriptional regulation).

As first shown by Brunn *et al.* [65], mTOR is capable of phosphorylating 4E-BP directly *in vitro*, preferentially at Thr 37 and 46 and possibly at all sites (Ser 65 and 82, Thr70) that undergo insulin/PI-3K-stimulated phosphorylation *in vivo* [66,67]. Subsequent work established the ability of mTOR to phosphorylate directly *in vitro* several functionally relevant sites on the p70 S6K [68,69]. The mTOR-catalyzed phosphorylation of 4E-BP, *in vitro* and probably *in vivo*, occurs in a hierarchical fashion, with phosphorylation of the sites (Thr37 and 46) amino-terminal to the 4E binding site (amino acids 54–60) occurring preferentially and those carboxy-terminal to the 4E-BP binding site occurring subsequently. The initial two phosphorylations have little impact on 4E-BP binding to eIF-4E; however, phosphorylation of the more carboxy-terminal sites, especially Ser65, potently inhibits eIF-4E binding. The phosphorylation at these carboxy-terminal sites also exhibits the greatest sensitivity to dephosphorylation in the presence of rapamycin and inhibitors of PI-3K; it is unclear whether this differential sensitivity is attributable to the properties of the relevant phosphatases, the dependence of TOR-catalyzed phosphorylation

at these more carboxy-terminal sites on the prior phosphorylation at Thr37 and 46, or the operation at these sites of a second, PI-3K-dependent protein kinase [66,67].

The properties of the mTOR-catalyzed *in vitro* phosphorylation of 4E-BP and p70 S6K differ in several ways. A striking difference is in the mTOR site selection on these two targets; all sites phosphorylated on 4E-BP by mTOR *in vitro* are Ser/Thr-Pro motifs [65,66]. In contrast, although mTOR catalyzes the phosphorylation at Ser/Thr-Pro sites on p70 S6K, in the SKAIPS domain and elsewhere (e.g., p70 S6K Ser394), the major site of mTOR-catalyzed phosphorylation is Thr412 in the motif FXXFTY [68,69]. Such diversity in site selection is unprecedented, and suggests the operation of a second, mTOR-associated protein kinase. Nevertheless, the mTOR-catalyzed phosphorylation of both p70 S6K and 4E-BP is inhibited *in vitro* by the addition of a rapamycin/FKBP12 complex, and mTOR mutated in its catalytic domain is unable to catalyze *in vitro* either phosphorylation. Thus, all these sites are phosphorylated *in vitro* directly by mTOR [69].

When extracted in a Tween-containing buffer [65], mTOR catalyzes detectable phosphorylation of both 4E-BP and p70 S6K. If, however, mTOR is extracted into a buffer containing Triton X-100, the ability of mTOR to phosphorylate 4E-BP *in vitro* is lost entirely, whereas mTOR-catalyzed phosphorylation of p70 S6K is unaltered or even increased [69]. Recent work [70,71] has established that the detergent-sensitive phosphorylation of 4E-BP by mTOR reflects the detergent-induced loss of an mTOR-associated scaffold protein, whose continued binding to mTOR is necessary for mTOR-catalyzed phosphorylation of 4E-BP *in vivo* and *in vitro*. This 150-kDa polypeptide (Raptor) is highly conserved from *S. cerevisiae* to humans and contains seven WD40 repeats in its carboxy-terminal segment. Raptor binds 4E-BP and p70 S6K as well as mTOR and is absolutely required for mTOR-catalyzed 4E-BP phosphorylation *in vitro*; Raptor binds selectively to hypophosphorylated forms of 4E-BP and physically links mTOR to eIF-4E. Insulin and amino acids promote the dissociation of the mTOR/Raptor complex from eIF-4E, in part by promoting the phosphorylation of 4E-BP [71]. The association of Raptor with mTOR may also be regulated by amino acid [70]. Remarkably, RNAi-induced inhibition of Raptor expression in *C. elegans* reproduces every phenotype seen with CeTOR deficiency, whether caused by CeTOR mutation or CeTOR RNAi [15,71], pointing to the likelihood that Raptor is central to all actions of TOR.

Another emerging chapter in the mTOR regulation of the p70 S6K is the role of the TSC1 and TSC2 gene products known as Hamartin and Tuberin, respectively. These two polypeptides, which occur as a heterodimer, together function as a tumor suppressor [72]. Mutation in either of the genes encoding these two polypeptides results in the syndrome tuberous sclerosis, which is characterized by the occurrence of multiple benign tumors, or hamartomas, particularly in the central nervous system, kidney, heart, lung, and skin, with the occasional emergence of a malignancy [73]. Deletion of the *Drosophila* homolog of TSC1 is the cause of the *gigas* mutant, characterized by organ and cellular overgrowth [74]. Overexpression in *Drosophila* of TSC1 and TSC2 together, but neither one singly, reduces cell size and cell proliferation [75,76] and is capable of suppressing the cellular overgrowth seen with deletion of DmPTEN or with overexpression of DmPKB [75–77]; reciprocally, loss-of-function mutations in TSC cause an increase in cell size [75,77] and can compensate for the failure of cell growth caused by hypomorphic mutations in *Drosophila* InsR or PI-3K but not the growth failure resulting from loss of function for Dm S6K [75]. Bialleic deletion of murine TSC1 results in the constitutive activation of p70 S6 kinase in serum-deprived mouse embryonic fibroblasts (MEFs), without activation of MAPK, whereas the serum-induced activation of PKB (but not MAPK) is greatly diminished [78]. TSC2 is phosphorylated *in vitro* and *in vivo* by protein kinase B (PKB), and mutation of these PKB phosphorylation sites to Ala greatly increases the inhibitory potency of recombinant TSC2 on coexpressed p70 S6K [79]. Thus, the TSC1/TSC2 complex appears to function as a negative regulatory element in mTOR signaling to p70 S6K; the role of the TSC complex in the regulation of PKB function (if any) is yet unclear. TSC inhibition of p70 S6K is reduced through an insulin/PI-3K stimulated, PKB catalyzed-TSC2 phosphorylation. Interestingly, homologs of either TSC1 or TSC2 are not identifiable in the *C. elegans* genome, a species in which the PI-3K and TOR pathways show no evident cross-regulation. TSC2 thus appears to be a major locus at which the PI-3K/PKB pathway positively regulates the output of mTOR, at least toward the p70S6K.

Despite the well-documented ability of mTOR to directly phosphorylate *in vitro* 4E-BP and p70 S6K at functionally relevant, rapamycin-sensitive sites, a considerable body of indirect evidence, derived from studies of the regulation of S6K1, indicates that the major pathway of mTOR regulation of the p70 S6K *in vivo* is indirect, through the negative regulation of a p70 S6K phosphatase, rather than by direct mTOR-catalyzed phosphorylation of p70 S6K [80]. The p70 S6 kinase is activated by a complex multisite phosphorylation, regulated jointly by the type 1 PtdIns(3′)OH kinase and mTOR. Activation is initiated by the phosphorylation of a cluster of Ser/Thr-Pro sites situated in a pseudosubstrate, autoinhibitory domain in the p70 S6 kinase noncatalytic carboxy-terminal tail. Although not activating *per se*, these phosphorylations, which can be catalyzed by a variety of proline-directed kinases as well as mTOR, enable the displacement of the tail from the centrally located catalytic domain, allowing access to the activating kinases. Activation is achieved by the phosphorylation of Thr252 [81,82], on the activation loop, and Thr412 [83], situated in a hydrophobic motif (FXXFTY) immediately carboxy-terminal to the canonical catalytic domain; although phosphorylation at either site gives some activation, the concomitant phosphorylation at both sites generates a strong positively cooperative activation of catalytic function [81]. The phosphorylation of Thr252 is catalyzed by the PtdIns(3,4,5)P_3-dependent kinase 1 (PDK1), although in a PtdIns(3,4,5)P_3-independent

manner, at least *in vitro* [81,82]. The identity of the kinase acting at Thr412 remains uncertain; mTOR itself can directly catalyze this reaction *in vitro* [68,69], as can PDK1 (although at <5% the rate of Thr252 [83]). ES cells lacking both alleles encoding PDK1 show no IGF-1-stimulated phosphorylation of either Thr252 or Thr412 [85]. Other elements that participate in p70 S6 kinase activation, although their roles are less well defined, include the atypical PKCs (λ or ζ) and Cdc42-GTP [80].

The p70 S6 kinase contains near its amino terminus a short (18 residues in S6K1) segment containing only acidic and hydrophobic amino acids. Deletion of this noncatalytic segment, when combined with deletion of the carboxy terminal noncatalytic tail, results in a mutant (p70Δ2-46/ΔCT104) that, like the wild type, has a low basal activity and is strongly activated *in vivo* by insulin and mitogens in a PI3-K-dependent manner; activation is accompanied by increased phosphorylation at the (remaining) p70 S6K sites critical to activation (i.e., Thr 252 in the activation loop and Thr 412 in the hydrophobic motif). In the wild-type p70 S6K, phosphorylation at Thr 412 is most sensitive to inhibition of PI3K (by wortmannin) or mTOR (by rapamycin) [83,86]. The p70 S6K 2-46Δ/ΔCT104 mutant remains fully sensitive to wortmannin, which results in dephosphorylation at Thr 412 and inactivation [86,87]. In contrast, the insulin/mitogen stimulated phosphorylation at Thr 412 and activation of the p70 Δ2-46/ΔCT104 mutant is entirely resistant to concentrations of rapamycin that far exceed those necessary to dephosphorylate Thr412 and inhibit the wild-type p70 S6K *in vivo* and that (in complex with FKBP12) inhibit the mTOR kinase activity assayed *in vitro* [86,87]. The unimpaired mitogen/PI-3K responsive activation of the Δ2-46/ΔCT104 variant in the presence of rapamycin establishes that mTOR kinase activity is not necessary for the insulin-mitogen activation by p70 S6K. Rather, it appears that the primary function of mTOR in p70 S6K regulation is to restrain a p70-S6K-inactivating element, most likely a p70 S6K phosphatase. Direct evidence in support of this idea is scant; however, Peterson *et al.* [88] have reported that the PP2A catalytic subunit can be coprecipitated with wild-type p70 S6K but not with the p70 S6K Δ2-46/ΔCT104 mutant, suggesting that the p70 S6K amino-terminal segment necessary for rapamycin sensitivity may serve as a binding site for the phosphatase or a phosphatase-associated regulatory protein. The function of this motif however is likely to be more complex, inasmuch as 4E-BP contains a motif similar in sequence to that in the p70 S6K amino terminus, and mutation of the motif in 4E-BP suppresses its insulin-stimulated 4E-BP phosphorylation [89]. In addition, overexpression of wild-type p70 S6K interferes with insulin-stimulated hyperphosphorylation of 4E-BP [89,90], but mutation or deletion of the p70 S6K amino-terminal rapamycin sensitivity segment abolishes the ability of coexpressed p70 S6K to suppress 4E-BP phosphorylation [89]. Thus, it appears that this motif is binding to some component critical for mTOR signaling; however, whether this is the mTOR polypeptide, a TOR scaffold protein, or a TOR-regulated phosphatase remains to be determined.

The idea that mTOR signaling is mediated in part through the modulation of protein phosphatase activity is entirely consistent with pathways elucidated in *S. cerevisiae*, wherein a mutation in the phosphatase regulatory protein TAP42 renders yeast significantly rapamycin resistant [91]. TAP42 associates with a small fraction of the yeast PP2A and SIT4 (homologous to mammalian PP6) phosphatase catalytic subunits and somehow restrains their activity toward specific substrates [92]. The TIP41 protein in turn negatively regulates TAP42 and TOR phosphorylates both. Nutrient deprivation or rapamycin inhibition of TOR causes dephosphorylation of TIP41, increasing its binding to TAP42, which releases the SIT4 phosphatase into an active state [93]. This results in the dephosphorylation of a number of mTOR target proteins (e.g., the cytoplasmic scaffold protein Ure2p, the transcription factor GLN3, or the protein [Ser/Thr] kinase NPR [94]). The mammalian homolog of TAP42, α4 [95], binds to the PP2A, PP4, and PP6 phosphatase catalytic subunits [96–99]; however, the rapamycin sensitivity of these complexes is disputed, and their role in the rapamycin-induced dephosphorylation of p70 S6K, 4E-BP, or other mTOR targets is unknown.

In addition to its positive regulatory input into the p70 S6K, mTOR also controls the activity of certain isoforms of PKC in an analogous manner. Thus, phosphorylation of the novel PKC isoforms δ and ε at sites near their carboxy terminus that are situated in hydrophobic motifs homologous to that surrounding S6K1 (Thr412) are also regulated in a serum-stimulated, rapamycin-sensitive manner [100,101]. Phosphorylation at these nPKC sites augments activity dramatically and is one step in a complex regulatory mechanism involving PDK1, an atypical PKC isoform and the ligand diacylglycerol [102]. As with the p70 S6K, the ability of rapamycin to cause dephosphorylation of the nPKCs is thought to be mediated by activation of a protein phosphatase. PKCδ can be coprecipitated with mTOR (as well as the DNA PK, another PIK-related kinase) and inactive variants of PKCδ can partially suppress serum-stimulated 4E-BP phosphorylation [103].

Regulation of mTOR Activity (Fig. 2)

Regulation of mTOR by RTK-PI-3K

Several reports indicate that insulin [104–106] induces a modest (~twofold) increase in the activity of immunoprecipitated mTOR; a more robust increase in mTOR activity is observed in response to neurotrophin (CNTF, BDNF) treatment of primary neurons and cell lines [107,108]. Overexpression of active forms of PI-3K or PKB results in increased 4E-BP phosphorylation at the rapamycin-/wortmannin-sensitive sites [109] and activation of p70 S6K [110], although PKB itself does not phosphorylate either 4E-BP or p70 S6K. Whether the effects of insulin, neurotrophins, recombinant PI-3K, and PKB reflect a direct modification and activation of TOR kinase or more indirect mechanisms (e.g., phosphorylation of and disinhibition

from TSC [79], PDK1 and/or PKB recruitment of other 4E-BP and/or p70 S6K kinases, negative regulation of protein phosphatase, or some combination of these actions) is not known. In addition to the modest (twofold) increase in the activity of immunoprecipitated mTOR, activation of PKB *in vivo* is associated with increased phosphorylation of mTOR at Ser2448 [106,111], a canonical PKB site. The functional significance of PKB-catalyzed mTOR phosphorylation is unclear, as mutation of Ser2448 to Ala on the rapamycin-resistant mTOR (Ser2035 Thr) does not alter its ability to rescue 4E-BP or p70 S6K from rapamycin-induced dephosphorylation [106]. Nevertheless, deletion of the mTOR segment surrounding Ser2448 [106] or the binding of a polyclonal Ab [66,105] to this site each increase the *in vitro* mTOR kinase activity by five- to tenfold, suggesting that a regulatory input (e.g., TSC inhibition) whose mechanism remains to be elucidated may be effected through this segment of mTOR.

Microinjection of a prokaryotic recombinant FRB fragment into MG63 osteosarcoma cells, a cell line whose growth is reliably arrested in G_1 by rapamycin, prevents entry into S [112], suggesting that the function of the FRB domain in maintaining TOR activity might be regulatory rather than structural. An important insight into the function of the FRB domain was the finding that this segment binds selectively to lipid vesicles that contain phosphatidic acid (PA) [113]. This binding can be inhibited by addition of an FKBP12/rapamycin complex or partially by mutation of mTOR Arg2109 to Ala; introduction of this mutation into a rapamycin-resistant mTOR (S2035T) mutant reduces by approximately 40% its ability to rescue coexpressed p70S6K from rapamycin inhibition. PA added exogenously can activate p70 S6K and 4E-BP phosphorylation. Mitogens, though activation of PLD, increase cellular PA levels, and butanol, which sequesters intracellular PA as an ester, inhibits the serum-induced activation of p70 S6K and the phosphorylation of 4E-BP without affecting phosphorylation of PKB or MAPK. Moreover, a mutant of p70 S6K resistant to inhibition by rapamycin (see above) is also resistant to inhibition by butanol. The noncovalent interaction of PA with the mTOR FRB domain appears to provide one component of the mitogen activation of mTOR signaling.

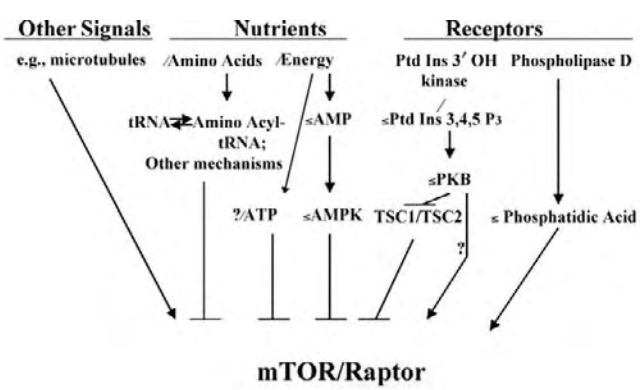


Figure 2

In summary, mTOR kinase activity is regulated in mammalian cells by tyrosine-kinase-linked receptors through a PI-3K–PDK1–Akt pathway, at least in some cell backgrounds and possibly for some, but not all, substrates. This activation persists to some degree after extraction and immunoprecipitation and is probably attributable to PI-3K/PB-induced mTOR phosphorylation. An additional important site of PKB regulation is through the phosphorylation of, and disinhibition from, TSC2 [79]. Moreover, as described above, an RTK–PLD-induced accumulation of phosphatidic acid [113] probably promotes mTOR activity through a noncovalent interaction; this activation is unlikely to survive cell extraction. An understanding of the TOR scaffold protein Raptor [70,71] as a site of regulation will be necessary for understanding the specific mechanisms that regulate the mTOR kinase activity toward each of its physiologic substrates. Finally, the role of gephyrin [114] in the receptor regulation of mTOR remains unclear. Gephyrin is a ubiquitously expressed tubulin-binding protein necessary for the postsynaptic clustering of glycine receptors in neurons. Gephrin binds to mTOR (AA1010–1128). Mutations in this region that abolish mTOR interactions with gephyrin abrogate the ability of a rapamycin-resistant mTOR (Ser2035Thr) to rescue p70 S6K and 4E-BP from rapamycin-induced dephosphorylation.

Regulation of mTOR by Amino Acids

The evidence that TOR retains its role as a nutrient-sensor in metazoans is entirely indirect; in contrast to the generally reproducible, if modest, stimulatory effects of insulin and neurotrophins, there has been no demonstration that alterations in the nutrient environment cause stable changes in mTOR kinase activity that can be measured by mTOR kinase assay *in vitro*, although it is reported that nutrient deprivation promotes an inhibitory interaction between Raptor and mTOR [70,71]. Nevertheless, there is persuasive evidence that TOR kinase activity *in vivo* is controlled by inputs related to amino acid and overall energy sufficiency. Thus, in a wide range of cultured mammalian cells, withdrawal of medium amino acids leads to progressive deactivation of the p70 S6K and dephosphorylation of eIF 4E-BP over 1 to 2 hours and completely inhibits the ability of insulin to promote these phosphorylations [87,115–117]. Amino acid withdrawal, however, does not significantly affect, at least over this initial interval, the upstream elements of the insulin signaling pathway (i.e., IR/IRS tyrosine phosphorylation, PI-3K activity, PKB or MAPK activation). Readdition of amino acids restores phosphorylation of p70 S6K on 4E-BP and their responsiveness to insulin. Moreover, elevation of ambient concentration of amino acids to higher than usual levels causes a progressive increase in p70 S6K activity and 4E-BP phosphorylation to levels observed with maximal insulin stimulation; at these high amino acid concentrations, addition of insulin gives no further stimulation of p70 S6K activity [87]. Thus, amino acid deficiency, like rapamycin, results in the selective

dephosphorylation of these two well characterized TOR targets in a manner that overrides the activating input of the RTK/PI-3K pathway. Notably, the doubly deleted rapamycin-resistant p70 S6K mutant, p70Δ2-46/ΔCT104, is also highly resistant to deactivation/dephosphorylation by amino acid withdrawal [87]. This finding establishes that amino acid withdrawal, like rapamycin, does not inhibit any of the steps necessary for the insulin/PI-3K phosphorylation of p70 S6K Thr252 (the PDK1 site) and Thr412 (unknown kinase), but rather promotes the dephosphorylation of these sites; mTOR and amino acid sufficiency thus appear to inhibit the same p70 S6K phosphatase. Although rapamycin inhibits the ability of amino acid readdition to restore p70 S6K phosphorylation, it has not been formally established whether amino acids require mTOR to inhibit this putative p70 S6K phosphatase. Wortmannin also inhibits amino-acid-induced p70 S6K phosphorylation, but concentrations higher than those sufficient to inhibit type 1a PI-3K are required [39] and correspond to those necessary for inhibition of mTOR itself [118]. Subsequent work demonstrated that the rapamycin-sensitive phosphorylation sites in the nPKCs (δ and ε) are also dephosphorylated in response to amino acid withdrawal [100,101].

The complete restoration of p70 S6K and 4E-BP phosphorylation in cell culture requires the readdition of all 20 amino acids; readdition of single amino acids is without effect, except for leucine, which enables a variable extent of partial restoration in many cell lines [39,88,115,119]. Similarly, whereas removal of any single amino acid usually results in some dephosphorylation, the most substantial inhibition is observed on removal of leucine and occasionally arginine. A large body of *in vivo* experiments by Jefferson *et al.* [120–123] indicate that leucine exerts significant stimulatory action on the phosphorylation of p70 S6K and 4E-BP and is uniquely effective in promoting the synthesis of ribosomal proteins, providing strong evidence that the amino-acid-activated, mTOR-dependent pathways evident in cell culture are operative *in vivo* and are one component of the multiple mechanisms by which amino acids and insulin coordinately regulate protein synthesis especially in skeletal muscle.

Although evidence has been provided for the existence of a membrane-localized leucine receptor, at least in regard to amino-acid-regulation of autophagy in hepatocytes [124], it is likely that the majority of amino-acid-dependent responses mediated by mTOR are initiated at an intracellular site [119]. Thus, inhibition of mRNA translation, either at the level of initiation (anisomycin) [125] or elongation (cycloheximide) [126], results in the activation of p70 S6K and hyperphosphorylation of 4E-BP [127] in a rapamycin-sensitive manner. Reciprocally, overexpression of eIF-4E (although transforming in many cell backgrounds) is accompanied by hypophosphorylation of 4E-BP and p70 S6K [127]. An attractive hypothesis is that the ability of protein synthesis inhibitors to stimulate the phosphorylation of p70 S6K and 4E-BP might reflect the activation of mTOR, induced by the accumulation of some intermediate in the translational process (e.g., a minor acylated tRNA) or by a byproduct of stalled translation, analogous to the synthesis of guanosine tetraphosphate during the "stringent" response in bacteria [128]. Support for such a mechanism is provided by the observation that amino acid alcohols, which inhibit cognate tRNA synthetase activity and protein synthesis, nevertheless cause dephosphorylation of 4E-BP and p70 S6K (suggesting a decrease in mTOR activity), in contrast to anisomycin [129]. Moreover, cycloheximide overcomes the dephosphorylation of p70 S6K/4E-BP caused by amino acid withdrawal. Similarly, CHO cells bearing a temperature-sensitive mutant histidyl tRNA synthetase, when shifted to the nonpermissive temperature, exhibit dephosphorylation of 4E-BP and p70 S6K [129]. If mTOR is regulated by the charging of tRNAs, this is accomplished through a mechanism distinct from that regulating the GCN2 kinase [130], as well as that underlying the bacterial stringent response [131].

Regulation of mTOR by Energy Sufficiency

Although the phosphorylation of 4E-BP is suppressed by amino acid withdrawal, some degree of insulin-stimulated 4E-BP phosphorylation persists under these conditions, to a degree sufficient to displace 4E-BP from 4E and to promote an increased association of eIF-4E with eIF-4G [132]. In CHO cells deprived of both amino acids and glucose, the basal- and insulin-stimulated phosphorylation of Thr 36/45 is severely inhibited; readdition of glucose alone, although insufficient to enable detectable phosphorylation of p70 S6K1 Thr412 or Ser444/447, allows substantial insulin-stimulated Thr36/45 phosphorylation and significant basal and insulin stimulated protein synthesis. This effect of glucose requires its metabolism and can be reproduced in part by lactate [132]. Although the identity of the kinase responsible for the glucose-dependent, insulin-stimulated phosphorylation of 4E-BP Thr36/45 is not known, a plausible candidate is mTOR, inasmuch as these are the primary sites of mTOR-catalyzed 4E-BP phosphorylation. The dependence of this response on glucose metabolism suggests that mTOR kinase activity is itself, to some extent, dependent on and regulated by some product of glucose metabolism, independent of amino acid sufficiency, PI-3K, and PKB. Several observations indicate that this input is related to the state of overall energy sufficiency, as reflected by the concentration of adenine nucleotides. Thus, inhibitors of glycosis such as 2-deoxyglucose (2DG) and inhibitors of mitochondrial oxidative phosphorylation, e.g., rotenone or CN^- both cause a marked inhibition of 4E-BP1 and p70 S6K phosphorylation, at concentrations that have little effect on PKB or MAPK activation [133]. Notably, a rapamycin-resistant mutant of p70 S6K previously shown to be resistant to inhibition on withdrawal of amino acids is also entirely resistant to the inhibitory effects of 2DG, strongly supporting the conclusion that the inhibitory effects of energy depletion on p70 S6K and presumably 4E-BP are mediated by inhibition of mTOR. It has been suggested that mTOR is directly sensing

the concentration of ATP itself, based on the apparently high ED_{50} for ATP (~1.0 m*M*) in the mTOR-catalyzed phosphorylation of 4E-BP *in vitro* [132]. This estimate of K_m for ATP, however, is probably compromised by the copurification with mTOR of protein phosphatases and other contaminants. A more plausible mediator of TOR inhibition in the setting of energy depletion is the AMP-activated kinase (AMPK) system [134,135]. AICAR, a precursor of the AMPK activator ZMP, can inhibit p70 S6K and 4E-BP phosphorylation in cell culture, at least in cells able to efficiently convert this precursor to ZMP, without inhibition of PKB and MAPK. In addition, mTOR directly and specifically associates with AMPK. AICAR given by subcutaneous injection to rats results in an inhibition in skeletal muscle of p70 S6K (Thr412) and 4E-BP (Thr37) phosphorylation, accompanied by a decrease in the association of eIF-4E with eIF-4G and an inhibition of protein synthesis [135]. The effects of AICAR in skeletal muscle may also be mediated by an inhibition of the PI-3K pathway inasmuch as AICAR injection *in vivo* also results in decreased phosphorylation of PKB (Ser473) and mTOR Ser2448, a canonical site of PKB-catalyzed phosphorylation *in vivo*; the latter responses are not seen upon glucose withdrawal from cultured cells. In summary, mTOR output is regulated by the cellular energy state, although this appears to be secondary in importance to regulation by amino acid sufficiency. Inhibition of mTOR by AMPK is likely to contribute an important component of the energy-dependent regulation of TOR.

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CHAPTER 88

AMP-Activated Protein Kinase

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Introduction

The AMP-activated protein kinase (AMPK) is the downstream component of a kinase cascade that has multiple cellular targets [1–3]. It is switched on by cellular stresses that deplete cellular adenosine triphosphate (ATP), causing increases in the cellular adenosine diphosphate (ADP) : ATP ratio. The AMP:ATP ratio is further amplified by adenylate kinase, the signal that activates the AMPK system [1]. Genome sequencing suggests that protein kinases related to AMPK may exist in all eukaryotes, including fungi and plants, as well as animals ranging from *Dictyostelium discoideum* to mammals. In budding yeast, the homolog of AMPK is the Snf1 complex, with the *snf1* gene (encoding the catalytic subunit) being originally characterized via mutations that caused failure to grow on carbon sources other than glucose [3]. A functional Snf1 complex is required for derepression of many glucose-repressed genes when glucose is removed from the medium. Similar protein kinases also exist in higher plants [4]. Although similar to the mammalian system in many respects, a puzzling feature is that the fungal and plant kinases do not appear to be allosterically activated by AMP.

Structure of the AMPK Complex

AMP-activated/SNF1 protein kinases are heterotrimeric complexes consisting of catalytic α subunits and regulatory β and γ subunits [5]. In mammals, each subunit is encoded by multiple genes (α1, α2, β1, β2, and γ1 to γ3), and these assemble into up to 12 different α, β, γ combinations [6]. Budding yeast contains single genes encoding the α (*snf1*) and γ (*snf4*) subunits and three genes encoding β subunits [7]. The α subunits contain N-terminal kinase domains and C-terminal regulatory domains that inhibit the kinase in its inactive state [8,9]. In yeast, the γ subunit is an activator by genetic criteria, and in mammals the γ subunits appear to be involved in binding the allosteric activator, AMP [6]. All γ subunits contain four tandem repeats of a sequence motif known as a CBS (cystathionine-β-synthase) domain. These occur in a variety of other proteins, from archaea to eukaryotes [10], and, although their exact function is unknown, in the enzyme cystathionine-β-synthase mutations in the CBS domain result in failure to be activated by the allosteric effector, S-adenosyl methionine [11]. Because both S-adenosyl methionine and AMP contain adenosine, it is tempting to speculate that the CBS domains of the γ subunits bind the adenosine moiety of AMP. The β subunits of AMPK act as scaffolds on which α and γ; assemble through interaction with the conserved KIS and ASC domains [7] and may also be involved in subcellular targeting [12] (Fig. 1).

Regulation of the AMPK Complex

AMPK/Snf1 complexes are inactive unless phosphorylated on a threonine residue within the activation loop of the α subunit [13,14] by upstream kinases that remain unidentified. AMPK complexes are allosterically activated by AMP, with the extent of activation (up to sevenfold) depending on the identity of the α and γ subunits [6]. AMP also promotes phosphorylation and activation of the kinase, via a three-fold mechanism of binding to AMPK and (1) making it a better substrate for the upstream kinase, (2) making it a worse substrate for the protein phosphatase, and (3) binding to and activating the upstream kinase. This multistep mechanism generates great sensitivity, such that over a critical range of concentrations a small change in AMP produces a large change in kinase activity [15]. Effects of AMP that are due

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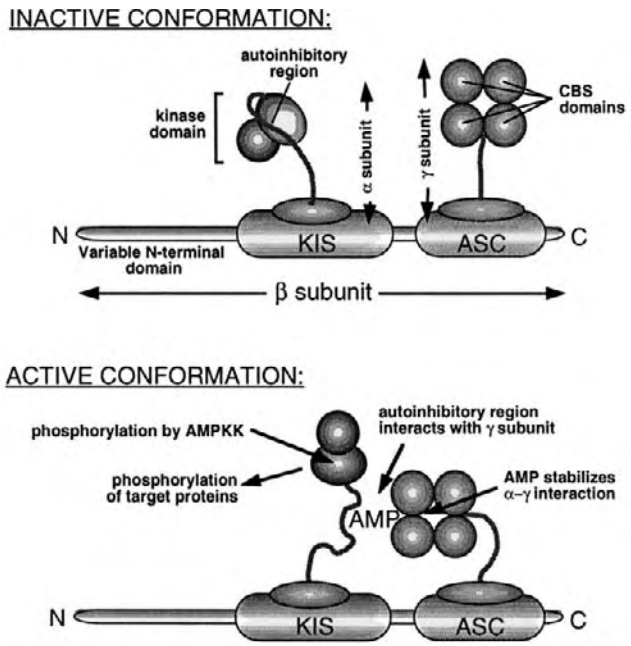


Figure 1 Model for the changes in interdomain interactions in the AMPK complex. In both the inactive and active conformations, the β subunit acts as a scaffold that binds α and γ via the conserved KIS and ASC domains. In the inactive conformation (top), the kinase domain of α is inhibited by interactions with the autoinhibitory region on the same subunit. In the active conformation (bottom), this interaction is prevented because the autoinhibitory region on α now interacts with the CBS domains on γ, instead of with the kinase domain. AMP promotes this conformation by stabilizing the α↔γ interaction, while ATP binding at the allosteric site disrupts it. In the active conformation, the kinase domain is free to be phosphorylated and activated by the upstream kinase and to phosphorylate downstream targets. (From Hardie, D. G. and Hawley, S. A., *BioEssays* 23, 1112–1119, 2001. With permission.)

to binding to AMPK itself are antagonized by high concentrations of ATP. The system therefore responds to rises in cellular AMP : ATP, leading to the concept that it is a sensor of cellular energy charge, or fuel gauge [16]. This view was reinforced by findings that the kinase is inhibited by phosphocreatine [17].

Regulation in Intact Cells and Physiological Targets

The AMPK system is activated by cellular stresses that inhibit ATP production or accelerate ATP consumption. Stresses of the former type include heat stress and metabolic poisons [18], ischemia and hypoxia [19,20], oxidative stress [21], and glucose deprivation [22], the latter also being the primary stress that activates the Snf1 complex in yeast [23]. A physiological stress that activates AMPK by increasing ATP consumption is muscle exercise [24]. Studies with transgenic mice expressing a dominant-negative mutant suggest that AMPK is wholly responsible for the effects of hypoxia and partly responsible for the effect of contraction on muscle glucose uptake [25].

A full description of known or putative physiological targets for AMPK is beyond the scope of this article, but readers may refer to a recent review [1]. In general, AMPK switches on catabolic pathways that generate ATP (e.g., glucose and fatty acid oxidation) while switching off cellular processes that consume ATP, especially anabolic (biosynthetic) pathways. This has led to the concept that it acts as a "metabolic master switch" [26]. It achieves this task both by direct phosphorylation of metabolic enzymes and via effects on gene expression. The mechanisms by which AMPK regulates transcription remain unclear, although it has been shown to phosphorylate p300, leading to reduced interaction of this co-activator with nuclear hormone receptors, such as that for PPAR-γ [27]. In addition, AMPK inhibits expression of two transcription factors (i.e., HNF-4α [28] and SREBP-1C [29]), thus indirectly regulating the transcription of entire classes of target genes.

Medical Implications of the AMPK System

Type 2 diabetes, which affects over 100 million people worldwide, is a hyperglycemic condition caused by reduced glucose uptake by muscle and increased glucose production by liver. Physical exercise is known to provide protection against its development, and because AMPK is activated by exercise it regulates the activity and expression of the insulin-sensitive glucose transporter GLUT4 and inhibits expression of enzymes of gluconeogenesis [30], this suggesting that AMPK could be a promising target for therapy of Type 2 diabetes [26]. This idea has been supported by recent findings that metformin, an important oral hypoglycemic agent used to treat Type 2 diabetes, activates AMPK *in vivo* [29].

Mutations in the AMPK γ2 gene cause hereditary conditions that lead to sudden death by heart failure, such as Wolf-Parkinson-White syndrome (a type of arrhythmia) with or without associated hypertrophy [31,32]. The effects of these mutations on AMPK activity remain unclear, although intriguingly they occur within the CBS domains, the putative AMP-binding regions.

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CHAPTER 89

Principles of Kinase Regulation

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Introduction

Protein kinases comprise one of the largest protein families, corresponding to ≈2% of eukaryote genes. Their prominence reflects the fact that protein phosphorylation is the most abundant form of cellular regulation, affecting essentially all cellular processes, including metabolism, growth, differentiation, motility, membrane transport, learning, and memory. To function as switches controlling all of these processes, kinases must be tightly regulated. Improper regulation leads to cancer and various other diseases. Regulation is an integral part of protein kinase function that controls the timing of catalytic activity and substrate specificity.

Protein kinase can be regulated in diverse ways, ranging from transcriptional control through subcellular localization and recruitment of substrates (using anchoring, adaptor, and scaffold proteins or domains [1]) to structural and chemical modifications of the proteins themselves. This short review focuses on the principles of regulation of protein kinase activity at the protein level (for further details, see related recent reviews [2,3]).

Protein Kinase Structure

Eukaryote protein kinase domains segregate into two large groups, phosphorylating either serine/threonine or tyrosine residues on target proteins. However, both groups have essentially similar three-dimensional structures comprised of two lobes with the active site located in the cleft between the small and large lobes [4,5]. The smaller, N-terminal (N-) lobe contains mainly beta structures and one important helix termed helix C, whereas the C-terminal (C-) lobe is largely alpha-helical. Important structural motifs include the glycine-rich motif that forms a phosphate-binding (P-) loop that anchors the ATP phosphates; the activation loop, often containing phosphorylation sites, provides a surface for peptide substrate binding (Fig. 1).

All protein kinases catalyze the same reaction—the transfer of the gamma-phosphate from ATP to the hydroxyl group of a Ser, Thr, or Tyr—and adopt strikingly similar structures in their active forms. The active structure positions the substrates within the constellation of catalytic residues. By contrast, the mechanisms used to maintain protein kinases in inactive forms show remarkable diversity. These range from allosteric to intrasteric and everything in between [6] and are used to modulate the conformations of the activation loop and the P-loop, the position of helix C, the access to ATP and substrate binding sites, and the relative orientation of the two lobes (Figs. 1 and 2). The control can be exerted by internal regions of the kinase catalytic domains, by sequences outside the catalytic domain, or by additional subunits or interacting proteins; these regions or proteins may respond to second messengers, and their expression may be controlled by the functional state of the cell. They can target the kinase to different substrates or subcellular locations or inhibit the kinase activity. These possible regulatory sites affect each other, resulting in a rich spectrum of possible regulatory pathways. We first review allosteric and intrasteric behaviors in protein kinases, and then address how individual sites are regulated.

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General Principles of Control

Allosteric Regulation

Allosteric regulation is a classic widespread mechanism of control of protein function; effectors bind to regulatory sites distinct from the active site, inducing conformational changes that profoundly influence the activity [7]. Allosteric effectors typically bear no structural resemblance to the substrate of their target protein. This form of regulation explains how end products of metabolic pathways could act at early steps of the pathway to exert feedback control. In protein kinases, allosteric control can be exerted by flanking sequences or separate subunits/proteins, such as, for example, the N-terminal sequence in EphB2 receptor tyrosine kinase or cyclin in cyclin-dependent kinases (CDKs) influencing the orientation of the lobes and rotation of helix C.

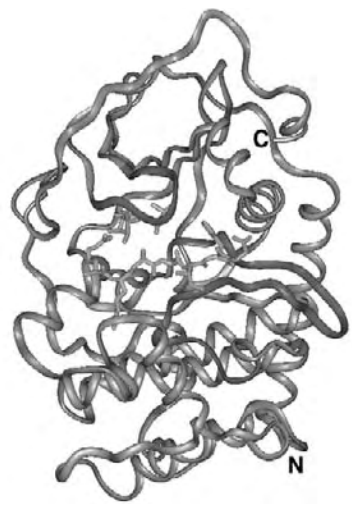


Figure 1 Ribbon diagram of the structure of the catalytic domain of PKA, with the various regulatory regions colored: activation loop, red; P-loop, blue; helix C, cyan. The bound ATP and peptide substrate (only seven residues are shown) are shown in stick representation in orange and green, respectively. The figure was generated using GRASP [32].

Intrasteric Regulation

The term *intrasteric regulation* was introduced to describe autoregulation of protein kinases and phosphatases by internal sequences that resembled substrate phosphorylation sites ("pseudosubstrates") and acted directly at the active site [8]. It is now clear that this form of control is used widely and extends to diverse enzyme classes as well as receptors and protein targeting domains [6]. Intrasteric interactions typically suppress protein functions, and diverse mechanisms can be used for activation, including protein activators or ligands, phosphorylation, proteolysis, reduction of disulfide bonds, or combinations of these. The best examples of intrasterically regulated protein kinases are the large subfamily activated either by calcium-binding proteins (e.g., calmodulin-dependent kinases [CaMKs], titin, twitchin) or calcium directly in the case of the plant calcium-dependent protein kinases that contain a calcium-binding domain fused with the kinase domain. In twitchin kinase, a C-terminal autoregulatory sequence threads through the active site cleft between the two lobes of the protein kinase domain, making a plethora of contacts with the peptide substrate binding site and ATP-binding residues as well as residues essential for catalysis [9] that completely shut down kinase activity. The binding site of the activator S100A1 has been mapped to one portion of the autoinhibitory sequence [9,10]. A very similar mechanism of inhibition occurs in the related giant kinase titin; however, here a combination of phosphorylation and calmodulin (CaM) binding (to a site analogous to the S100A1 binding site in twitchin) is required to activate the enzyme [11]. The more distantly related CaMK-I is also activated by CaM binding to an autoregulatory sequence, but the structure shows a modified mode of

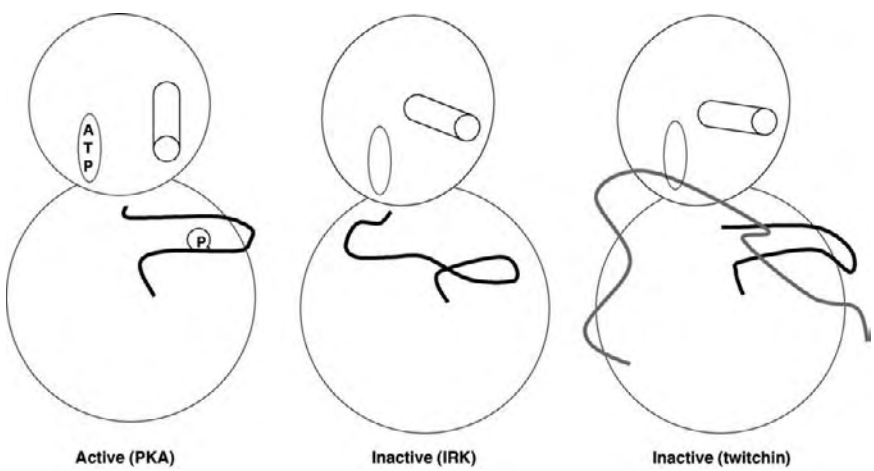


Figure 2 A schematic diagram showing prototypic kinase active structure (PKA) and inactive structures (IRK and twitchin), highlighting the regulatory regions. The two lobes of the catalytic domain, activation loop (thick line), helix C (cylinder), the ATP binding site (ATP), and the autoregulatory sequence (thick gray line) are shown.

inhibition where the autoregulatory sequence exits the active site before the P-loop and not over the activation loop, as is the case for twitchin kinase [12]. In p21-activated kinase (PAK), the autoregulatory sequence is located on a distinct "inhibitory switch domain" binding to the C-lobe of the kinase which both blocks the substrate binding site and causes various distortions to the kinase domain [13]. PAK is activated by the GTP form of the Rho family of G proteins. Other protein kinase families predicted to be intrasterically regulated include glycogen synthase kinase 3β, which is proposed to be autoinhibited. In this case, phosphorylation of Ser-9 causes the N-terminus to bind to the small lobe and direct the autoregulatory sequence into the active site [14]. The protein kinase C family and cGMP-dependent protein kinases also have autoregulatory sequences N-terminal to the catalytic domain; the activators are phospholipids and diacylglycerol, and cGMP, respectively [15].

Insulin receptor tyrosine kinase (IRK) has revealed a more subtle autoinhibitory mechanism with a tyrosine residue bound to the active site [5]. This tyrosine may be considered a transient pseudosubstrate and is ultimately autophosphorylated in response to insulin binding to the extracellular part of the receptor. Phosphorylation of this and two other tyrosine residues results in a rearrangement, allowing access to the active site [16]. A similar blocking of the active site by a tyrosine is also observed in the inactive structure of the MAP kinase ERK2 [17]. In this case, the tyrosine is one of the two residues phosphorylated by a distinct upstream kinase to yield the active enzyme [18].

Regulatory Sites in Protein Kinase Domains

Activation Loop

The activation loop represents the most complex element of protein kinase structure and shows a great variety of behaviors. The loop has evolved a remarkable ability to rearrange in response to phosphorylation. Many kinases that require activation by phosphorylation in the activation loop contain an arginine residue immediately preceding the catalytic aspartate and have therefore been termed RD kinases [19]. The activation loop represents a part of the active site and also has an influence on the position of helix C; it must be in an open and extended conformation to allow substrate binding. The most dramatic examples of the modulation of activity by phosphorylation of the activation loop include the previously mentioned IRK and MAP kinase ERK2. The phosphorylation even modulates the oligomerization and nuclear localization of ERK2 [20]. The inactive conformation of the activation loop has even been exploited medically as a specific binding site for the anticancer drug Gleevec in Bcr–Abl [21].

Helix C

This helix modulates kinase activity because it is coupled through intramolecular contacts to both the ATP binding site and the activation loop, by moving as a rigid body in response to intra- and intermolecular regulators. The best understood example is CDK2, where cyclin binding directly to the helix and its vicinity induces a rotation that reconstitutes the ATP binding site [22]. In Src family tyrosine kinases, the binding of the adjacent SH3 domain from the protein holds helix C in a conformation similar to the inactive state of CDK2; ligands to the SH3 domain (and the SH2 domain also present in the protein) allow helix C to resume the active conformation [23–25]. Both CDKs and Src family kinases simultaneously require phosphorylation in the activation loop for full activity.

P-Loop

The conformation of the P-loop differs subtly between the active kinases. It is likely flexible so it can both accommodate ATP binding despite subtle changes in the orientation of the two lobes and respond to regulators.

ATP Binding Site

Blocking the conserved ATP binding site is a common mechanism to regulate protein kinase activity, and the specificity stems from intermolecular protein–protein interfaces (e.g., p16-CDK6 interaction [26,27]) or complex intramolecular interactions (e.g., IRK, twitchin kinase).

Substrate Binding Site

The substrate peptide binding site is located in the groove between the N- and C-lobes with the activation loop constituting a part of it. It is most often blocked via intrasteric interactions or modulated via the conformation of the activation loop. In the case of CaMK-I, potent synthetic peptide substrates have been found that appear to induce the activation loop into a productive conformation without the need for phosphorylation [28], but it remains to be seen whether this phenomenon extends to protein substrates.

Flanking Segments

Polypeptide segments flanking the protein kinase domain either N- or C-terminally are responsible for autoinhibition in most kinases exhibiting intrasteric regulation (e.g., twitchin, CaMK-I, PAK). In some cases, the flanking regions are not directed to the active site but inhibit the catalytic activity allosterically through conformational change alone. EphB2 kinase is activated by phosphorylation in both the activation loop and the N-terminal flanking sequence. In the dephosphorylated state, this N-terminal sequence binds to the N-lobe and stabilizes helix C and the activation loop in a catalytically unproductive conformation [27]. Phosphorylation of the N-terminal segment also activates the type I TGFβ receptor (TGFβ-I). This N-terminal GS region inhibits the kinase activity when bound to the inhibitory protein FKBP12, distorting the N-lobe and particularly

helix C [29]. The activators of EphB2 and TGFβ-I are various SH2 domains and Smad proteins, respectively.

Conclusions

The structures of different protein kinases in active and inactive states have revealed some fundamental principles of kinase regulation, as well as numerous variations on the major themes. It is expected that further variations exist, and additional structural information will be crucial in understanding them; currently, structural information is only available for less than 1% of the protein kinases. Attempts to gain further insights into the mechanisms of regulation are being made by combining structural analysis with computational [30], biophysical [31], and other approaches.

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CHAPTER 90

Calcium/Calmodulin-Dependent Protein Kinase II

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Introduction

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is the most complex in structure and regulation of the family of calmodulin-dependent protein kinases. It was first described as a calcium/calmodulin-dependent protein kinase with a relatively broad substrate specificity and highly expressed in brain [1–3]. We now know that CaMKII is expressed in many tissues, including spleen, heart, and skeletal muscle [2,4]; however, its level of expression in neurons of the forebrain is extraordinarily high. It constitutes approximately 2% of total protein in homogenates of the adult rat hippocampus and approximately 1% of total protein in rat forebrain homogenates [5]. This high level of expression suggested that it might carry out specialized functions in neurons and, indeed, this appears to be the case. Many studies now implicate CaMKII in regulation of multiple determinants of synaptic strength in excitatory neurons in the brain, as well as in regulation of homeostasis of synaptic components. This chapter discusses the structure of CaMKII and its regulation by autophosphorylation, as well as current ideas about how its characteristics are used to regulate synaptic function.

Structure of CaMKII

CaMKII was first purified from rat brain homogenates [6,7] and shown by study of its hydrodynamic properties to be a dodecameric hetero-oligomer of two subunits, termed alpha (50 kDa) and beta (60 kDa) [6]. These subunits are highly homologous to each other and are both catalytic. They appear to assemble together into dodecamers that contain the same average proportions of α- and β-subunits as are present at the time of synthesis. Thus, the holoenzyme composition in a given cell is not homogenous but is instead distributed randomly according to the proportion of available subunits at the time of assembly. There does not appear to be an energetic preference for one holoenzyme composition over another; but this subject has not been studied exhaustively.

Subunits

Cloning of cDNAs encoding subunits of CaMKII demonstrated that the rat genome contains four different genes encoding subunits of CaMKII, each of which has a distinctive pattern of tissue-specific expression (see Table 1). These subunits all apparently associate into dodecameric holoenzymes, as do the α and β subunits [4]. The most significant differences in sequence among the subunits are found in a region between the amino-terminal catalytic domain (≈300 residues) and the carboxyl-terminal association domain (≈160 residues) [8]. In this variable region, each of the subunits contains unique sequences ranging from 0 to 70 residues in length; sometimes this region is also alternatively spliced. The variable sequences are believed to confer unique properties. For example, the β-subunit has a higher affinity for calmodulin than does the α-subunit [9,10], perhaps endowing it with greater sensitivity to cytosolic calcium. The β-subunit also displays a much stronger affinity for actin filaments than does the α-subunit [11]. On the other hand, the α-subunit has a higher affinity for the potential postsynaptic density docking protein, densin [12].

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Table I Distinct Mammalian Genes Encode Four CaMKII Subunits

Subunit	Molecular weight	Tissue distribution	Refs.
α	54.1 kDa	Only in neurons, at very high levels in forebrain neurons	[8,15]
β	60.4 kDa	Only in neurons, at moderate levels in most neurons	[16]
γ	59 kDa	In most tissues, at moderate levels	[17,18]
δ	60.1 kDa	In most tissues, at moderate levels	[17]

Hence, the small differences in properties of the subunits may confer important differences in regulation and subcellular localization.

In the brain, the message encoding the α-subunit is transported into dendrites, whereas that encoding the β-subunit is confined to the soma [13]. Transport of the α-subunit message permits its synthesis in dendrites and, by deduction, assembly of new dendritic "α-only" holoenzymes [14].

Structure of the holoenzyme

Association Domain

Individual subunits associate with each other through their carboxyl-terminal association domains [19]. The two domains mediate formation of an antiparallel dimer. Six dimers then associate to form the dodecameric holoenzyme. The holoenzyme is extremely stable; there is no evidence for the existence of significant amounts of dimers or other intermediate structures in cells, nor is there any indication that holoenzymes can exchange subunits.

Structure Determined by Cryoelectron Microscopy

The individual catalytic domains and the holoenzyme of CaMKII have not been crystallized, but a great deal of insight has come from determination of the structure of a homomeric α-subunit dodecamer at about 3-nm resolution by cryoelectron microscopy [20] (see Fig. 1). This unique structure consists of a hollow, gear-shaped, central cylinder approximately 20 nm in diameter and 10 nm thick. Six slanted flange-like "teeth" project from the surface of the cylinder and confer a six-fold rotational symmetry. Each of the teeth appears to be formed by an antiparallel dimer of the association domains of two subunits. The catalytic domains extend from each end of the teeth to form two parallel rings of six enzymes separated by the cylindrical central structure. The variable regions of the different subunits would form part of the central structure. Thus, specific subcellular association sites located in the variable region would be situated on the central cylinder. The symmetry of the structure suggests that each holoenzyme would contain six pairs of similar binding domains that can mediate subcellular localization. It will be interesting to learn whether this domain arrangement permits the kinase holoenzyme to act as a structural node within the postsynaptic density or other cytoskeletal structures.

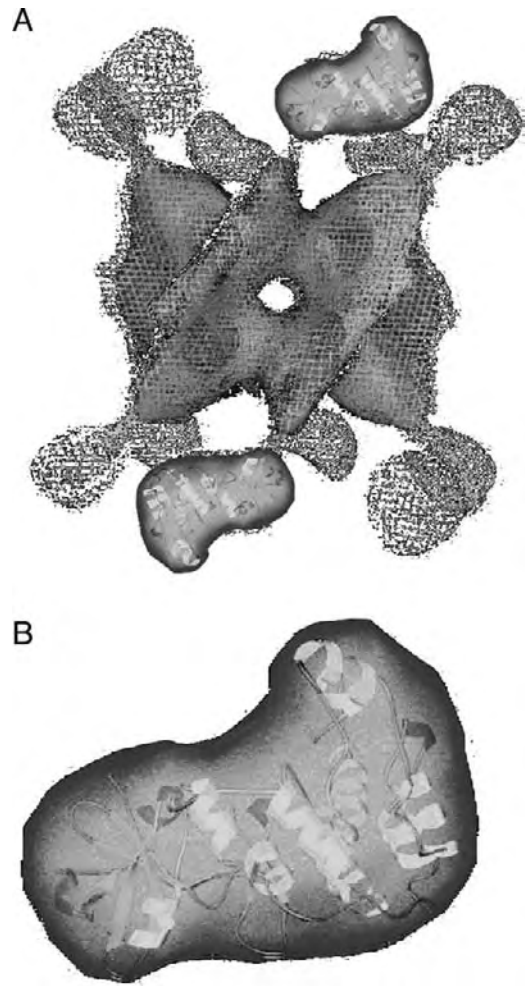


Figure 1 Structure of the holoenzyme of CaMKII. The structure of a holoenzyme of α subunits of CaMKII was determined by cryoelectron microscopy by Kolodziej *et al.* [20]. A surface representation (adapted from Fig. 5 of their paper) is shown in (A). The red central portion is the cylinder formed by the 12 association domains. The yellow, foot-like structures are individual catalytic subunits. Superimposed on two of the catalytic domains is a scaled representation (blue) of the fit of the x-ray structure of the catalytic domain of the cAMP-dependent protein kinase into the surface representation of the "feet." A larger view of this fit (also from Fig. 5 of Kolodjiez *et al.*) is shown in (B).

Regulation by Autophosphorylation

Production of Ca^{2+}-Independent Activity

CaMKII is extensively regulated by autophosphorylation, and this property appears to be critical for its role in regulation of neuronal properties. Rapid autophosphorylation of a single threonine residue in the regulatory domain of the catalytic subunit (Thr286; or 287 in the beta subunit) causes the catalytic unit to remain active until it is dephosphorylated by cellular phosphatases [21–24]. The autophosphorylation occurs only within single holoenzymes [21,25,26] but requires intersubunit catalysis [25,26]. Calmodulin must bind to two neighboring subunits within one of the six-membered rings before either of the subunits can become autophosphorylated. Thus, calmodulin must be bound to both the substrate

subunit and the catalytic subunit [25]. Autophosphorylation of Thr286 allows activation of the subunit to outlast the triggering calcium transient [21,27], but it also decreases the rate of dissociation of calmodulin from the subunit by as much as 100-fold in the absence of calcium [28]. This latter property causes the kinase holoenzyme to respond more sensitively to high-frequency calcium transients than to low-frequency calcium transients [25], perhaps influencing which patterns of synaptic activity lead to changes in synaptic strength.

Desensitization

A second regulating autophosphorylation event takes place more slowly. If calmodulin dissociates from an activated subunit, one of two threonines (Thr305 or Thr306) that reside near the catalytic site becomes autophosphorylated [29]. Because these threonines are located in the calmodulin-binding domain, their phosphorylation blocks further binding of calmodulin, thus preventing reactivation of the subunit until the site is dephosphorylated [29–31]. Phosphorylation of these sites can thus desensitize the kinase to calcium signals. The physiological function of this second stage of autophosphorylation is not clear.

Regulatory Roles of CaMKII in Neurons

Although CaMKII is present throughout the neuronal cytosol, it is highly enriched in the postsynaptic density (PSD) [32,33], a specialization of the submembrane cytoskeleton that lies just underneath glutamatergic postsynaptic receptors across from the presynaptic active zone [34]. This finding was an early indication that CaMKII might play a special role at synapses. The conjecture was born out by the phenotype of mice from which the abundant α-subunit had been deleted. These mice are epileptic, do not display normal long-term potentiation at their hippocampal synapses, and perform poorly on learning paradigms [35,36]. Interestingly, mutant mice in which the critical autophosphorylated Thr286 is mutated to alanine have an equally severe phenotype [37].

CaMKII appears to be a major target of calcium ion flowing through activated *N*-methyl-D-aspartate (NMDA)-type glutamate receptors in dendritic spines [14,38,39]. Its activation there can lead to phosphorylation and upregulation of AMPA-type glutamate receptors [39] or addition of new AMPA receptors to the synapse through a distinct process that does not require direct phosphorylation of the AMPA receptor [40].

Additional targets for CaMKII in the PSD include the ras GTPase-activating protein synGAP [41] and the NMDA receptor itself [42,43]. Regulation of synGAP by CaMKII may provide a link to the many Ras-regulated processes within dendrites. Thus, CaMKII is situated at the hub of a variety of synaptic control mechanisms influenced by activation of the NMDA receptor. We have just begun to understand the complexity of its influence in neurons [44].

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CHAPTER 91

Glycogen Synthase Kinase 3

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Introduction

Glycogen synthase kinase 3 (GSK3) was identified over 20 years ago as a protein kinase that phosphorylated and inhibited glycogen synthase [1], the enzyme that catalyzes the transfer of glucose from UDPG to glycogen. Subsequently, two separate isoforms were cloned, termed GSK3α and GSK3β [2]. These enzymes have extremely similar catalytic domains, the major distinguishing feature being the glycine-rich N terminus present in the α-isoform. Over the past few years, GSK3 has re-entered center stage because it has become clear that it is a key player in two distinct signal transduction pathways: (1) the phosphatidylinositol (PtdIns)-3-kinase-dependent pathway that is triggered by insulin and growth factors, and (2) the Wnt signaling pathway that is required for embryonic development. The following account provides a short summary of the structure, substrate specificity, functions, and regulation of this protein kinase. For more detailed accounts, readers are referred to several recent reviews [3–5].

The Substrate Specificity of GSK3

Soon after its discovery, it was noted that GSK3 could only phosphorylate glycogen synthase efficiently if glycogen synthase had already been phosphorylated by CK2 [6]. Phosphorylation by CK2 did not inhibit glycogen synthase, but primed this enzyme for phosphorylation by GSK3. Elegant studies by Roach and his colleagues then established that the substrate specificity requirements of GSK3 are unique: the protein kinase phosphorylating serine and threonine residues that lie in Ser/Thr–Xaa–Xaa–Xaa–pSer/pThr, where pSer is phosphoserine, pThr is phosphothreonine, and Xaa is any amino acid [7]. In the case of glycogen synthase, the phosphorylation of Ser656 by CK2 forms the recognition site for the GSK3-catalyzed phosphorylation of Ser652. This, in turn, acts as the recognition site for the phosphorylation of Ser648 and so on, leading to the sequential phosphorylation of Ser644 and Ser640, the phosphorylation of the last two residues having the major effect on activity [7].

GSK3 phosphorylates many proteins *in vitro*, some of which are likely to be physiological substrates. For example, it phosphorylates Ser535 on the ε-subunit of eukaryotic protein synthesis initiation factor eIF2B [8,9], which is the guanosine triphosphate (GTP)/guanosine diphosphate (GDP)s exchange factor that converts eIF2 to its active GTP-bound form, thereby allowing it to form a ternary complex with Met-tRNA and the 40S ribosome. The phosphorylation of Ser535 inactivates eIF2B, resulting in an inhibition of protein synthesis. The phosphorylation of Ser535 by GSK3 is dependent on the prior phosphorylation of Ser539. This residue is not phosphorylated by CK2 but, at least *in vitro*, is phosphorylated specifically by the dual-specificity, tyrosine-phosphorylated and -regulated kinase (DYRK) [10]. However, whether a DYRK isoform phosphorylates eIF2B at Ser539 *in vivo* has not yet been established. GSK3 is also reported to phosphorylate ATP-citrate lyase at Thr446 and Ser450 [11,12] and the cAMP-response element binding protein (CREB) at Ser129 [13]. In these cases, phosphorylation of ATP-citrate lyase and CREB depends on the prior phosphorylation of Ser454 and Ser133, respectively, by protein kinases such as cAMP-dependent protein kinase A (PKA). Thus, it is clear that the nature of the priming kinase varies from substrate to substrate. In the case of glycogen synthase and eIF2B, the level of phosphorylation of the priming site is high, even in quiescent cells, because CK2 and DYRK are constitutively active protein kinases. However, in the case of ATP-citrate lyase and CREB, the level of phosphorylation of the priming site increases in response to several extracellular signals, such as those that elevate cAMP and activate PKA.

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The site on GSK3 that binds the priming phosphate of substrates has been identified. It is located in the N-terminal lobe of the catalytic domain near the activation loop present in many protein kinases and contains three crucial basic residues (Arg96, Arg180, and Lys205 in the β-isoform) that interact directly with the priming phosphate [14–16]. Interestingly, the three-dimensional structure of GSK3 most closely resembles that of mitogen-activated protein kinase (MAPK) family members. The activation of MAPKs requires the phosphorylation of a threonine and a tyrosine residue located in a Thr–Xaa–Tyr sequence in the activation loop, which is catalyzed by dual specificity MAPK kinases (MKKs). Intriguingly, the phosphothreonine residue in the activation loop of MAPKs interacts with the same three basic residues that bind the priming phosphate in substrates of GSK3 [15]. Moreover, GSK3 is itself phosphorylated at a tyrosine residue located in a position equivalent to that of the phosphotyrosine residue in MAPKs [17]. Thus, the way in which the active form of GSK3 is generated may be analogous to that of MAPKs, except that the active conformation is induced when the priming phosphate of the substrate binds to GSK3 [15]. Unlike the MAPKs, the phosphotyrosine residue in GSK3 (Tyr279 of GSK3α, Tyr216 of GSK3β) appears to be phosphorylated constitutively in most mammalian cells [17,18]. GSK3β expressed in *Escherichia coli* (a bacterium thought to lack protein tyrosine kinase activity) is phosphorylated at Tyr216 and wild-type GSK3β but is not a catalytically inactive mutant and becomes phosphorylated at Tyr216 when transfected into human HEK 293 cells (Frame and Cohen, unpublished data). These observations suggest that the phosphorylation of the tyrosine residue in GSK3 is catalyzed by GSK3 itself. In contrast, there is evidence that in the slime mold *Dictyostelium discoideum* GSK3 is activated by tyrosine phosphorylation, which is catalyzed by the protein kinase ZAK1 [19]. However, the tyrosine residues that become phosphorylated have not yet been identified, and ZAK1 homologs do not appear to be present in the human genome. Nevertheless, the possibility that the tyrosine phosphorylation of GSK3 may be catalyzed by another protein kinase in some mammalian cells cannot be excluded, because the phosphorylation of GSK3β at Tyr216 has been reported to increase in neuronal cells after cerebral damage or after withdrawal of nerve growth factor (NGF) from the culture medium [20,21].

The Regulation of GSK3 Activity by Insulin and Growth Factors

GSK3 can be inhibited via the phosphorylation of a serine residue near the N terminus of the protein (Ser21 in GSK3α, Ser9 in GSK3β) [22]. This serine lies in an Arg–Xaa–Arg–Xaa–Xaa–Ser sequence, which is a consensus motif for phosphorylation by several protein kinases that are components of different signal transduction pathways (Fig. 1). Protein kinase B (PKB, also called Akt) inhibits GSK3 in response to signals that activate class I phosphatidylinositol (PtdIns) 3-kinases and elevate the level of PtdIns(3,4,5)P3 [22]; MAPK-activated protein kinase 1 (MAPKAP-K1, also called RSK) inhibits GSK3 following stimulation by signals that activate the classical MAPK cascade [23, 24]; and S6K1 inhibits GSK3 in response to amino acids acting via the protein kinase mTOR [25].

In embryonic stem cells that do not express 3-phosphoinositide-dependent protein kinase 1 (PDK1), an essential upstream activator of both PKB and MAPKAP-K1, the PKB-mediated inhibition of GSK3 (induced by insulin-like growth factor 1) and the MAPKAP-K1-mediated inhibition of GSK3 (induced by the tumor-promoting phorbol ester TPA) do not occur [26]. This genetic evidence supports the view that GSK3 can be inhibited by PKB and MAPKAP-K1 *in vivo*.

The mechanism by which phosphorylation inhibits GSK3 has been elucidated. The phosphorylated N terminus becomes a pseudosubstrate occupying the same binding pocket as the priming phosphate of substrates [14,16]. This suggests that

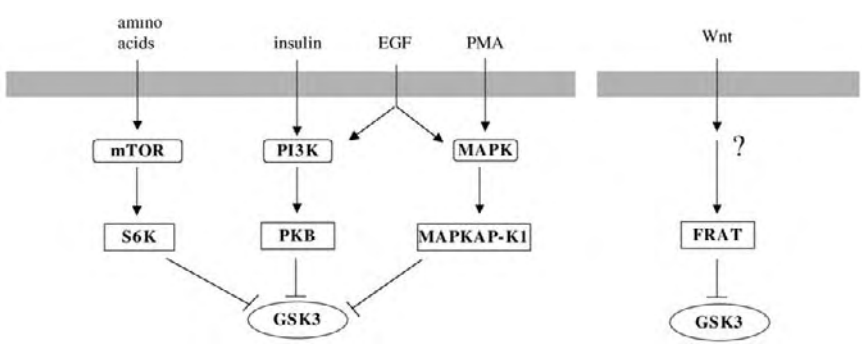


Figure 1 GSK3 can be inhibited by several different agonists. The inhibition of GSK3 by growth factors, amino acids, and hormones, such as insulin, occurs by a different mechanism than does inhibition of GSK3 by Wnts. Protein kinases that are activated by these agonists, such as PKB, MAPKAP-K1, and S6 kinase (S6K), phosphorylate the N terminus of GSK3 on a serine residue (Ser9 of GSK3β and Ser21 of GSK3α). In contrast, Wnt signaling does not lead to an increase in Ser9/Ser21 phosphorylation and instead may involve the displacement of Axin and β-catenin from GSK3 via the binding of FRAT and Dishevelled to GSK3.

the extent to which phosphorylation inhibits GSK3 activity *in vivo* may vary between substrates and will depend on the affinity of any particular substrate for GSK3.

GSK3 as a Drug Target

Insulin induces the activation of glycogen synthase, mainly by stimulating the dephosphorylation of the serine residues in glycogen synthase that are targeted by GSK3 [27] and stimulates the activation of eIF2B by promoting the dephosphorylation of Ser535 [9]. These dephosphorylation events, which are likely to be mediated (at least in part) by the PKB-catalyzed inhibition of GSK3, contribute to the insulin-induced stimulation of glycogen and protein synthesis. For these reasons, and because the level of GSK3 is elevated in animal models of diabetes [28], there has been considerable interest over the past few years in trying to identify GSK3 inhibitors for the treatment of Type 2 diabetes. Small-cell-permeant inhibitors of GSK3 have now been developed. These are relatively specific and can activate glycogen synthase and stimulate the conversion of glucose to glycogen in liver cells [29]. Related compounds have also been reported to lower blood glucose levels *in vivo* [30]. The efficacy of these compounds *in vivo* may be partly explained by the finding that they mimic the ability of insulin to repress transcription of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase [31]. Thus, GSK3 appears to play a role in the insulin-regulated transcription of the genes encoding these enzymes, although the underlying molecular mechanism is unknown. In summary, inhibitors of GSK3 may lower the levels of blood glucose *in vivo* by suppressing the production of glucose as well as by enhancing the conversion of glucose to glycogen.

There is considerable evidence that the inhibition of GSK3 triggered by growth factors contributes to the anti-apoptotic effects of these signals. Moreover, lithium ions (which inhibit GSK3 relatively specifically) and the cell-permeant GSK3 inhibitors SB 216763 and SB 415286 protect cerebellar granule neurons from apoptosis resulting from the lowering of the concentration of potassium ions in the medium [32]. The same compounds also protect chicken dorsal-root-ganglion sensory neurons from apoptosis caused by the withdrawal of nerve growth factor from the medium or by the inhibition of NGF-induced PtdIns 3-kinase activity with the compound LY 294002 [32]. Reducing neuronal apoptosis is an important therapeutic goal in the context of head trauma, stroke, epilepsy, and motor neuron disease; therefore GSK3 is also an attractive therapeutic target for the design of inhibitory drugs to treat these diseases.

The Role of GSK3 in Embryonic Development

It is well established that GSK3 plays a key role in embryonic development as a central player in the Wnt signaling pathway. In this pathway, GSK3 is present in a complex that contains at least three other proteins: Axin, β-catenin, and the adenomatous polyposis coli (APC) protein [33]. Axin acts as a scaffold by binding to GSK3, β-catenin, and APC, while APC also interacts with β-catenin. In this complex, GSK3 is active and phosphorylates Axin, β-catenin and APC. The phosphorylation of Axin stabilizes this protein, while the phosphorylation of APC appears to facilitate its interaction with β-catenin. In contrast, the phosphorylation of β-catenin targets it for destruction by the proteasome. In response to secreted glycoproteins (Wnts), the activity of GSK3 towards Axin and β-catenin is inhibited, resulting in the dephosphorylation of these proteins. The precise molecular mechanism is not fully elucidated but may involve the displacement of Axin (and hence β-catenin and APC) from GSK3 by a protein known as FRAT (frequently rearranged in advanced T-cell lymphomas; also called GSK3-binding protein (GBP) which is complexed to another protein called Dishevelled [34–36] (Fig. 1). The dephosphorylation of β-catenin leads to its stabilization, accumulation, and translocation to the nucleus, where it stimulates the transcription of genes that are critical for embryonic development.

The evidence implicating GSK3 in the Wnt signaling pathway was originally obtained in *Drosophila*, which expresses a single form of GSK3 that is very similar to GSK3β. For these reasons, it has been widely assumed that GSK3β is the only isoform that participates in the Wnt signaling pathway in mammalian cells. However, GSK3β knockout mice develop normally to the late embryonic stage, implying that GSK3α can compensate for GSK3β in this pathway. GSK3β knockout mice die at a late embryonic stage due to liver apoptosis, which appears to be caused by hypersensitivity to the proinflammatory cytokine tumor necrosis factor α [37].

GSK3 and Cancer

Many of the components of the Wnt signaling pathway are over-expressed or mutated in different tumors [38]. For example, virtually all colon tumors arise from an initiating mutation in the APC gene (85%) or in the β-catenin gene (10–15%) that makes β-catenin resistant to degradation. Axin mutations occur in hepatocellular carcinomas and FRAT1 in T-cell lymphomas. Alterations in these components would be predicted to lead to inappropriate accumulation of β-catenin.

These observations have implications for the development of GSK3 inhibitors to treat diabetes and other diseases, as compounds that target the ATP-binding site, such as SB 216763 and SB 415286, inhibit the phosphorylation of all GSK3 substrates, including Axin and β-catenin. They therefore mimic the Wnt signaling pathway and stimulate the accumulation of β-catenin [32]. The development of GSK3 inhibitors that do not have the potential to be oncogenic may therefore require the identification of compounds that prevent the phosphorylation of glycogen synthase but which do not inhibit the phosphorylation of Axin and β-catenin. This may be possible because Axin and β-catenin appear to bind to GSK3 at sites distinct from glycogen synthase

and eIF2B. Mutations in GSK3 have been identified that prevent the phosphorylation of glycogen synthase and eIF2B but not the phosphorylation of Axin and β-catenin, and vice versa [14, 39].

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CHAPTER 92

Protein Kinase C: Relaying Signals from Lipid Hydrolysis to Protein Phosphorylation

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Introduction

Protein kinase C (PKC) has been in the spotlight since the discovery a quarter of a century ago that, through its activation by diacylglycerol, it relays signals from lipid hydrolysis to protein phosphorylation [1]. The subsequent discovery that PKCs are the target of phorbol esters resulted in an avalanche of reports on the effects on cell function of phorbol esters, nonhydrolyzable analogs of the endogenous ligand, diacylglycerol [2–4]. Despite the enduring stage presence of PKC and tremendous advances in understanding the enzymology and regulation of this key protein, an understanding of the function of PKC in biology is still the subject of intense pursuit. Its uncontrolled signaling wreaks havoc in the cell, as epitomized by the potent tumor-promoting properties of phorbol esters. In fact, the pluripotent effects of phorbol esters, compounded with the existence of multiple isozymes of PKC, has made it difficult to uncover the precise cellular function of this key enzyme [5]. Studies with knockout mice have underscored the problem, with knockouts of most isozymes having only subtle phenotypic effects [6]. This chapter summarizes our current understanding of the molecular mechanisms of how protein kinase C transduces information from lipid mediators to protein phosphorylation.

Protein Kinase C Family

The 10 members of the mammalian PKC family are grouped into three classes based on their domain structure, which, in turn, dictates their cofactor dependence (Fig. 1). All members comprise a single polypeptide that has a conserved kinase core carboxyl-terminal to a regulatory moiety. This regulatory moiety contains two key functionalities: an autoinhibitory sequence (pseudosubstrate) and one or two membrane-targeting modules (C1 and C2 domains). The C1 domain binds diacylglycerol and phosphatidylserine specifically and is present as a tandem repeat in conventional and novel PKCs (C1A and C1B); the C2 domain nonspecifically binds Ca^{2+} and anionic phospholipids such as phosphatidylserine. Non-ligand-binding variants of each domain exist: atypical C1 domains do not bind diacylglycerol and novel C2 domains do not bind Ca^{2+}.

Conventional PKC isozymes (α, γ, and the alternatively spliced βI and βII) are stimulated by diacylglycerol and phosphatidylserine (C1 domain) and Ca^{2+} (C2 domain); novel PKC isozymes (δ, ε, η/L, θ) are stimulated by diacylglycerol and phosphatidylserine (C1 domain); and atypical PKC isozymes (ζ, ι/λ) are stimulated by phosphatidylserine (atypical C1 domain) [5–7]. (Note that PKC μ and ν were considered to constitute a fourth class of PKCs but are now generally regarded as members of a distinct family

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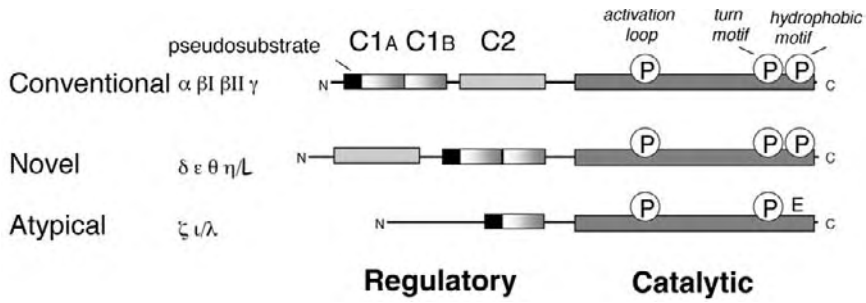


Figure 1 Domain composition of protein kinase C family members showing autoinhibitory pseudosubstrate, membrane-targeting modules (C1A and C1B and C2 domains), and kinase domain of the three subclasses: conventional, novel, and atypical isozymes. Also indicated are the positions of the three processing phosphorylation sites, the activation loop and two carboxyl-terminal sites, the turn motif, and hydrophobic motif. (Adapted from Newton, A. C. and Johnson, J. E., *Biochem. Biophys. Acta*, 1376, 155–172, 1998.)

called protein kinase D.) The role of the novel C2 domain in novel PKCs and that of the atypical C1 domain in atypical PKCs is not clear, but each may regulate the subcellular distribution of these isozymes through protein–protein interactions.

Regulation of Protein Kinase C

The normal function of PKC is under the coordinated regulation of three major mechanisms: phosphorylation/ dephosphorylation, membrane targeting modules, and anchor proteins. First, the kinase must be processed by a series of ordered phosphorylations to become catalytically competent. Second, it must have its pseudosubstrate removed from the active site to be catalytically active, a conformational change driven by engaging the membrane-targeting modules with ligand. Third, it must be localized at the correct intracellular location for unimpaired signaling. Perturbation at any of these points of regulation disrupts the physiological function of PKC [7].

Phosphorylation/ Dephosphorylation

The function of PKC isozymes is controlled by phosphorylation mechanisms that are required for the maturation of the enzyme. In addition to the processing phosphorylations, the function of PKC isozymes is additionally fine-tuned by both Tyr and Ser/Thr phosphorylations [8,9]. The conserved maturation phosphorylations are described below.

PHOSPHORYLATION IS REQUIRED FOR THE MATURATION OF PROTEIN KINASE C

The majority of PKC in tissues and cultured cells is phosphorylated at two key phosphorylation switches: a loop near the active site, referred to as the *activation loop*, and a sequence at the carboxyl terminus of the kinase domain [10,11]. The carboxyl-terminal switch contains two sites: the turn motif, which by analogy with protein kinase A is at the apex of a turn on the upper lobe of the kinase domain, and the hydrophobic motif, which is flanked by hydrophobic residues (note that, in atypical PKCs, a Glu occupies the phospho-acceptor position of the hydrophobic motif). It is the phosphorylated species that transduces signals. While it had been appreciated since the late 1980s that PKC is processed by phosphorylation [12], the mechanism and role of these phosphorylations are only now being unveiled [7,9].

The first step in the maturation of PKC is phosphorylation by the phosphoinositide-dependent kinase, PDK-1, of the activation loop. This enzyme was originally discovered as the upstream kinase for Akt/protein kinase B [13] and was subsequently shown to be the activation loop kinase for a large number of AGC kinases, including all PKC isozymes [14–16]. The name PDK-1 was based on the phosphoinositide-dependence of Akt phosphorylation and is an unfortunate misnomer because the phosphorylation of other substrates (for example, the conventional PKCs) has no dependence on phosphatidylinositol 3-kinase (PI3K) lipid products [17]. Rather, PDK-1 appears to be constitutively active in the cell, with substrate phosphorylation regulated by the conformation of the substrate [18–20].

Completion of PKC maturation requires phosphorylation of the two carboxyl-terminal sites, the turn motif and hydrophobic motif. In the case of conventional PKCs, this reaction occurs by an intramolecular autophosphorylation mechanism [21]. Autophosphorylation also accounts for the hydrophobic motif processing of the novel PKCε [22]; however, it has been suggested that another member of this family, PKCδ, may be the target of a putative hydrophobic motif kinase [23].

Research in the past few years has culminated in the following model for PKC phosphorylation. Newly synthesized enzyme associates with the plasma membrane, where it adopts an open conformation with the pseudosubstrate exposed, thus unmasking the PDK-1 site on the activation loop [17,24]. It is likely held at the membrane by multiple weak interactions with the exposed pseudosubstrate, the C1 domain, and the C2 domain (because the C1 and C2 ligands are absent, these domains are weakly bound via their interactions with anionic phospholipids). PDK-1 docks onto the carboxyl terminus of PKC, where it is positioned to phosphorylate the activation loop [25]. This phosphorylation is

the first and required step in the maturation of PKC; mutation of the phospho-acceptor position at the activation loop to Ala or Val prevents the maturation of PKC and results in accumulation of unphosphorylated, inactive species in the detergent-insoluble fraction of cells [26,27].

Completion of PKC maturation requires release of PDK-1 from its docking site on the carboxyl terminus. Physiological mechanisms for this release have not yet been elucidated, but it is interesting that over-expression of peptides that have a high affinity for PDK-1 promotes the maturation of PKC [25]. One such peptide is PIF, the carboxyl-terminus of PRK-2, which has a hydrophobic phosphorylation motif with a Asp at the phospho-acceptor position [28]. Release of PDK-1 unmasks the carboxyl terminus of PKC, allowing phosphorylation of the turn motif and the hydrophobic motif [7,10].

DEPHOSPHORYLATION: DEACTIVATION SIGNAL

While the phosphorylation of conventional PKCs is constitutive, the dephosphorylation appears to be agonist stimulated [29]. Both phorbol esters and ligands such as tumor necrosis factor α (TNFα) result in PKC inactivation and dephosphorylation [29–31]. In addition, serum selectively promotes the dephosphorylation of the activation loop site in conventional PKCs, thus uncoupling the phosphorylation of the activation loop from that of the carboxyl-terminal sites [17]. The hydrophobic site of PKCε has also been reported to be selectively dephosphorylated by a rapamycin-sensitive phosphatase [32]. It is likely that the uncoupling of the dephosphorylation of these sites has contributed to confusion as to whether the hydrophobic site is regulated by its own upstream kinase rather than autophosphorylation [23].

Membrane Translocation

The translocation from the cytosol to the membrane has served as the hallmark for PKC activation since the early 1980s [33,34]. The molecular details of this translocation have emerged from abundant biophysical, biochemical, and cellular studies showing that diacylglycerol acts like molecular glue to recruit PKC to membranes, an event that, for conventional PKCs, is facilitated by Ca^{2+} [35–37].

Both *in vitro* and *in vivo* data converge on the following model for the translocation of conventional PKC in response to elevated Ca^{2+} and diacylglycerol [35,38]. In the resting state, PKC bounces on and off membranes by a diffusion-limited reaction. However, its affinity for membranes is so low that its lifetime on the membrane is too short to be significant. Elevation of Ca^{2+} results in binding of Ca^{2+} to the C2 domain of this soluble species of PKC. This Ca^{2+}-bound species has a dramatically enhanced affinity for the membrane, with which it rapidly associates. The membrane-bound PKC then diffuses in the two-dimensional plane of the membrane, searching for the much less abundant ligand, diacylglycerol. This search for diacylglycerol is considerably more efficient from the membrane than one initiated from the cytosol. Following collision with, and binding to, diacylglycerol, PKC is bound to the membrane with sufficiently high affinity to allow release of the pseudosubstrate sequence and activation of PKC. Decreases in the level of either second messenger weaken the membrane interaction sufficiently to release PKC back into the cytosol. Note that if PMA is the C1 domain ligand, PKC can be retained on the membrane in the absence of elevated Ca^{2+} because this ligand binds PKC two orders of magnitude more tightly than diacylglycerol [39]. Similarly, if Ca^{2+} levels are elevated sufficiently, PKC can be retained at the membrane in the absence of a C1 ligand.

Novel PKC isozymes translocate to membranes much more slowly than conventional PKCs in response to receptor-mediated generation of diacylglycerol because they do not have the advantage of pre-targeting to the membrane by the soluble ligand, Ca^{2+} [40]. Atypical PKC isozymes do not respond directly to either diacylglycerol or Ca^{2+}.

Anchoring Proteins

The control of subcellular localization of kinases by scaffold proteins is emerging as a key requirement in maintaining fidelity and specificity in signaling by protein kinases [41]. PKC is no exception, and a battery of binding partners for members of this kinase family have been identified [42–45]. These proteins position PKC isozymes near their substrates, near regulators of activity such as phosphatases and kinases, or in specific intracellular compartments. Disruption of anchoring can impair signaling by PKC, and *Drosophila* photoreceptors provide a compelling example. Mislocalization of eye-specific PKC by abolishing its binding to the scaffold protein, ina D, disrupts phototransduction [46].

Unlike protein kinase A binding proteins (AKAPs) [47], there is no consensus binding mechanism for interaction of PKC with its anchor proteins. Rather, each binding partner identified to date interacts with PKC by unique determinants and unique mechanisms. Some binding proteins regulate multiple PKC isozymes, while others control the distribution of specific isozymes. There are binding proteins for newly synthesized unphosphorylated PKC, phosphorylated but inactive PKC, phosphorylated and activated PKC, and dephosphorylated, inactivated PKC [43,45]. Anchoring proteins for PKC have diverse functions—some positively regulate signaling while others negatively regulate it. An emerging theme is that many scaffolds bind multiple signaling molecules in a signaling complex; for example, AKAP 79 binds PKA, PKC, and the phosphatase calcineurin [48]. The physical coupling of kinases and phosphatases underscores the acute regulation that each must be under to maintain fidelity in signaling.

Model for Regulation of Protein Kinase C by Phosphorylation and Second Messengers

Figure 2 outlines a model for the regulation of PKC by phosphorylation, second messengers, and anchoring proteins. Newly synthesized PKC associates with the membrane in a conformation that exposes the pseudosubstrate

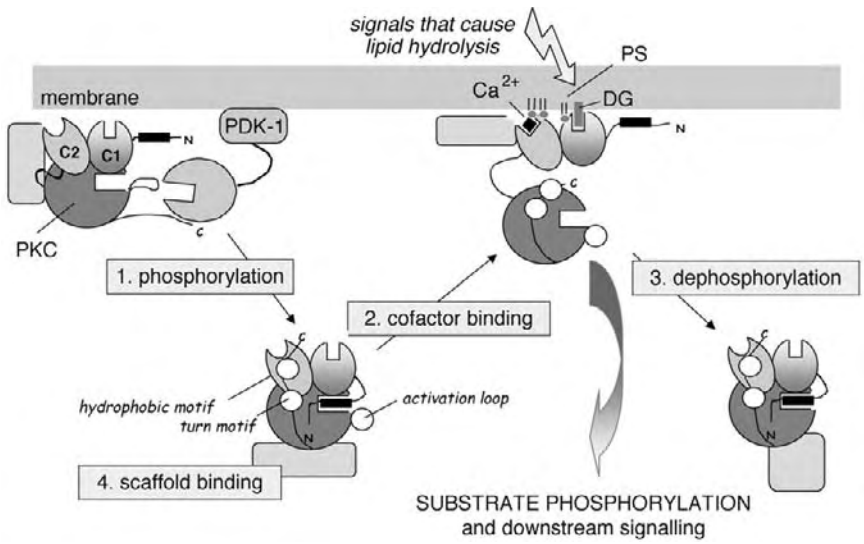


Figure 2 Model showing the major regulatory mechanisms for PKC function: (1) processing by phosphorylation, (2) activation by lipid mediators, (3) deactivation by dephosphorylation, and (4) spatial control by scaffold proteins. See text for details. (Adapted from Newton, A. C., *Chem. Rev.*, **101**, 2353–2364, 2001.)

(black rectangle), allowing access of the upstream kinase, PDK-1, to the activation loop. PDK-1 docks onto the carboxyl terminus of PKC. Following its phosphorylation of the activation loop and release from PKC, the turn motif and hydrophobic motif are autophosphorylated. The mature PKC is released into the cytosol, where it is maintained in an auto-inhibited conformation by the pseudosubstrate (middle panel), which has now gained access to the substrate-binding cavity (open rectangle in the large circle representing the kinase domain of PKC). It is this species that is competent to respond to second messengers. Generation of diacylglycerol and, for conventional PKCs, Ca^{2+} mobilization provide the allosteric switch to activate PKC. This is achieved by engaging the C1 and C2 domains on the membrane (Fig. 2, right panel), thus providing the energy to release the pseudosubstrate from the active site, allowing substrate binding and catalysis. In addition to the regulation by phosphorylation and cofactors, anchoring/scaffold proteins (stippled rectangle) play a key role in PKC function by positioning specific isozymes at particular intracellular locations [43,45]. Following activation, PKC is either released into the cytosol or, following prolonged activation, dephosphorylated and downregulated by proteolysis.

Function of Protein Kinase C

Despite over two decades of research on the effects of phorbol esters on cell function, a unifying mechanism for the role of PKC in the cell has remained elusive. An abundance of substrates have been identified, and the reader is referred to reviews summarizing these and potential signaling pathways involving PKC [5,6,49–52]. However, a unique role for PKC in defining cell function is lacking. This is epitomized by the finding that there is no severe phenotype associated with knocking-out specific PKC isozymes in mice.

Closer analysis of the phenotypes of knockout animals of various PKC isozymes does suggest a common theme: animals deficient in PKC are deficient in adaptive responses. For example, PKCε $^{-/-}$ mice have reduced anxiety and reduced tolerance to alcohol [53], PKC$\gamma^{-/-}$ mice have reduced pain perception [54], and PKCβII$^{-/-}$ mice have reduced learning abilities [55]. This theme carries over to the molecular level, where many of the substrates of PKC are receptors that become desensitized following PKC phosphorylation.

Summary

PKC plays a pivotal role in cell signalling by relaying information from lipid mediators to protein substrates. The relay of this information is under exquisite conformational, spatial, and temporal regulation, and extensive studies on the molecular mechanisms of this control have provided much insight into how PKC is regulated. With novel approaches in chemical genetics, analysis of crosses of PKC isozyme knockout mice, and proteomics, the PKC signaling field is poised to move to the next level of making headway into the *raison d'être* of this ubiquitous family of kinases.

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CHAPTER 93

The PIKK Family of Protein Kinases

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Introduction

In the mid-1990s, a series of cloning papers announced the arrival of the phosphatidylinositol 3-kinase (PI3K)-related protein kinase (PIKK) family of proteins [1,2]. These reports and subsequent studies revealed two defining features of this protein family. First, all known members are very large, being between 280 and 470 kDa in size. Second, despite being protein serine/threonine kinases, the kinase domains of PIKK family members are markedly different from those of other protein serine/threonine or tyrosine kinases and, instead, are more related in sequence (≈20 to 25% identity) to the kinase domain of the PI3K family of phospholipid kinases. Members of the PI3K family play diverse roles in intracellular signaling triggered by mitogenic and other stimuli through phosphorylating the inositol ring of phosphatidylinositol derivatives, thus generating second messengers for downstream effector pathways [3]. Nevertheless, the available evidence indicates that PIKK family proteins have specificity toward proteins rather than lipid targets. Although the primary specificities of the PIKK and PI3K families therefore appear to be different, it is likely that they bring about catalysis by very similar mechanisms.

Over the past few years, it has become clear that members of the PIKK family exist in all eukaryotes studied, although none has so far been found in prokaryotes. Six human PIKK family members have been identified to date, and genome scanning suggests that this number is unlikely to increase. Since publication of the original biochemical and cloning papers of the PIKKs, extensive research on the members of this family of kinases has taken place, leading to significant insights into their biological roles and biochemical functions. One emerging unifying concept for this class of kinases is that they may directly, or indirectly through partner proteins, be involved in monitoring or modulating of polynucleic acids. Here, we provide an overview of the PIKK family with particular emphasis on the human proteins.

Overview of PIKK Family Members

Members of the PIKK family are involved in a diverse set of biological functions. The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) plays a crucial role in site-specific V(D)J recombination in the developing immune system and in the nonhomologous end-joining (NHEJ) pathway of DNA repair [4]. The protein product of the gene mutated in ataxia-telangiectasia (ATM) and the ATM Rad3-related (ATR) protein have key roles in the signaling of DNA damage [5–7]. SMG-1, originally identified in a *Caenorhabditis elegans* screen as a suppressor of morphogen gradient, is involved in the process of nonsense-mediated RNA decay (NMD) [8], while the mammalian target of rapamycin (mTOR; also termed FRAP or RAFT) is involved in controlling cellular growth in response to nutrients and amino acids by playing a pivotal role in controlling the translational machinery [9,10]. Another member of the PIKK family, TRRAP (transformation/transcription associated protein), is an essential cofactor for

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both the c-MYC and the E1A/E2F transcription factor pathways through its interactions with the SAGA and PCAF histone acetyltransferase complexes [11–14].

Overall Architecture of PIKK Family Proteins

Figure 1 illustrates the domain architecture of the PIKK family. The PIKK kinase domain is located within the carboxy-terminal, ≈400-amino-acid residues of these proteins, with the exception of hSMG-1, whose kinase domain lies more centrally within the polypeptide. Consistent with the crystal structure of the kinase domain of porcine PI3K (p110γ), which shows gross overall structural similarity to the structures of classical protein Ser/Thr kinases [15], the kinase domains of PIKK family members generally contain residues that can be aligned with those playing key roles in adenosine triphosphate (ATP) coordination in other classes of kinase. A notable exception to this is TRRAP, which does not contain the DXXXXN and DFG motifs that are critical for ATP coordination within a kinase catalytic site [11]. The available data suggest that this renders the kinase domain of TRRAP catalytically inactive [14]. Nevertheless, the significant sequence homology between the C-terminal region of TRRAP and other members of the PIKK family suggests that this region of TRRAP will retain the overall structural features of the PIKK kinase domain.

In all cases, the kinase active site of PIKK family proteins is flanked N-terminally by a region of ≈500-amino-acid residues, which has been named the FAT domain (derived from FRAP, ATM, and TRRAP) and C-terminally by a small (≈35-amino-acid residue) FAT C-terminal (FAT-C) domain [16]. To date, FAT and FAT-C domains have only been found in proteins in combination. No function has been ascribed to these domains but they could be involved in intermolecular protein–protein interactions. Alternatively, they could modulate kinase activity either by intramolecular interactions with the PIKK kinase domain or by binding kinase substrates and thus bringing them into the proximity of the ATP binding site. Notably, the FAT-C domain found in SMG-1 is separated from the kinase domain by almost 1200 amino acid residues. Nevertheless, it is possible that these regions come together in the protein tertiary structure [8].

In mTOR, conserved HEAT repeats are found in two groupings in the first half of the protein [9]. The HEAT repeat is a tandemly repeated module of 37 to 47 amino acid residues occurring in a range of cytoplasmic proteins (which include the four proteins from which the acronym was derived: Huntingtin, EF3, the alpha regulatory subunit of PP2A, and TOR). These motifs may be involved in protein–protein interactions within multiprotein complexes [17]. The FRB (FKBP12–rapamycin binding) domain has so far been found only within the mTOR and SMG-1 sequences [8,9]. When bound to this region of mTOR, the FKBP12–rapamycin complex is able to inhibit mTOR function [9]. The crystal structure of this beta-barrel-like domain in a complex with the rapamycin–immunophilin complex has been solved [18]. Other than this clearly soluble and surface domain of mTOR, no truly distinct or soluble domains have so far been identified in other members of the PIKK family. Finally, a small region of homology between ATM and ATR upstream of the FAT domain has been noted [6].

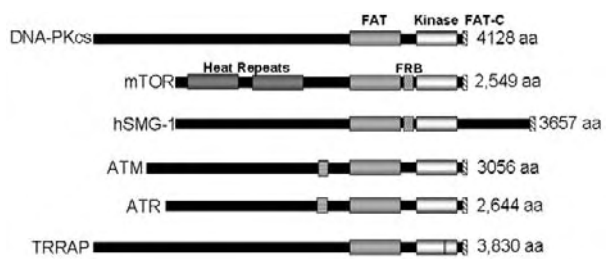


Figure 1 The domain architecture of the PIKK family; human proteins are shown (see text for details).

mTOR: A Key Regulator of Cell Growth

The first member of the PIKK family to be cloned was the target of rapamycin (TOR), which was identified through a yeast screen to identify mutants that were resistant to the growth inhibitory effect of rapamycin [19]. The mammalian protein is termed mTOR, FRAP (FKBP–rapamycin associated protein), and also RAFT (rapamycin and FKBP target) [20–23]. Recent research has indicated that mTOR is a central controller of cell growth. By targeting mTOR, rapamycin blocks T cells in the G_1 phase of the cell cycle [9]. This prevents T-cell activation and proliferation in response to mitogenic stimuli and results in immunosupression [10]. Signaling to mTOR is thought to be mediated, in part, by activation of a PI3K-dependent pathway that may involve PKB/Akt (Fig. 2). Once activated, mTOR is believed to modulate cap-dependent translation and ribosome biogenesis through phosphorylation and regulation of 4E-BP1 and S6 kinase, respectively [9,10]. The TOR proteins have also been shown to be somehow involved in controlling other sets of cellular growth events including transcription, actin organization, membrane traffic, and protein degradation [9,10]. mTOR is also now regarded as an attractive target for chemostatic anticancer therapy, and an ester derivative of rapamycin (CCI-779) is currently undergoing evaluation as an anticancer agent [24].

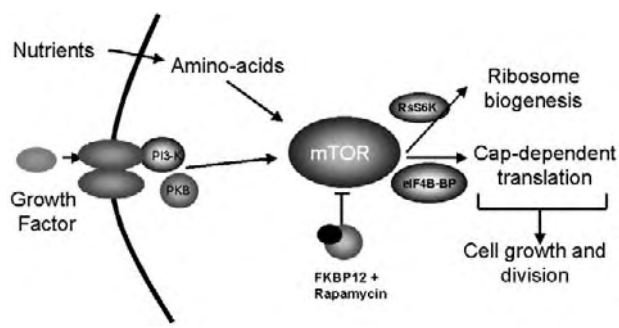


Figure 2 A model of mTOR signaling pathways that modulate translational control as elucidated from studies on mammalian cells.

DNA-PKcs: At the Heart of the DNA Nonhomologous End-Joining Machinery

Along with the high-affinity DNA end-binding protein Ku, DNA-PKcs forms the heterotrimeric holoenzyme termed DNA-dependent protein kinase (DNA-PK) [4,25]. DNA-PK was initially identified biochemically as a relatively abundant nuclear protein whose serine/threonine kinase activity is greatly stimulated by linear double-stranded DNA [4]. Subsequently, a series of radiosensitive mutant cell lines defective in DNA double-strand break repair were found to possess mutations in DNA-PKcs or in one of the two Ku subunits [26]. Thus, DNA-PKcs now has a clearly established role in the NHEJ pathway of DNA double-strand break repair. The immune-deficiency of mice lacking functional DNA-PK is due to a defect in V(D)J recombination—a cut-and-paste genome rearrangement process that occurs in developing B and T lymphocytes to generate the antigen-binding diversity of the immunoglobulin and T-cell receptor genes, respectively [27].

Recently, it has became clear that DNA-PK is also physically localized to telomeres, the physical caps at the ends of linear eukaryotic chromosomes, and functions there to help prevent the formation of chromosomal end-to-end fusions [28]. Importantly, the kinase activity of DNA-PKcs is essential for its biological functions [29]. When it becomes assembled at a DNA double-strand break, DNA-PK is believed to recruit and/or phosphorylate additional NHEJ factors, such as DNA ligase IV/XRCC4 and the recently identified Artemis protein, to bring about repair of the lesion (Fig. 3) [30,31]. Another *in vivo* target for DNA-PK that has been identified recently is interferon regulatory factor-3 (IRF-3), which is phosphorylated by DNA-PK during paramyxovirus infection [32].

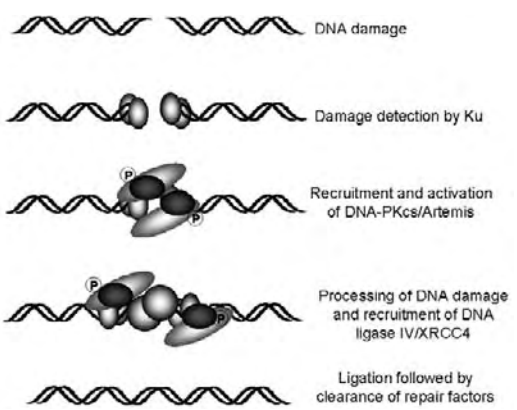


Figure 3 Model of NHEJ. Upon induction of a DNA double-strand break, the high-affinity DNA end-binding protein Ku is targeted to the site of damage. Ku end binding then permits the recruitment and activation of DNA-PKcs along with the newly identified Artemis. This complex is then believed to act as a scaffold at the site of damage and, through Artemis activation via DNA-PK phosphorylation, process the DNA ends for subsequent ligation by the DNA-ligase IV/XRCC4 complex. Once ligation is complete the complex is cleared from the site of damage by an unknown mechanism that may involve DNA-PK-mediated phosphorylation.

ATM and ATR: Signalers of Genome Damage

A homozygous deficiency of ATM in humans leads to ataxia-telangiectasia (A-T), a debilitating disorder in which progressive loss of motor coordination (ataxia) is brought about by the gradual loss of Purkinje cells in the cerebellum [33]. In addition, A-T patients have an increased cancer incidence, and cells derived from these individuals are hypersensitive to ionizing radiation and to chemical agents that induce DNA double-strand breaks [33]. Notably, whereas normal cells delay progression through the cell cycle after treatment with such agents, A-T cells are defective in these "checkpoint" responses [5,6]. Indeed, A-T cells are deficient in the G_1/S, G_2/M, and S phase checkpoints. Over the past few years, a large number of research papers have addressed these checkpoint defects and it is now clearly established that ATM phosphorylates, and therefore appears to modulate the activities of, the key cell-cycle control proteins p53, BRCA1, NBS1, MDM2, RAD17, and CHK2. Recent review articles give a clear and detailed analysis of downstream targets of ATM [5,6].

Unlike ATM, which appears to be primarily involved in responding to DNA DSBs, ATR acts to delay cell-cycle progression in response to other types of DNA damage, such as those induced by ultraviolet light. It also has a particularly important role in recognizing DNA damage or difficulties in genome replication during S phase [6,7]. Loss of ATR function leads to early embryonic lethality in mice, and ATR-deficient cells are incapable of successfully traversing S phase in culture. Although these phenotypes have highlighted the crucial role of ATR in genome maintenance, they have hampered studies to understand the precise functions of this enzyme. Nevertheless, by the use of cell lines over expressing a kinase-dead ATR, the functions of ATR in cellular responses to DNA damage produced by ultraviolet or infrared light and to DNA replication inhibitors has been established [34,35]. *In vivo* targets for ATR include p53, RAD17, BRCA1, and CHK1 [6]. Orthologs of both ATM and ATR have been identified in all eukaryotic species so far analyzed. In particular, work on the ATR orthologs in the genetically amenable organisms *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Mec1p and Rad3, respectively) has been enormously helpful in elucidating the functions of these PIKKs in DNA-damage sensing and signaling [36].

SMG-1: A Regulator of Nonsense-Mediated mRNA Decay

The most recently cloned and probably the final member of the PIKK family is SMG-1, which plays a key role in the regulation of nonsense-mediated RNA decay (NMD) [8,37]. NMD serves to recognize and eliminate mRNA species that contain premature translation termination codons and thus code for nonfunctional or potentially harmful polypeptides [38]. It seems that SMG-1 is able to associate with other components of the mRNA surveillance pathway and,

in particular, acts to directly phosphorylate one of these proteins, hUPF1/SMG-2 [8]. Inhibition of SMG-1 by overexpression of a kinase-dead SMG-1 or by inhibition using small molecule inhibitors results in a marked suppression of the degradation of mRNAs with premature stop codons and an increase in truncated protein production [8].

TRRAP: A Crucial Transcriptional Co-Activator

TRRAP was first identified through its interaction with the N terminus of the product of the c-MYC oncogene and with the E2F-1 transcriptional activation domain, suggesting that TRRAP is an essential cofactor for both the c-MYC and E1A/E2F oncogenic transcription factor pathways [11]. Work in mammalian cells and in *S. cerevisiae* has shown that TRRAP (Tra1p in yeast) acts as a transcriptional cofactor that recruits histone acetyltransferase (HAT) complexes to sequence-specific transcriptional activators [12–14]. TRRAP appears not to act as a protein kinase, and its primary amino-acid sequence gives clues as to why this is the case [11]. However, the kinase domain has been shown to have a key noncatalytic role in that it is required for forming a structural core for the assembly of a functional HAT complex [39]. Null mutation of TRRAP has shown it to be essential for early mouse development and has revealed that it functions in the mitotic checkpoint and during normal cell-cycle progression [40].

PIKK Family Members as Guardians of Nucleic Acid Structure, Function, and Integrity?

It has been proposed that a common feature of the PIKK family may be an ability to interact with nucleic acids [41,42]. This is clearly established for DNA-PKcs, which stably binds to DNA upon interacting with DNA-bound Ku. Through this DNA–protein–protein interaction, the protein kinase potential of DNA-PK is released [4,25]. A role for DNA-PKcs in binding to telomeres has also been proposed [28]. In addition, ATM has been shown to bind *in vitro* to DNA with a preference for DNA termini or DNA that has been treated with ionizing radiation [43,44]. Also, within minutes of treating cells with ionizing radiation, ATM kinase activity is significantly increased [45,46] and the protein becomes associated with chromatin [47]. Likewise, stimulation of ATR activity by DNA *in vitro* has been observed [48,49], suggesting a direct interaction with DNA or potentially an indirect interaction through its newly discovered partner ATRIP [50]. Indeed, recent work has revealed that the *S. cerevisiae* ATR ortholog, Mec1p, is recruited to sites of DNA damage by Lcd1p, an ortholog of ATRIP [51]. SMG-1 is involved in cellular responses to mRNAs with premature termination codons, raising the possibility that it may be targeted to such mRNAs in order to modulate the process of NMD [8]. TRRAP acts as a scaffold for the SAGA/histone deacetylase complex and hence may interact with packaged DNA to allow the chromatin-modulating enzymes with which it is associated to access their substrates. Finally, a mechanism has been proposed whereby mTOR senses amino-acid levels via its ability to detect the amino-acylation status of tRNAs [41]. Whether directly or indirectly through a protein partner, there is a growing body of evidence that PIKKs somehow detect and respond to cellular polynucleic acids. Could this activity be found in the N-terminal portions of these proteins that show no detectable sequence homologies with each other, thus representing a new set of nucleic acid binding domains?

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CHAPTER 94

Histidine Kinases

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Autophosphorylating histidine protein kinases are predominantly found in bacterial organisms and to a more limited extent in archaebacteria and nonvertebrate eukaryotic organisms where they function in two-component signal transduction pathways. These signaling systems typically consist of a sensor histidine kinase (HK) and a response regulator (RR) (Fig. 1A). HKs are responsive to extracellular stimuli and can undergo adenosine triphosphate (ATP)-dependent autophosphorylation of a conserved histidine residue. The phosphoryl group is then transferred to a conserved aspartic acid residue in the RR protein, which then acts as a molecular switch for activating an associated effector domain or triggering downstream signaling events. A large majority of RRs contain C-terminal effector domains that have DNA-binding activity and thus function in regulating gene expression. In some cases, expanded versions of the two-component system have evolved whereby additional response regulator domains and histidine-containing phosphotransfer (HPt) proteins have been incorporated, thus forming a multistep phosphorelay (Fig. 1B). It has been estimated that nearly 20% of all HKs are hybrid proteins that have a RR domain fused at the C-terminus [1]. These additional signaling modules presumably allow for more regulatory checkpoints and flexibility of the signaling pathway.

The importance of two-component regulatory systems is illustrated by the wide variety of essential cellular processes that are governed by HKs and RRs. In bacteria, for example, two-component systems regulate cell–cell communication, cell differentiation, pathogenesis, and adaptive responses to environmental stress. In plants, fungi, and slime molds, multistep histidine-to-aspartate phosphorelay systems play important regulatory roles in hormone signaling pathways, stress responses, virulence, cell-cycle progression, and cell differentiation. Our aim here is to provide an overview regarding the general functional properties and structural organization of histidine protein kinases. We also refer the interested reader to several recent review articles [1–6] and monographs [7,8] that more comprehensively cover the subject of HKs and their role in two-component signal transduction systems.

A large majority of HKs are multidomain, membrane-bound proteins, which contain an N-terminal extracellular sensing domain and a cytoplasmic kinase domain. In contrast to many eukaryotic receptor tyrosine kinases that are induced to form dimers upon ligand binding, sensor HKs exist in a dimeric state within the bacterial inner membrane or plasma membrane of eukaryotic cells. The transmembrane signaling mechanism is proposed to involve a conformational change that affects juxtaposition of the two monomers relative to each other, which in turn affects kinase or phosphotransfer activities of the HK. It should be noted that not all HKs are associated with membranes; rather, there are also soluble cytoplasmic HKs that lack transmembrane spanning regions but interact with membrane-bound, receptor-like proteins. ATP-dependent phosphorylation of the HK monomers occurs intermolecularly. Subsequently, the phosphoryl group is transferred to a downstream RR. Some HKs have also been shown to exhibit phosphatase activity toward their cognate RR proteins. Thus, HKs can have at least three to four functional roles: sensory perception, autophosphorylation, phosphoryl transfer, and RR phosphatase activity. The effect of external stimuli on HK function can be to alter the rate of autophosphorylation or affect the relative ratios between autokinase, phosphotransfer, and RR phosphatase activities. Regardless of the particular mode of HK regulation, the net result is a change in the level of phosphorylated RR in the cell and the output response is elicited.

Histidine kinases can be identified on the basis of sequence similarities and to date comprise a large superfamily of more than 350 proteins [1,9,10]. HKs typically have two (or more)

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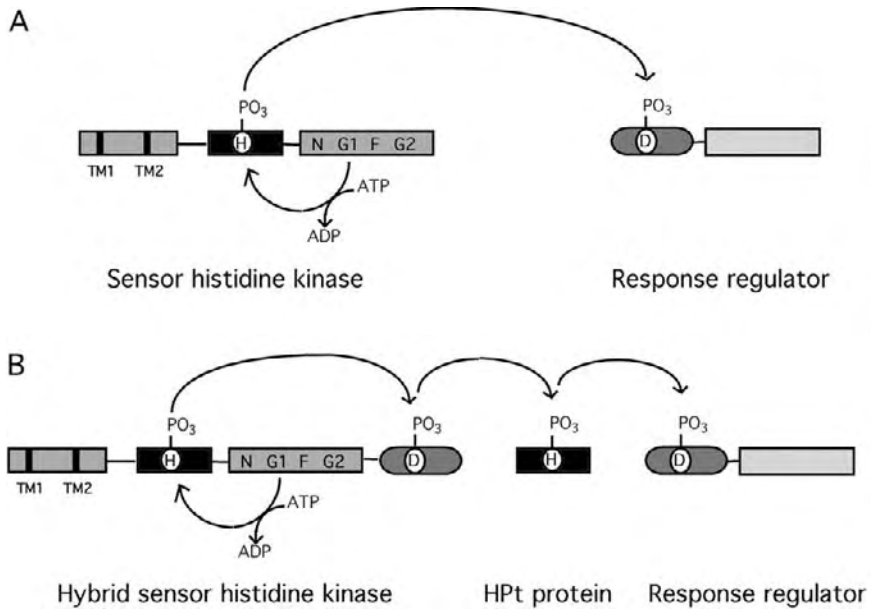


Figure 1 Two-component phosphotransfer systems. (A) Simple two-component phosphotransfer systems consist of a homodimeric membrane-bound sensor histidine kinase (shown here as a monomer) and a response regulator. The histidine kinase (with transmembrane segments indicated as TM1 and TM2) autophosphorylates on a conserved histidine (H) residue using ATP. The phosphoryl group (PO_3^{2-}) is then transferred to a conserved aspartic acid (D) residue in the cognate response regulator. The output response of the signaling pathway is regulated by phosphorylation/dephosphorylation of the response regulator protein. (B) Multistep phosphorelay systems often consist of a hybrid HK that contains an additional response regulator domain at the C terminus and a histidine-containing phosphotransfer (HPt) protein that signals to a downstream response regulator protein.

N-terminal membrane-spanning regions that effectively place a sensing domain outside the cell. As might be expected, the sequences of the sensing domains vary considerably, which is indicative of the wide variety of signals perceived by different HKs. Several conserved sequence motifs can be found within the cytoplasmic domains of HKs, and these are designated the H, N, G1, F, and G2 boxes [11,12]. The H box contains the phosphorylatable histidine residue, which is often found within the dimerization domain, whereas the N, G1, F, and G2 boxes are the hallmarks of the kinase domain and comprise the ATP-binding site. The linker region between the transmembrane domain and the cytoplasmic H box is predicted to form a coiled-coil structure consisting of two amphipathic helices (also known as a HAMP domain) [13]. This region has been demonstrated to be critical for signal transmission and most likely affects intermolecular and/or domain-domain interactions [14–16].

Three-dimensional structure information is available for domains of two HK proteins, the bacterial osmosensor EnvZ and the bacterial chemotaxis protein CheA, and has revealed several similarities and differences among HK proteins [17–20]. The histidine kinase domain is structurally distinct from other kinases for which structural information is available. However, the histidine kinase domain resembles other proteins that catalyze ATP hydrolysis such as DNA topoisomerase II, the chaperone Hsp90, and the DNA mismatch repair enzyme MutL [21,22]. Several structures are now available for full-length RR proteins and even activated RRs, but full-length HKs have posed significant problems for structural biologists due to their multidomain architecture and membrane localization [4].

Chemically, phosphorylated histidines are one of the least stable of the known phosphoamino acids. Phosphorylation of the histidine residue in HKs results in the formation of a high-energy phosphoramidate (N–P) bond. In contrast, phosphorylation of hydroxyamino acids such as serine, threonine, and tyrosine results in formation of relatively stable phosphoester (O–P) bonds. The large standard free energy of hydrolysis of the N–P bond helps to explain why phosphohistidine-containing proteins are ideal as phosphodonors in two-component signaling pathways, bacterial sugar–phosphate transport systems, and as phosphoenzyme intermediates [6,23,24]. Phosphorylation of the aspartic acid residues in response regulators also produces a high-energy acyl phosphate linkage, and it has been proposed that this energy is used to drive long-range conformational changes in proteins [25].

Overall, HKs function to modulate the level of phosphorylation of their cognate RRs in response to environmental stimuli; therefore, a discussion of HKs would be incomplete without at least a brief description of RRs. Most RRs have a two-domain architecture in which an N-terminal regulatory domain controls the activity of a C-terminal effector domain in a phosphorylation-dependent manner. The majority of bacterial RRs have effector domains that specifically bind DNA and, therefore, function to activate or repress gene transcription.

These can be further divided into OmpR, NarL, NtrC, and LytTR subfamilies based on phylogenetic relatedness of the DNA-binding domain [26–28]. Other RRs have effector domains with enzymatic activity—for example, the methylesterase CheB [29] and the cAMP-dependent phosphodiesterase RegA [30]. Some RRs, like the chemotaxis protein CheY, do not have an effector domain but rather signal to downstream effector proteins via protein–protein interactions.

Extensive biochemical and structural characterization of RR proteins have revealed some general features and functional properties of the conserved regulatory domain (also referred to as a receiver domain) [4]. These domains consist of about 125 amino acid residues and have a common doubly wound $\beta_5\alpha_5$ tertiary fold that supports an active site composed of several highly conserved carboxylate-containing side chains and an invariant lysine. The regulatory domain catalyzes phosphoryl group transfer from the phosphohistidyl residue of the cognate HK (or HPt protein) to a conserved aspartyl side chain within the active site of the RR in a Mg^{2+}-dependent manner. RR proteins most likely exist in a dynamic equilibrium between two (or possibly more) conformational states, and phosphorylation is thought to affect this equilibrium by promoting or stabilizing one particular conformation, the activated state. In some cases, such as CheB and PhoB, phosphorylation of the RR relieves inhibitory interactions between the regulatory and effector domains [31,32]. For other RRs, phosphorylation serves to increase the affinity of the RR for DNA, promote RR dimerization, or affect downstream protein–protein interactions. The phosphorylated lifetimes of RRs can vary substantially from one another, often reflecting the duration of the cellular response. RRs have an intrinsic (or self-catalyzed) phosphate hydrolysis rate, which in some cases can be influenced by auxiliary protein phosphatases. In addition, some RRs are subject to HK-catalyzed dephosphorylation.

Although HKs and RRs have been studied extensively and some two-component systems are well understood at the biochemical and structural levels, there are many unanswered questions in the field. For example, what is the mechanism of transmembrane signal transmission? How are different HKs regulated? How are signals transmitted and processed within individual two-component signaling systems? How do cognate HK and RR pairs interact with each other? These questions will undoubtedly be answered through combinatorial and interdisciplinary approaches. Given the vast number of critical processes that two-component signaling pathways control in the cell, HKs and RRs will continue to be a popular subject of many investigators in the years to come.

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CHAPTER 95

Atypical Protein Kinases: The EF2/MHCK/ChaK Kinase Family

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Introduction

The "classical" family of protein kinases represents one of the largest eukaryotic protein superfamilies, with at least 500 distinct members that are involved in a wide variety of roles in signal transduction [1]. These enzymes, which phosphorylate serine, threonine, and tyrosine residues, contain a conserved catalytic domain of ≈300 amino acids that is easily recognized by the presence of conserved motifs (20–40% amino acid identity). Based initially on the detailed structural analysis of protein kinase A [2] and now extended by the determination of the crystal structures of many catalytic domains [3], a great deal of information is available concerning the conserved fold of this enzyme class and the catalytic mechanism. In addition, a significant amount is known about the structural features that define their substrate specificity and individual functions.

Despite the knowledge that other structurally distinct proteins in eukaryotic genomes could catalyze protein phosphorylation—for example, the histidine kinases and related enzymes—recent studies have unexpectedly revealed the presence of a number of seemingly atypical serine/threonine protein kinases that have no obvious amino acid sequence similarity to the classical kinases. The best characterized of these atypical kinases are EF2 kinase, a family of myosin heavy-chain kinases, and a transient receptor potential (TRP)-related ion channel, termed ChaK (channel kinase). In addition, a number of related gene products have been identified through database searches. Despite the lack of detectable sequence similarity, the structure of the catalytic domain of the atypical kinases reveals surprising homology to that of the classical protein kinases. Moreover, the atypical kinase domain is also related to metabolic enzymes that contain the so-called "ATP-grasp" domain. The discovery of this family of atypical kinases highlights the likely evolutionary link between protein kinases and metabolic enzymes. Moreover, these recent studies raise the possibility that other families of protein kinases exist that are not easily recognized from analysis of sequences alone.

Identification of an Atypical Family of Protein Kinases: EF2 Kinase, Myosin Heavy Chain Kinase and ChaK

EF2 kinase was originally identified as a Ca^{2+}/calmodulin-dependent enzyme (previously termed CaM kinase III) capable of phosphorylating eukaryotic elongation factor 2 (EF2) [4,5]. Biochemical studies indicated that EF2 kinase is a monomeric, elongated protein of ≈95 kDa [6,7]. Redpath *et al*., isolated a cDNA that apparently encoded EF2 kinase, and it was suggested that a subdomain of the kinase was related to the catalytic region of the classical protein kinases [8]. However, based on additional analysis, the EF2 kinase sequence was clearly distinct in primary structure from the classical protein kinases. This lack of relationship was further clarified when Egelhoff and colleagues identified a novel *Dictyostelium* myosin heavy chain kinase (MHCK) that contained an ≈250 amino acid domain that exhibited a high degree of similarity with a central region of EF2 kinase [9].

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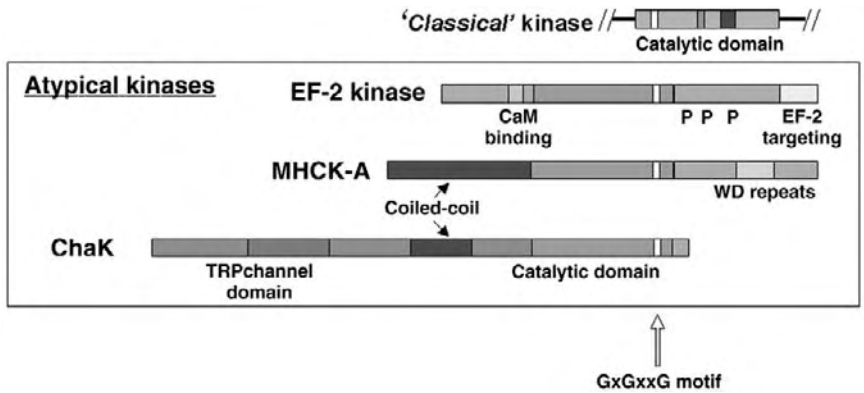


Figure 1 Domain organization of an atypical family of protein kinases. The illustration shows a comparison of EF2 kinase, MHCK-A, and ChaK, with the catalytic domain of the classical protein kinases. A GXGXXG motif that is involved in MgATP binding in classical kinases is not conserved in the atypical kinases. In contrast, the GXGXXG motif at the C-terminal end of the atypical kinases is likely to be involved in peptide substrate binding. In EF2 kinase, the CaM-binding domain is located N-terminal to the catalytic core; phosphorylation by PKA and other kinases at sites C-terminal to the catalytic core is involved in regulation of kinase activity. A region at the extreme C terminus of EF2 kinase is involved in binding eEF2. In MHCK-A, a C-terminal WD-repeat domain is involved in interaction with myosin II in *Dictyostelium*; the N-terminal coiled-coil domain is involved in binding to F-actin in lammelipodia. In ChaK, a long TRP-related channel is located toward the N terminus. The kinase domain of ChaK forms a stable dimer, presumably with another subunit within the presumed tetrameric channel; the function of the coiled-coil domain is unknown but could possibly also be involved in channel subunit interactions.

This conserved domain was unrelated in sequence to the catalytic domain of the classical kinases and was subsequently found to represent the catalytic domain of the atypical protein kinases (Fig. 1).

Additional cloning of EF2 kinase from rat, mouse, human, and *Caenorhabditis elegans* [10] and analysis of expressed sequence tag (EST) databases [11,12] revealed a homologous conserved domain in several additional genes distinct from either EF2 kinase or MHCK. Subsequent work by Egelhoff and colleagues identified three myosin heavy-chain kinases (A, B, and C) in *Dictyostelium* [13,14]. Several groups using different approaches identified ChaK (for channel kinase; also termed TRP-PLIK, LTRPC7, and TRPM7) [15–18]. In ChaK, the conserved atypical kinase domain is at the extreme C-terminus of a large polypeptide that contains a centrally located Ca^{2+} channel domain that is homologous to the family of transient receptor potential (TRP) channels [19]. The TRP family of channels are generally Ca^{2+} permeable, are often regulated via activation of G-protein-coupled receptors, and have been linked to a variety of cellular processes, including photoreception in *Drosophila* and detection of physical and chemical stimuli. Three subtypes of TRP channels—short, long, and Osm-9—have been identified based on the relationship of their N- and C-terminal extensions and their mechanisms of regulation. ChaK (and a related but slightly longer gene product, ChaK2) are members of the long TRP subgroup and are most highly related to melastatin 1, a long TRP channel that is expressed in melanocytes and is downregulated in metastatic melanoma cells [20,21]. While previous studies have indicated that ion channels are highly regulated by protein phosphorylation and may associate within complexes that contain protein kinases and phosphatases, ChaK is unique in that it contains a protein kinase domain fused to an ion channel. Finally, Ryazanov and colleagues have begun to characterize several other polypeptides that contain atypical kinase domains, although nothing is known about their function at the present time [11,22]. These include a gene product cloned from lymphocytes that also contains predicted membrane-spanning regions and gene products cloned from heart and muscle. Genes encoding atypical kinases are also found in *Neurospora*, *Trypanosoma*, and possibly in other protozoans.

The Structure of the Atypical Kinase Domain Reveals Similarity to Classical Protein Kinases and to Metabolic Enzymes with ATP-Grasp Domains

The kinase domain of ChaK forms a dimer as a consequence of a domain-swapping exchange of an N-terminal 27-residue "dimerization segment" [17]. The isolated ChaK kinase domain is also a dimer in solution, and the dimeric property of ChaK may be relevant to the biological function or regulation of the kinase, as TRP channels, like other voltage-gated ion channels, are likely to be tetrameric. However, EF2 kinase and MHCK are monomers and would require an alternative extended polypeptide sequence to replace the buried dimerization segment. When considered as a monomer, the catalytic domain of ChaK is strikingly similar to that of the classical protein kinase (Fig. 2). As in protein kinase A (PKA), there are two lobes separated by the catalytic cleft. In particular, the N-terminal lobe consisting mainly of β-strands, is very similar in topology to the same

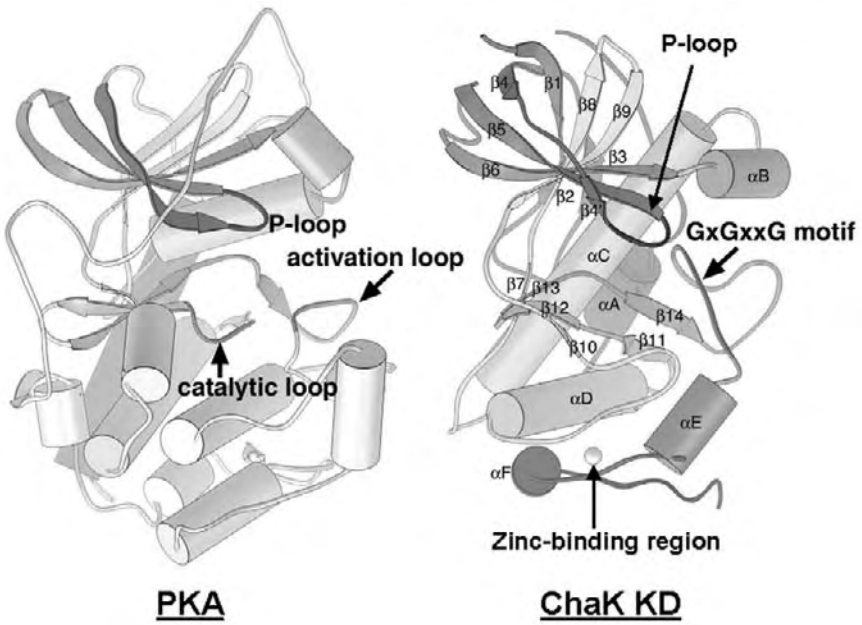


Figure 2 Structural comparison of the kinase domains of ChaK (ChaK KD) and protein kinase A (PKA). The upper N-terminal lobes of ChaK and PKA are largely comprised of β-strands and are related in structure. MgATP binds in a cleft formed between the upper and lower lobes of both classical and atypical kinases and involves the conserved P-loop. The catalytic loop in the classical kinases is not conserved in the atypical kinases. The GXGXXG motif in ChaK is contained in an extended loop that may play a similar role as the activation loop in the classical protein kinases. The zinc-binding region of the atypical kinases is unique to this class of enzymes.

region of PKA and contains a conserved phosphate-binding P-loop that is involved in adenosine triphosphate (ATP) binding in both atypical and classical kinases. The major difference between the ChaK and PKA catalytic domains is found in the C-terminal lobes, where ChaK contains a zinc-binding module that is likely to play an important role in the structural integrity of the kinase domain. A loop that connects the zinc-binding module to the catalytic cleft is poorly resolved in nucleotide-free ChaK but assumes a more defined structure in the presence of ADP or AMP-PNP (an ATP analog). This loop, which contains a glycine motif (GXGXXG) that is highly conserved in the atypical kinases, may be equivalent to the activation loop, a region important for regulation of classical kinases. Alternatively, the flexible loop in the atypical kinases may play a more fundamental role in peptide substrate recognition.

Despite the lack of amino acid sequence similarity, key residues involved in nucleotide binding and catalysis are conserved in the atypical kinase domain. These include the strictly conserved lysine residue that interacts with the α- and β-phosphates of ATP and which is invariant in classical protein kinases. In addition, three residues (Asp-1765, Gln-1767, and Asp-1775) are situated in positions equivalent to three essential residues in the classical kinases (Asp-166, Asn-171, and Asp-184 in PKA). Thus, the general features of catalysis are closely conserved between atypical and classical protein kinases and strongly suggest that they share a common evolutionary origin. Site-directed mutagenesis studies of several of these key residues has provided direct support for their proposed roles in catalysis (Yamaguchi *et al.*, unpublished data). However, mutation of conserved cysteine residues in the zinc-binding module would be expected to seriously affect structural integrity of the protein rather than have a specific effect on kinase activity [15,23]. Mutation of a glycine in the GXGXXG motif has also been found to inhibit ChaK activity, presumably by affecting peptide substrate binding [15].

Notably, there are some significant differences between the atypical and classical kinases in terms of the detailed features of the nucleotide binding site. Moreover, the hydrophobic ATP-binding pocket of ChaK is not strictly conserved in other atypical kinases. The distinct features of the ATP binding region of the atypical kinases provides an explanation for the fact that small-molecule inhibitors of the classical kinases have little or no effect on EF2 kinase (Matsushita and Nairn, unpublished results), whereas a novel class of selenocarbonyl compounds are specific inhibitors of EF2 kinase but not of several members of the PKA family [24]. It seems likely that it should be possible to exploit the specific properties of the ATP binding site to develop molecules that selectively inhibit the different atypical kinases.

A notable feature of the active site of ChaK is the absence of the so-called catalytic loop (Fig. 2). This feature distinguishes the atypical kinase active site from that of the classical kinases, and highlights the similarity of the C-terminal lobe of ChaK to the family of metabolic enzymes that contain the ATP-grasp fold. The elucidation of the structure of the ChaK kinase domain, as well as other ATP-utilizing enzymes such as phosphatidylinositol phosphate kinase IIβ [25], lends

additional support to the idea that there is an evolutionary linkage among these various groups of proteins and the classical protein kinases [26,27].

Substrate Specificity of Atypical Kinases

Based on the general structural similarities and the conserved features of ATP binding and catalysis, it seems likely that peptide substrates will interact in a similar fashion with atypical and classical kinases. Possibly, the peptide substrate might replace a string of water molecules that is observed in the ChaK crystal structure between the terminal phosphate of ATP and the loop containing the glycine motif. Ryazanov and colleagues have speculated that EF2 kinase and MHCK might recognize target phosphorylation sites located in α-helices within their respective substrates, as MHCK phosphorylates residues within the α-helical coiled-coil tail of myosin II, and the phosphorylation sites in EF2 may possibly assume an α-helical conformation [11,22]. However, there is no direct biochemical evidence to support this model, and the structural features of ChaK suggest that the atypical kinases will recognize peptide substrates in an extended manner with multiple contacts in the catalytic cleft.

Notably, both EF2 kinase and MHCK phosphorylate threonine residues within their respective substrates [4,28,29]. In addition, studies using various protein substrates and model peptide analogs indicated that MHCK exhibited a consistent preference for threonine [14]. This result raised the possibility that atypical kinases might be selective for threonine. However, studies with ChaK have indicated that it both autophosphorylates and phosphorylates exogenous substrate proteins at serines and threonines [18]. Moreover, EF2 kinase and MHCK readily phosphorylated mutant substrates containing serine in place of the normally phosphorylated threonine [12,29]. These latter results suggest that atypical protein kinases are unlikely as a group to show preference for threonine in their respective substrates.

Structure–function studies of EF2 kinase and MHCK have suggested that binding to substrate requires additional protein–protein interactions outside of the immediate contacts at the active site [12,23,30–32]. Using a variety of deletion mutants of EF2 kinase, these studies found that the catalytic domain (≈residues 1 to 350) maintained the ability to autophosphorylate itself, but C-terminal truncated proteins did not phosphorylate EF2. Indeed, removal of as few as 19 amino acids from the C-terminus of the enzyme resulted in an almost total loss of EF2 kinase activity, but not of autophosphorylation. Comparison of EF2 kinase from different species, including mammals and *C. elegans* indicates that a stretch of ≈100 amino acids at the C terminus of the protein is highly conserved [10]. A C-terminal domain containing the last ≈300 residues can pull down EF2 [30]. Moreover, addition of a recombinant C-terminal fragment inhibited EF2 phosphorylation (Matsushita *et al.*, unpublished results). Together, these studies support the idea that EF2 interacts directly with the extreme C terminus of EF2 kinase, a region removed from the catalytic domain by ≈300 amino acids. In the case of MHCK-A, the catalytic domain is flanked at the N terminus by a coiled-coil region and at the C terminus by a seven-fold WD repeat motif. Removal of the WD repeat domain decreased significantly the rate of phosphorylation of full-length myosin but had no effect on the kinetics of phosphorylation of a short synthetic peptide that served as a substrate for the kinase [31,32]. Presumably, these additional targeting interactions stabilize the interaction of EF2 or myosin with their respective kinase and perhaps orient the region containing the phosphorylation sites of EF2 or myosin in the correct position in the active sites of either kinase.

Regulation of Atypical Kinases

A substantial amount of evidence indicates that EF2 kinase is highly regulated by second messengers, in particular Ca^{2+}, and serves to integrate the effects of several signaling pathways on the regulation of protein synthesis [12]. EF2 kinase is essentially inactive in the absence, but highly active in the presence, of Ca^{2+}/calmodulin. In the subfamily of classical kinases that are regulated by Ca^{2+}/calmodulin, the enzymes are maintained in an inactive autoinhibited state through the interaction of a "pseudosubstrate" sequence within a C-terminal regulatory domain with the active site of the kinase [33,34]. Binding of calmodulin to the regulatory domain relieves the autoinhibitory interaction and allows access of MgATP and peptide substrate to the active site. In EF2 kinase, site-directed mutagenesis, as well as peptide binding and competition studies, have identified a fairly typical amphiphatic α-helical calmodulin-binding domain within residues 80 to 100 [12,23,30]. However, there is no evidence to suggest that EF2 kinase contains an autoinhibitory domain. Thus, the domain organization of EF2 kinase is distinct from that of the classical Ca^{2+}/calmodulin-dependent protein kinases, and possibly the autoinhibitory mechanism is also different. Consideration of the structure of the catalytic domain of ChaK indicates that the calmodulin-binding domain of EF2 kinase would be in a position equivalent to the helical region of the dimerization segment. Conceivably, the calmodulin-binding domain could interact in an autoinhibitory manner with the active site of EF2 kinase, or, alternatively, binding of calmodulin could stabilize an active conformation of the enzyme.

In the presence of Ca^{2+}/calmodulin, EF2 kinase autophosphorylates through an intramolecular mechanism, and as many as five serine or threonine residues are phosphorylated [7]. EF2 kinase is also phosphorylated at multiple sites by PKA [6,35]. Autophosphorylation or phosphorylation by PKA is associated with generation of a partially Ca^{2+}/calmodulin-independent enzyme activity. Ser365 and Ser499 were identified as the major PKA sites and mutagenesis studies indicated that phosphorylation of probably both sites is involved in generation of Ca^{2+}/calmodulin-independent activity [12,36]. The phosphorylation of EF2 kinase by PKA

suggests a mechanism whereby increased cAMP levels would be linked to inhibition of protein synthesis [37]. Moreover, activation of PKA is frequently associated with activation of Ca^{2+} channels and would, in turn, lead to direct activation of EF2 kinase. However, chronic activation of PKA is associated with downregulation of EF2 kinase protein levels (for example, PC12 cells), where it may play a role in the action of nerve growth factor [12,38]. Thus, the regulation of EF2 kinase by cAMP appears complex.

EF2 kinase has been found to be phosphorylated and regulated by several other kinases. Previous studies had shown that insulin could reduce EF2 phosphorylation via inactivation of EF2 kinase [39]. A recent follow-up study has now found that Ser365 is phosphorylated by p70 S6 kinase leading to inactivation of the enzyme [40]. Thus insulin inactivates EF2 kinase via a pathway that involves phosphatidylinositol 3-kinase (PI3K), phosphoinositide-dependent protein kinase (PDK1), and mTor (the mammalian target of rapamycin). Decreased EF2 phosphorylation is associated with an increased rate of polypeptide elongation, and dephosphorylation of EF2 presumably contributes together with other processes to the positive effect of insulin on protein synthesis. Interestingly, the same study showed that Ser365 of EF2 kinase was also phosphorylated by p90RSK as part of a separate signal transduction pathway that lies downstream of the classical mitogen-activated protein (MAP) kinase cascade. Finally, EF2 kinase is phosphorylated at Ser359 by SAPK4/p38δ, another member of the MAP kinase family [41]. Phosphorylation of EF2 kinase is increased by anisomycin, an activator of stress-activated protein kinase 4 (SAPK4), and leads to inhibition of enzyme activity. It is notable that these various phosphorylation events involve residues in a similar region of EF2 kinase that is 20 to 150 amino acids C-terminal to the catalytic domain. It not immediately obvious how phosphorylation of these sites, which has both positive and negative effects, influences kinase activity. Indeed, phosphorylation of Ser365 has been found to have opposite effects on enzyme activity in different studies. Clearly, additional studies will be needed to clarify the complex pattern of regulation of EF2 kinase by other protein kinases.

Recent studies suggest that the kinase domain, but not activity, of ChaK (termed TRP-PLIK) may be subject to regulation. The kinase domain of ChaK was identified in one study as a result of its ability to interact with phospholipase C (PLC) in a yeast two-hybrid system [15]. An additional study has confirmed this interaction, and indicated that the C2 domain of PLC-β1, -β2, and -β3, but probably not -β4, interacts with the kinase domain [42]. In the same study, ChaK (also termed TRPM7) permeation by Ca^{2+} was suggested to be regulated by a circuitous mechanism that involves constitutive activation by phosphatidylinositol 4,5-bisphosphate (PIP_2), and hydrolysis of PIP_2 by PLC. Presumably, the pool of PLC that interacts with ChaK is responsible for the localized hydrolysis of PIP_2 and is responsible for the observed inhibition of ChaK in response to activation of several Gαq-coupled receptors. In other recent studies directed at examining the potential relationship of ChaK to store-operated Ca^{2+} currents (CRAC), it has been found that ChaK exhibits an unique feature whereby ChaK channel activity (but not CRAC) is inhibited by Mg^{2+} [43,44]. It is unclear if the regulation by Mg^{2+} is of any physiological significance, and it seems unlikely to be related to the Mg^{2+}-dependence of ChaK kinase activity.

Functions of the Atypical Family of Protein Kinases

The functions of EF2 kinase, MHCK, and presumably ChaK are diverse, and it seems likely that further studies of the uncharacterized atypical kinases will bring surprises. Of the atypical kinases, the functional role of EF2 kinase is the most thoroughly characterized (for review, see Nairn *et al*., [12]). Phosphorylation of EF2 results in inhibition of the elongation step of protein synthesis, and activation of EF2 kinase by Ca^{2+}/calmodulin inversely couples the intracellular level of Ca^{2+} to rates of protein synthesis. The precise physiological role of Ca^{2+}-dependent regulation of protein synthesis is not fully understood but appears to be involved in selective translation of specific mRNAs [45]. These studies also highlight a potential role for phosphorylation of EF2 in the regulation of "local protein synthesis." Within cells, Ca^{2+} levels are regulated in a precise spatial and temporal fashion. Thus, localized phosphorylation of eEF2 could modulate protein synthesis in specific subcellular compartments in cells.

Three MHCKs have been identified in *Dictyostelium*, where they are all likely to phosphorylate the heavy chain of myosin II. Phosphorylation of the helical coiled-coil tail of myosin II inhibits its assembly and is a major mechanism that limits the function of myosin II in *Dictyostelium* [46]. Recent studies indicate that the N-terminal coiled-coil domain, which is unique to MHCK-A, is involved in binding to F-actin in lammelipodial protrusions in *Dictyostelium* [47]. Moreover, MHCK-A redistributes to actin-rich membrane protrusions following stimulation with a chemoattractant. This targeting mechanism for MHCK-A, therefore, may be responsible for the lack of myosin II filament assembly within F-actin-containing lammelipodia [48].

Although little is known concerning the physiological role of ChaK, the unusual inclusion of an enzyme domain within the same polypeptide as an ion channel suggests that the intrinsic kinase activity will be involved in regulation of some aspect of the properties of the channel. However, the kinase activity does not appear to be necessary for channel function [16,18]. An initial study that suggested that kinase activity was associated with channel activity most likely is explained by a failure of mutated proteins to be properly expressed [15]. The kinase domain of ChaK is capable of autophosphorylating multiple sites, and these conceivably might be involved in inactivation of channel activity. Alternatively, the kinase activity might play a role in channel assembly or localization. An intriguing possibility is that the kinase is also able to phosphorylate substrates that

are associated with the channel or are involved in some novel manner in responding to ion permeation of the channel.

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CHAPTER 96

Casein Kinase I and Regulation of the Circadian Clock

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Introduction

A multitude of physiological responses and behavioral outputs are expressed with a daily rhythm reflecting adaptation to day/night changes in environmental conditions [1–6]. In the fruit fly circadian clock, a complex of two proteins, CLOCK (CLK) and CYCLE (CYC), activate expression of two genes, *period* (*per*) and *timeless* (*tim*). PERIOD (PER) and TIMELESS (TIM) proteins associate physically in the cytoplasm and after several hours translocate to the nucleus, where they interfere with the transactivation potential of CLK/CYC. Delays are built into this circuit to generate sustained cycling with a period of ~24 hours. For example, TIM is degraded upon light exposure, and PER is degraded in the absence of TIM. Two serine/threonine protein kinases, *double-time* (*dbt*)/casein kinase I (CKI) and *shaggy*/GSK3, phosphorylate PER and TIM, respectively, establishing the time-course of their activities [7,8]. Thus, protein phosphorylation plays a critical role in circadian rhythmicity.

double-time: A Casein Kinase I Homolog in *Drosophila*

Several mutations have been characterized that affect the *Drosophila* casein kinase I-like gene, *double-time*. These lengthen, shorten, or completely abolish fly locomotor activity rhythms [8,10,11]. Analysis of the molecular clock of *Drosophila* reveals that the phenotypes of the mutants are correlated with changes in stability and phosphorylation of the PER protein [8,9]. In the period shortening dbt^S mutant, PER appears to be phosphorylated and degraded prematurely in the nucleus, decreasing the duration of the effects of PER on transcription, and promoting an earlier start of a new circadian cycle. The period lengthening mutations dbt^L, dbt^g, and dbt^h conversely increase stability of PER and lengthen the interval of repression, leading to a delay in the start of the next cycle [8,10]. In these long-period flies, the pattern of PER phosphorylation and its abundance do not change when compared to wild-type flies, until morning, when PER degradation in the nucleus is delayed by about 4 to 6 hours. dbt^g- and dbt^h-like mutations strongly reduce activity of a yeast CKI (HRR25) when measured using a synthetic peptide as substrate [10].

Molecular analysis of a strongly hypomorphic allele, dbt^P, indicates little or no PER phosphorylation and highly increased stability of the protein [8]. PER protein levels in dbt^{ar}, a less severe but similarly arrhythmic mutant, are also elevated. dbt^{ar} flies show an intermediate level of PER phosphorylation, never reaching the maximum level necessary for cyclical degradation [11]. dbt^{ar} is a His 126-to-Tyr missense mutation, a change found naturally in the closely related CKIγ isoform, suggesting that the specificity of dbt^{ar} kinase may be changed but not its activity. The arrhythmicity in dbt^{ar} can be rescued (to long-period rhythmicity) by short-period *per* mutations that are known to destabilize the PER protein [11]. This indicates that both mutations affect the same molecular step that controls PER degradation in the nucleus. Together, these findings have led to the conclusion that one of the major functions of DBT in the circadian clock is to destabilize PER by phosphorylation.

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In biochemical assays, DBT has been shown to bind PER directly *in vivo* and *in vitro* and phosphorylate PER *in vitro* (unpublished data). The kinase domain of DBT, the region in which all mutations affecting rhythmicity reside, binds to PER [9]. It is unclear however, how DBT activity is regulated in the circadian cycle. In wild-type cells, DBT is produced at a constant rate, unlike TIM and PER, which oscillate in their abundance. However, the subcellular localization of the bulk of the DBT protein oscillates in phase with PER in *Drosophila* lateral neurons, the pacemaker cells of the brain that regulate locomotor activity rhythms [12]. This cycling between cytoplasm and nucleus is apparently driven by PER, as DBT constitutively localizes to the nucleus in PER-deficient cells. PER phosphorylation is temporally regulated; PER becomes progressively phosphorylated and its stability decreases after TIM leaves nuclear PER–TIM–DBT complexes in the late night. Phosphorylation increases through the early morning [13]. Thus, DBT phosphorylates PER and marks it for degradation. Binding of TIM to the PER–DBT complex may regulate the accessibility of PER as a substrate for DBT (Fig. 1).

DBT has also been implicated in the regulation of nuclear entry of PER/TIM dimers. Nuclear translocation of PER is reportedly delayed in dbt^S flies. Cytoplasmic PER in the dbt^S flies appears to have a normal half-life, unlike nuclear PER in this mutant, thus the function of DBT in nuclear entry may not be mediated by regulating the stability of PER [14].

Casein Kinase I in the Mammalian Clock

Two highly related casein kinase I isoforms, ε and δ, are components of the mammalian circadian oscillator. These two kinases are 97% homologous and have over 80% homology to DBT in the kinase domain; their C-terminal tails are unrelated to the corresponding region in DBT. CKIε and CKIδ bind mammalian PER1 and PER2 proteins in tissue culture and *in vivo* and also phosphorylate both PER proteins *in vitro* [15,16,17,18].

In cultured cells CKIε and CKIδ regulate nuclear entry and stability of the mammalian PER proteins. CKIε has been implicated in regulating the subcellular localization of PER1 by trapping otherwise nuclear PER1 protein in the cytoplasm in cultured human embryonic kidney cells, but translocating PER1 and PER3 to the nucleus in COS-7 cells [17,18]. Contradictory results from tissue-culture experiments may reflect cell-line-dependent variability in the expression of other factors required for CKI-regulated PER nuclear translocation. CKI also seems to regulate CLK/BMAL-dependent transcription. Reduction of CKI activity inhibits transcriptional activation by CLK/BMAL [19,20]. Immunoprecipitations from mouse liver show both CKI isoforms interacting with PER1 and PER2 proteins, specifically with the phosphorylated forms of the PER proteins [21]. In mice, the subcellular location of CKIε also varies with circadian time, as originally found in *Drosophila*.

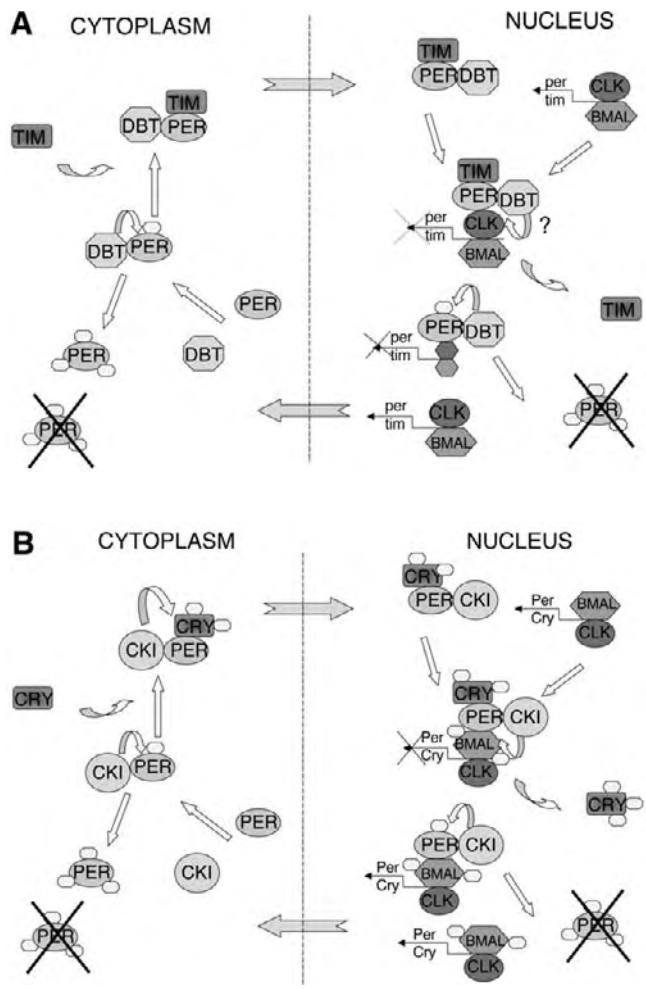


Figure 1 Regulatory activities of DBT/CKI within models of the *Drosophila* (A) and Mammalian (B) circadian clocks. PER is found in a complex with CKI throughout the circadian cycle. In the middle of the day cytoplasmic, newly synthesized PER interacts with CKI and is phosphorylated and degraded. In the early evening, TIM in *Drosophila* and CRY in mammals, accumulate and associate with PER. Such complexes are no longer destabilized by CKI. In *Drosphila* cells, PER bound to TIM and DBT translocates to the nucleus. In mammals, PER forms cytoplasmic complexes with CRY and CKI preceding nuclear translocation. CRY is phosphorylated in the latter complexes by CKI in a PER dependent manner. In the nucleus PER-containing complexes bind two transcription factors, CLK and BMAL. When expressed alone, CLK and BMAL activate *per* and *tim* (files) or *Per* and *Cry* (mammals) expression, but further association with PER-containing complexes suppresses this activity. These interations also suppress clock controlled genes that function downstream of the central oscillator. In the multiprotein complex CKI phosphorylated BMAL in mammalian cells, which increases its activity and may be required to overcome the repression in the end of the circadian cycle. In the early morning, at the peak of transcriptional inhibition in *Drosophila*, TIM is degraded and CKI phosphorylated PER. Hyperphosphorylated nuclear PER is ubiquitinated and degraded in the early morning allowing a new transcriptional cycle to start.

The majority of the kinase is located in the cytoplasm but a fraction translocates to the nucleus during the night when PER proteins are expected to enter the nucleus [21]. Nuclear localization of CKIε and δ is dramatically reduced in *cryptochrome* 1 and 2 double-knockout mice where PER proteins show a strong reduction in nuclear translocation. In the

nucleus, both kinases appear to be part of a larger protein complex that contains all known clock proteins [21].

Genetic evidence for CKI involvement in the mammalian clock comes from the identification of two mutations that cause shortened behavioral rhythms in the Syrian golden hamster mutant *tau* and in humans with familial advanced sleep phase syndrome (FASPS). The *tau* mutation in the Syrian golden hamster leads to a fast-running circadian clock with a period of 20 hours [22]. The mutation lies in the hamster homolog of CKIε. *In vitro*, this mutation leads to a decrease in kinase activity to about 15% of the wild-type level. *In vivo*, there is also a reduction in the abundance of CKIε to half the wild-type level [23]. PER proteins are fully phosphorylated in the mutant hamster, indicating that CKIδ may compensate for reduced CKIε activity and suggesting a dominant-negative function for the *tau* mutant CKIε [21]. FASPS in humans is an inherited, dominant circadian clock disorder that involves slightly reduced period length to 23 hours and a significant phase advance. Patients with FASPS typically awaken prematurely (~4:00 a.m.) and sleep onset is similarly advanced by 3 to 4 hours [24]. The FASPS-causing mutation in one recently studied kindred lies in the human ortholog of the mouse *per2* gene changing Ser 662 to Gly in a potential CKI phosphorylation site [23]. The mutant protein is phosphorylated *in vitro* with a reduced rate by CKIε [23]. Sequences immediately downstream of the mutation contain several potential CKI phosphorylation sites that may be phosphorylated in a cascade starting from the first site. Human PER2 does indeed become progressively phosphorylated over time in an *in vitro* kinase assay supporting the idea of consecutive phosphorylation of these sites [23].

Casein Kinase I in the *Neurospora* Clock

The bread mold *Neurospora crassa* may also use CKI as a part of the molecular clock. In *Neurospora*, most protein components of the clock are apparently unrelated to those of *Drosophila* and mammals but perform similar functions. The Frequency (FRQ) protein contributes to a feedback loop by negatively regulating its own transcription. Activation of *frq* transcription is carried out by two factors White Collar-1 and White Collar-2 [2,4]. FRQ undergoes progressive phosphorylation during the circadian cycle, similar to PER proteins in the fly and mammalian clocks [26]. As in animals, phosphorylation appears to be required for subsequent FRQ degradation [26]. Two protein destabilization sequences or PEST domains have been recognized in the FRQ protein which serve as substrates *in vitro* for *Neurospora* CKI [27]. One of these regions, PEST-1, is phosphorylated *in vitro* by CK-1a, the DBT ortholog in *Neurospora*. Deletion of the PEST-1 sequence in FRQ leads to a more stable FRQ protein. The increased stability of FRQ correlates with its reduced phosphorylation and is phenotypically manifested in a slow-running molecular oscillator with a lengthened period of approximately 28 hours [27].

Similarities and Differences of CKI Function in Different Clock Systems

Experimental results described above suggest similar functions for *Drosophila* DBT, mammalian CKIε and CKIδ, and *Neurospora* CK-1a in the molecular clock. In these three systems, a central clock protein appears to be the major target for the kinases. In flies and mammals, two distinct steps in the circadian cycle are affected by CKI. In the first step, CKI regulates the nuclear entry of the PER proteins in the early night, providing a delay in nuclear accumulation that is likely required for sustained molecular cycling. In mammalian cells, PER1 nuclear translocation is delayed in an enzyme-dependent manner. CKI seems to mask the nuclear localization signal of PER1 by phosphorylation of a nearby region in the protein [16]. Although in *Drosophila* action of DBT in the cytoplasm should delay PER accumulation through effects on PER degradation rate, a short-period mutation of *dbt* leads to a delay in PER nuclear import by another mechanism that does not seem to involve protein stability [14]. One common function of CKI in the three model systems is the destabilization of nuclear PER proteins and FRQ. CKI-dependent phosphorylation reduces the half-life of PER proteins, helping to overcome the transcriptional repression by PER and thus starting a new circadian cycle. The mode of regulation of CKI activity in the clockworks is less clear. CKI proteins are constantly synthesized, but the subcellular localization of the kinase and its binding to other proteins in complex with PER (such as TIM) is under circadian control. This may lead to circadian phosphorylation of some substrates located in the nucleus by rhythmically regulating substrate accessibility.

In spite of many similarities between the *Drosophila* and mammalian enzymes, there are important differences. Structurally the C-terminal tails of the enzymes differ significantly, suggesting a possible difference in enzyme regulation. Mammalian CKIε and CKIδ have been shown to autoinhibit their activity by phosphorylating their C-terminal tails [28]. The specific sequences required for this autophosphorylation are as poorly conserved between the mammalian and *Drosophila* enzymes as the rest of the C-terminal region. Regulation of DBT activity may also be affected by post-translational modifications as evidenced by dramatic differences in the activity of the enzyme purified from bacterial and eukaryotic sources (unpublished data). The mammalian and fly kinases may also differ in substrate selectivity. In the mammalian clock, in addition to PERs, other clock proteins appear to be important substrates for CKI. In cultured mammalian cells, Cryptochrome (CRY) is phosphorylated by CKI in a PER-dependent fashion, where PER acts as a scaffolding protein by simultaneously binding the kinase and its additional (CRY) substrate [20].

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CHAPTER 97

The Leucine-Rich Repeat Receptor Protein Kinases of *Arabidopsis thaliana*: A Paradigm for Plant LRR Receptors

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Introduction

The importance of receptor protein kinases as mediators of cellular communication in plants is illustrated by the plethora of genes encoding these proteins [1]. Although only a few of these receptors have been analyzed in detail, it is clear that they function in the coordination and integration of numerous developmental and adaptive responses. The sequence of *Arabidopsis thaliana* offers a view of the genes and proteins found in plants and establishes a foundation for a complete understanding of plant cellular communication. This overview focuses on the leucine-rich repeat (LRR) receptors in *Arabidopsis* because of the wealth of information derived from analysis of *Arabidopsis* mutants and from the genome sequence. A comprehensive review of structures, expression, and functions of the plant receptor protein kinases is available to those interested in additional information on these important signaling proteins [1].

LRR Receptor Protein Kinases: The Genomic Point of View

One of the surprising findings from analysis of the *Arabidopsis* genome is the large number of genes encoding proteins kinases. There are almost 1100 genes in *Arabidopsis* that are predicted to encode proteins related to the eukaryotic serine/threonine/tyrosine protein kinases ([2]; http://PlantsP.sdsc.edu). This represents approximately 4.2% of the genome. Even more remarkable is the observation that 610 [3] to 669 [2] of these genes belong to a large, yet distinct, family of related protein kinases. This large family, designated the receptor-like kinases (RLKs), is most closely related to the Pelle and interleukin-1 (IL-1)-receptor-associated kinases and shares a common ancestry with the animal receptor tyrosine kinases, receptor serine kinases, and Raf protein kinases [1,3].

The RLK family is divided into two types, the RLKs that have an extracellular domain and the receptor-like cytoplasmic kinases (RLCKs) that do not have an extracellular domain. The relationship of the RLKs and the RLCKs is reminiscent of the similarity between the receptor and nonreceptor tyrosine kinases and suggests a shared origin. However, little is known about the *Arabidopsis* RLCKs, and it is not yet clear how the sequence similarities shared between the RLCKs and RLKs reflect shared biochemical mechanisms of action.

Over 400 RLK genes contain an extracellular domain [3]. Thus, the RLKs represent the largest group of cell surface receptors in plants, as there are only 27 G-protein-coupled receptor (GPCR)-related domains detected in *Arabidopsis*, and components of many other common signaling pathways found in animals, flies, and worms are not found in *Arabidopsis* [4]. Moreover, the number of *Arabidopsis* RLKs is six to ten times higher than the 40 to 60 receptor tyrosine kinases observed in worms and humans [5]. The large number of RLKs in *Arabidopsis* in part reflects the gene redundancy seen in this organism; it is estimated that only 35% of the predicted proteins in the *Arabidopsis* genome are unique. However, 21 classes of RLKs in *Arabidopsis* differ in their

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extracellular domains, including 12 groups of LRR RLKs. These 12 groups of LRR RLKs include 235 genes that have 1 to 32 LRRs [3].

LRR Receptor Protein Kinases: The Functional View

Although some information has been obtained from expression studies and biochemical analysis of the RLKs [1], genetic approaches have provided the most information about the roles of the LRR RLKs in cellular communication. Of particular significance for this review is the fact that the functions of only five *Arabidopsis* RLKs have been described, all of which encode LRR RLKs. These *Arabidopsis* RLKs of known function include ERECTA (ER), CLAVATA 1 (CLV1), HAESA (HAE), BRASSINOSTEROID-INSENSITIVE 1 (BRI1), and FLAGELLIN SENSING 2 (FLS2).

ERECTA

The *er* mutant plants have a compact inflorescence, with shorter, blunt fruits and round leaves. The *er* phenotype is expressed throughout much of the plant's life [6], and the common developmental pattern observed among affected organs suggests that *er* controls cell expansion. ER kinase assays show that this RLK, like all of the LRR RLKs examined to date, is a serine/threonine protein kinase [7]. Numerous *er* alleles have been identified, and phenotypic variation among alleles suggests both the extracellular domain and the protein kinase domains are critical for function. Essentially all mutants that have been identified have a strong *er* phenotype very similar to that observed in an *er*-null plant [7]. Although little is known about other components of an ER signaling pathway, the recent identification of a mutation in a putative heterotrimeric G-protein β subunit gene (*agb1*) that has some phenotypic similarities with *er* suggests a connection between LRR RLK and GPCR signaling [8]. This connection is supported by an analysis of *agb1-er* double mutants that indicates these two genes may function in a common developmental pathway that controls fruit shape; however, a biochemical connection between *abg1* and *er* has not been established.

CLAVATA 1

CLV1 is thought to promote differentiation of cells in shoot and floral meristems. Many alleles of CLV1 accumulate undifferentiated cells in the meristem of the *Arabidopsis* shoot. This leads to the formation of enlarged shoot and floral meristems, fasciated stems, extra floral organs, and fruit with extra cells at the tip, which give these fruits their characteristic club-like appearance [9]. Biochemical and genetic analyses of CLV1 have led to the identification of several other proteins that may mediate CLV1 signaling. Size exclusion chromatography of immunoprecipitated CLV1 from plant extracts indicates that CLV1 is part of a multimeric complex [10], which is consistent with genetic data on dominant interference of some mutant alleles [11]. Analysis of the CLV1 complex has also led to the identification of an associated Rho-like GTPase and the type 2C protein phosphatase KAPP (kinase-associated protein phosphatase). Small G proteins have well-established roles in many signaling cascades [12], but their relevance to plant signal transduction is uncertain. KAPP was initially identified as a protein that binds several RLKs [13,14] in a phosphorylation-dependent manner via a fork head-associated domain [15]. Overexpression of KAPP in a wild-type background caused a weak *clv* phenotype [16], and cosuppression-based loss of KAPP expression in a *clv1* background suppresses the *clv* phenotype [17]. These results suggest a role for KAPP as a negative regulator of CLV1 signaling.

Another partner for CLV1 is the CLV2 protein. *clv2* is the second of three mutants that express the *clv* phenotype. CLV2 encodes a transmembrane LRR protein with a short cytoplasmic domain but lacks the protein kinase domain found in CLV1 [18]. Analysis of protein extracts prepared from *clv2* mutants shows an overall reduction of CLV1 protein and a shift in the distribution of CLV1 from the wild-type high-molecular-weight complex to a lower molecular weight complex [18]. Because CLV2 has no protein kinase domain or other identifiable enzymatic function, CLV2 may be required to form stable CLV1 signaling complexes.

A third mutant gene, *clv3,* shares many of the same traits expressed by *clv1* mutants, and genetic analyses of *clv1-clv3* double mutants place these genes in the same pathway [11]. *CLV3* is predicted to encode a small secreted polypeptide [19] and is a member of a large family of genes that are putative secreted signaling peptides in plants [20]. Two lines of evidence support the role of CLV3 as a ligand for CLV1. CLV3 has been shown to be required for the formation of an active CLV1 signaling complex, and CLV3 will bind to yeast cells expressing CLV1 and CLV2 [21].

HAESA

HAESA is an LRR receptor protein kinase that belongs to the same family as CLV1 [3], yet it appears to have a very different function. Like CLV1, HAE is a transmembrane serine/threonine protein kinase located in the plasma membrane [22]. HAE is expressed in abscission zones of floral organs, as well as at the base of petioles and pedicels. Expression in flowers is stage dependent and first observed in maturing flowers, coinciding with competence to self-pollinate. Transgenic plants expressing a constitutive antisense HAE construct have defective floral organ abscission, which suggests HAE plays a role in abscission [22].

BRASSINOSTEROID-INSENSITIVE 1

The *bri1* mutant plants are dwarfs that do not respond to plant steroid hormones known as brassinosteroids. This insensitive phenotype indicates BRI1 is a brassinosteroid receptor or signaling component [23]. This hypothesis was supported with the cloning of *bri1* which showed that *bri1* encodes a plasma-membrane-localized LRR receptor protein kinase. This was an unexpected finding because in animals steroid hormones act by diffusing across the plasma membrane and

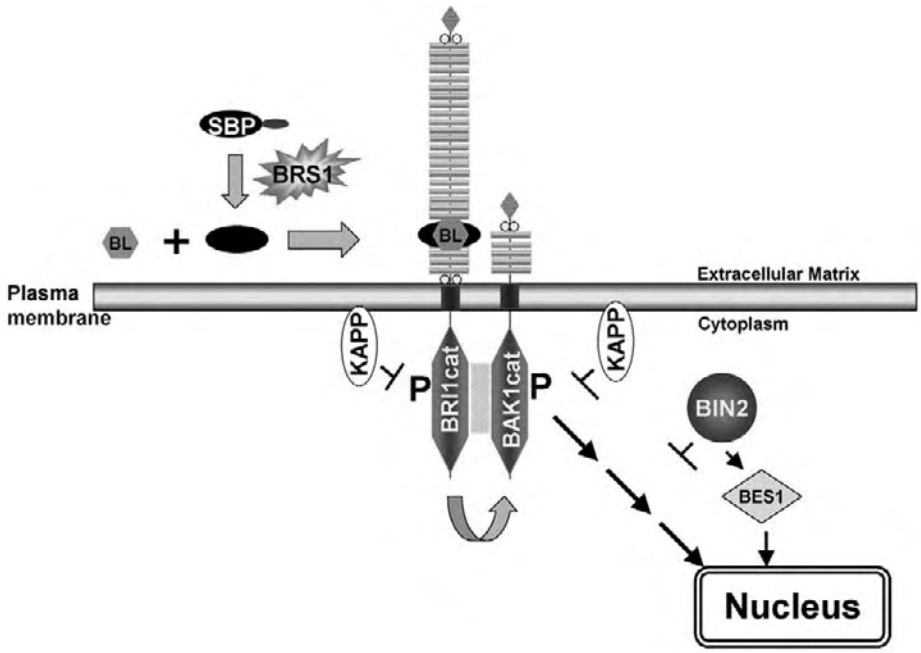


Figure 1 Model for BRI1 signaling. A putative steroid binding protein (SBP) is processed by BRS1, a secreted serine carboxypeptidase. Activated SBP binds brassinolide (BL) and the ligand complex then can interact with BRI1. Upon binding, the BRI1 protein kinase is activated and stimulates the activity of BAK1, a co-receptor, which can further trigger a phosphorylation cascade. Both KAPP and BIN2 are negative regulators in the BRI1-mediated signaling pathway. BES1 is a potential transcription factor that is substrate of BIN2. A direct connection between BIN2 and BRI1/BAK1 is still unclear.

binding to transcription factors in the cytoplasm or nucleus, resulting in a change in gene expression [24].

Evidence supporting the role of BRI1 in brassinosteroid signaling includes the observation that a chimeric receptor containing the LRR domain of BRI1 and the protein kinase domain of XA21 (an LRR RLK from rice that functions in disease resistance) triggers defense responses when treated with brassinosteroids [25]. In addition, brassinosteroids stimulate BRI1 autophosphorylation in plants and bind to immunoprecipitated BRI1 [26]. Thus, a combination of genetic and biochemical analyses show the LRR receptor BRI1 is a critical element in steroid hormone signaling in *Arabidopsis* (Fig. 1).

Recent genetic screens have revealed additional components of brassinosteroid signaling, *BIN2* and *BRS1*. Plants with mutant alleles of *BRASSINOSTEROID-INSENSITIVE 2* (*BIN2*) are semidominant dwarfs with phenotypes similar to *BRI1*. *BIN2* encodes a GSK2/SHAGGY-like protein kinase and when overexpressed BIN2 interferes with BRI1 signaling [27]. This suggests that BIN2 is a negative regulator of brassinosteroid signaling. *bri1 SUPPRESSOR 1* (*BRS1*) encodes a carboxypeptidase that was identified in an activation tagging screen of a weak *bri1* allele [28]. Overexpression of BRS1 suppresses the *bri1* phenotype and results in increased growth. BRS1 is predicted to be a secreted type II carboxypeptidase, and overexpression of BRS1 suppresses two different weak alleles of *bri1* that have extracellular domain lesions, but not a *bri1* allele with a mutation in the protein kinase domain. Thus, BRS1 is thought to be involved in proteolytic processing of a protein involved in an early step in BRI1 signaling.

FLAGELLIN-SENSING 2

Peptides derived from flagellin, a bacterial flagellar protein, activate plant defense responses, such as callose deposition, production of reactive oxygen species, and induction of pathogenesis-related gene expression in many plants. In *Arabidopsis*, flagellin peptides also alter growth of seedlings. Using this altered growth response as a screen, flagellin-insenstive plants have been identified. One of these, FLS2, encodes a LRR receptor protein kinase [29], and a *fls2* allele with an extracellular domain mutation does not bind flagellin [30]. FLS2 is related to two other plant disease resistance genes, *XA21* from rice and *Cf9* from tomato [31]. As described above, XA21 is a LRR receptor that has a protein kinase domain, while Cf9 is similar to CLV2 because it is an LRR receptor with a short cytoplasmic domain and no protein kinase domain [31].

Summary

The LRR receptors represent the largest group of cell surface receptors in *Arabidopsis thaliana* and probably in all plants. Although this review has focused on the LRR receptor protein kinases, another 30 genes in *Arabidopsis* encode LRR receptors with a short cytoplasmic domain [31]. Although the functions of only of few of the *Arabidopsis* LRR receptors have been established, it is clear that these proteins have diverse roles in coordinating cellular signaling in plants. CLV1 and HAE belong to the same family of LRR RLKs yet have very different patterns of expression and are

involved in distinct developmental processes. BRI1 and ER, which are also related to CLV1 and HAE, are expressed in most parts of the plant but have dissimilar mutant phenotypes. In contrast, FLS2 is involved in binding an elicitor of plant defense responses and thus reflects the role of some LRR receptors as disease resistance genes.

A combination of genetics and biochemistry has advanced our understanding of the signaling molecules associated with the LRR receptors, including ligands and downstream effectors. Although we are not yet able to describe an entire signal transduction cascade for any one of the plant LRR receptors, approaches such as functional genomics and proteomics promise to provide important insights into the molecular mechanisms by which the LRR receptors control development and adaptive responses in plants.

UPDATE

Several recent publications have identified the functions of additional Plant LRR receptors. The receptors for Phytosulfokine, a five amino acid sulfated peptide involved in plant growth and Systemin, an 18 amino acid peptide involved in triggering wound responses, are LRR-RLKs related to BRI1 (Matsubauashi *et al*., 2002 *Science* **24**:1470; Scheer and Ryan, 2002 *Proc. Natl. Acad. Sci*. **99**:9585). SYMRK and NORK are related LRR-RLKs involved in the regulation of root nodule symbioses in plants (Stracke *et al*., 2002 *Nature* **417**:959; Endre *et al*., 2002 *Nature* **417**:962). BAK1 is an LRR-RLK involved in modulating brassionsteroid signaling (Li *et al*., 2002 *Cell* **110**:in press; Nam and Li, 2002 *Cell* **110**:in press). In addition, BES1 and BRZ1 have recently been identified as nuclear localized proteins that function in brassionsteroid signaling (Yin *et al*., 2002 *Cell* **109**:181; Wang *et al*., 2002 *Dev Cell* **2**:505).

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CHAPTER 98

Engineering Protein Kinases with Specificity for Unnatural Nucleotides and Inhibitors

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A major challenge of the post-genomic era is to derive a complete map of all signaling networks in mammalian cells. One difficulty in mapping signaling pathways is determination of the substrates of each protein kinase [1,2]. Kinase substrates are difficult to identify chiefly because of the large size of the kinase superfamily (over 500 human kinases) and the large number of low-abundance substrates that kinases are predicted to phosphorylate. Current methods for identification of kinase substrates include: (1) searching protein sequence databases for known phosphorylation motifs [3], (2) screening for efficient substrates in expression libraries [4], and (3) detecting stable association between kinase and substrates by screening yeast two-hybrid libraries [5]. Each of these methods has identified interesting candidate substrates, some of which have been validated using various biochemical tests.

The vast majority of kinase substrates remain to be identified, however, because these methods do not take into account key aspects of kinase substrate recognition, such as subcellular localization, colocalization of kinase and substrate in large supramolecular protein complexes, and temporal activation/deactivation of kinase activity. In particular, tyrosine kinases do not possess high intrinsic specificity for short linear sequences flanking substrate phosphorylation sites [6]; this severely limits the application of sequence database searching for kinase substrates. Most biochemical and genetic screens require putative substrate proteins to be isolated from their cellular context. The physiological relevance of these *in vitro* substrates is questionable because it is known that the formation of signaling complexes (which colocalize kinases and their substrate proteins via many associated proteins) is critical to the observed signaling specificity *in vivo* [7]. To address the challenge of identifying direct kinase substrates, our laboratory has developed a novel chemical approach to accelerate the process of kinase substrate identification (*vide infra*).

A related, yet distinct hurdle in mapping kinase function is the generation of selective inhibitors for individual kinases. Small molecule inhibitors have advantages for deciphering the function of a kinase in that they can be used at any point in development (and thus do not suffer from poor temporal control over gene function as do knockout approaches), and they can be readily removed to restore kinase function, thereby providing rapid conditional on/off control [8]. Furthermore, many protein kinases are considered potential drug targets for treatment of various human diseases [9]. The key challenge in the design of kinase inhibitors for research or drug development has been to achieve target specificity. In order to be used as a therapeutic agent, a small molecule inhibitor should be very specific for its target kinase in order to minimize the off-target effects, which can be substantial in such a large and highly conserved family of enzymes. Despite the tremendous efforts spent in developing selective kinase inhibitors, only a few relatively specific inhibitors for

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protein kinases have been found to date [10–12]. One notable example is PD 98059, which specifically prevents the activation of MAPK/ERK kinase 1 (MEK1) by the upstream kinase MEKK due to the rare mode of action of the inhibitor [13]. Clinically, Gleevec, an inhibitor that is known to inhibit BCR-ABL as well as at least three other kinases, was approved by the U.S. Food and Drug Administration (FDA) for the treatment of chronic myeloid leukemia disease. This is significant because it suggests that perfect specificity may not be essential for the development of kinase inhibitors as drugs [14,15]. However, truly mono-specific kinase inhibitors would greatly facilitate functional studies of individual protein kinases.

To address this need, our lab has developed a chemical/genetic approach, combining chemical synthesis with protein engineering, that circumvents the difficulties of both substrate identification and specific inhibitor design associated with this important gene family [16–18]. The approach we developed is to engineer one kinase in the cell to be structurally unique from all other kinases, yet functionally identical to its wild-type allele. By identifying a highly conserved active site residue that can be mutated to render it distinct from all other protein kinases in nature, the idea is to make specific binding easy to achieve. Of course, the mutation to the kinase must be silent in terms of phospho-acceptor specificity recognition, regulation of catalytic activity, etc.

By examining the crystal structures of protein kinases for residues within the adenosine triphosphate (ATP) binding site, yet distant from catalytically essential residues, one residue (in the hinge region between the N terminal lobe and C terminal lobe) was noted to be in close contact with the N6 amino group of ATP. (The N6 position of ATP is distant from the gamma-phosphate group that is transferred in the catalytic step of the reaction.) This residue is located in subdomain V of the kinase domain in the primary sequence and is always occupied by a large hydrophobic residue in the kinase superfamily based on sequence alignment (Fig. 1). This residue has been termed the *gatekeeper* because it governs access to a large hydrophobic pocket in the ATP binding site. When the gatekeeper residue is mutated to a small residue, Gly or Ala (such mutant alleles are referred to as as1 or as2 for analog-sensitized allele 1 or 2), a much larger pocket is created in the active site of the mutant kinase (which is not found in any wild-type protein kinase because no natural kinases contain a Gly or Ala gatekeeper residue). This feature can

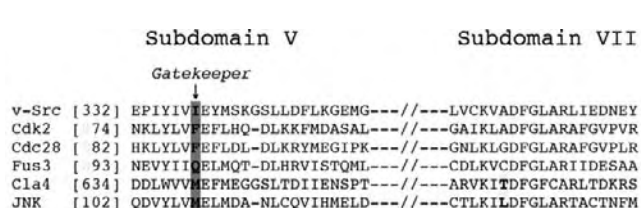


Figure 1 Sequence alignment of several engineered kinases in subdomains V and VII. The gatekeeper residue in each kinase and the second mutation (N-terminal to the DFG motif) in Cla4 and JNK are highlighted.

then be exploited to distinguish the mutant from all wild-type kinases. For example, the engineered kinase can efficiently use ATP analogs with large substituents at the N6 position that are very poorly used (orthogonal ATP analogs) by wild-type kinases [16,19]. Importantly, the mutation has been shown not to alter the structure and specificity profile of the kinase [20] and is functionally silent in most kinases examined [18]. Therefore, the engineered kinase together with [γ-^{32}P] orthogonal ATP analogs can be used to radiolabel the *bona fide* substrates of the target kinase in the background of any pool of proteins including whole cell lysates (Fig. 2).

The strategy was first explored with the oncogenic tyrosine kinase v-Src where it was shown that v-Src I338G (v-Src-as1) could use orthogonal ATP analogs efficiently, while wild-type v-Src cannot [16,19]. Next, a screen using NIH3T3 cell lysates containing v-Src-as1 and [γ-^{32}P]N^6-benzyl ATP identified several novel candidate substrates of v-Src including Cofilin, Calumenin, and Dok-1 [21]. Further studies suggested that v-Src phosphorylation sites on the scaffold protein Dok-1 are critical for its binding to negative regulators of the Src signaling pathway (RasGAP and Csk), which led to a model of the precise order of assembly of a retrograde signaling pathway in v-Src transformed cells [21]. A wide variety of protein kinases (including both tyrosine and serine/threonine kinases) were later shown to be amenable to this approach, highlighting its generality [16,22–24]. Habelhah *et al*. [22] used the approach to identify direct substrates of the stress-activated protein kinase, JNK. In this case, a second mutation in the active site, adjacent to the DFG motif (Fig. 1), was required for the engineered JNK (JNK-as3) to efficiently use N^6-phenylethyl ATP. JNK-as3 was then used together with [γ-^{32}P]N^6-phenylethyl ATP to identify heterogeneous nuclear ribonucleoprotein K, hnRNP-K, as a direct substrate of JNK. Interestingly, hnRNP-K was also found to be a substrate of another MAP kinase, ERK, and further study established the role of MAPK/ERK in phosphorylation-dependent cellular localization of hnRNP-K, which is required for its ability to silence mRNA translation [23]. Several dozen other kinases are currently being investigated using the same approach for substrate identification, including CDK2 [24] and Cdc28 (D. O. Morgan, unpublished results).

In addition to their ability to accept orthogonal ATP analogs, the analog-sensitized kinases can be selectively inhibited by inhibitor analogs with enlarged groups at the proper position based on the same principle [17,18,25]. The pyrozolo [3,4-*d*]pyrimidine-based molecule PP1 is a known potent inhibitor of Src family kinases. The crystal structure of a PP1/Hck complex revealed that the pyrozolo[3,4-*d*]pyrimidine ring of PP1 occupies the active site in essentially the same orientation as the adenine ring of ATP, and that the phenyl group at the C3 position is in direct contact with the gatekeeper residue [26]. Therefore a series of PP1 analogs with large substituents at the C3 position were synthesized and screened against an array of kinases (wild-type and analog-sensitized) from several divergent families (v-Src, c-Fyn,

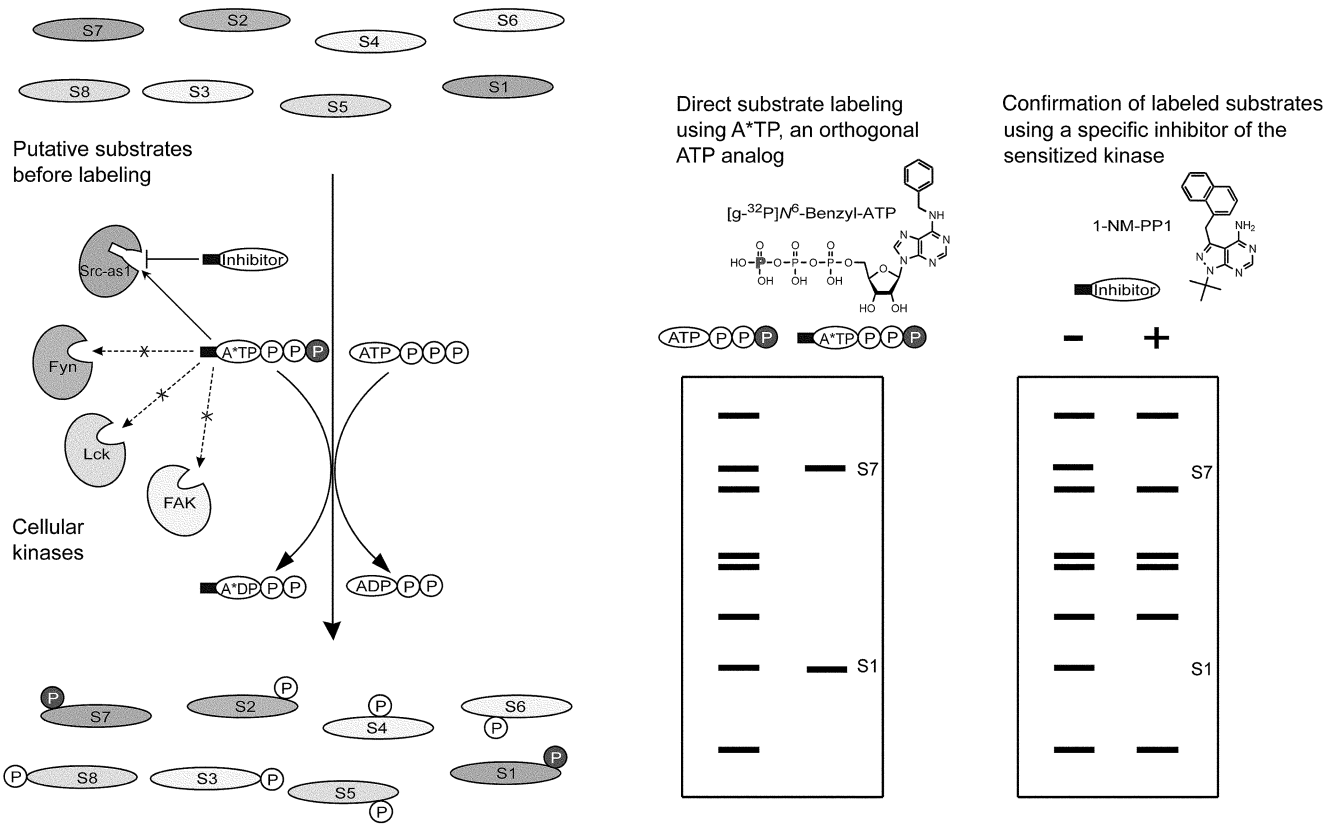


Figure 2 Schematic diagram of two complementary methods to identify substrates of Src-as1. A [γ-^{32}P]-orthogonal ATP analog (A*TP) radiolabels only substrates of Src-as1 (represented by S1 and S7 proteins) as can be visualized by autoradiography. A specific inhibitor (Inhibitor) of Src-as1 can then be used to confirm the phosphorylation using autoradiography or western blot.

c-Abl, CDK2, CAMKII) [17,18]. It was found that derivatives of PP1 could effectively inhibit the sensitized alleles of each of these kinases with high target selectivity. This chemical genetic approach provided inhibitors of the sensitized kinases with both high potency and unparalleled specificity, which enabled their use to reveal unexpected kinase-activity-dependent functions of various kinases, including v-Src [17], Cdc28 [18], Fus3 [18], Cla4 [27], Cbk1 [28], PKA (G. S. McKnight, unpublished results), v-erbB (W. Weiss, unpublished results), CAMKIIα (J. Z. Tsien, unpublished results), Ime2 (I. Herskowitz, unpublished results), and Apg1 (D. J. Klionsky, unpublished results).

Bishop *et al.* used kinase sensitization to study the effects of target-specific inhibition of the Cdc28p kinase from *Saccharomyces cerevisiae* [18]. Cdc28p is the major CDK in budding yeast and is essential for advancement through multiple stages of the cell cycle [29]. 1-Naphthylmethyl-PP1 (1-NM-PP1) was found be a potent and specific inhibitor of Cdc28-as1 (Cdc28 F88G) from studies both *in vitro* and *in vivo*. Interestingly, a low concentration of 1-NM-PP1 (500 n*M*) caused *cdc28-as1* cells to arrest at G2/M with a 2*C* DNA content and large hyperpolarized buds, while a higher concentration of 1-NM-PP1 (5 μ*M*) caused *cdc28-as1* cells to arrest in G_1 with a 1-*C* DNA content. This suggested that the G_2/M transition is more sensitive to inhibition of Cdc28p activity than the G_1/S transition in the yeast cell cycle. This result was completely unexpected because previous studies showed that most temperature-sensitive *cdc28* mutants arrested as unbudded cells in G_1 at elevated temperatures, suggesting that the G_1/S transition is more sensitive to the inhibition of Cdc28p activity [30]. The discrepancy between genetically (*ts*) and pharmacologically induced phenotypes can be explained by the possibility that Cdc28p has other critical cellular functions that are not directly attributable to its enzymatic activity. This demonstrates that chemical studies using small molecules complement traditional genetic studies in cell biology by revealing otherwise hidden functions of proteins.

Weiss *et al.* [27] utilized this kinase-sensitization strategy to study the physiological functions of Cla4p in budding yeast. Cla4p is an effector for Rho-family GTPase Cdc42p and is known to be involved in various actin-polarization-related processes [31]. A genetic approach using temperature-sensitive mutants for the study of Cla4 is not ideal because actin-polarization-related processes are intrinsically temperature sensitive [32]. Weiss *et al.* generated an inhibitor-sensitized Cla4 allele (Cla4-as3), which (similar to JNK) contained a second mutation (Fig. 1) necessary for high sensitivity to 1-NM-PP1. It was observed that the identity of the residue N-terminal to the DFG motif strongly affects sensitivity to inhibitor analogs: Thr or a larger residue at this position often abolishes binding of analogs, presumably due

to a clash with the naphthyl moiety in 1-NM-PP1 [26]. The inhibitor was then used to block Cla4-as3 activity at different stages of the cell cycle to reveal the roles of Cla4 during these different processes. Again, this revealed functions of the target kinase different from those revealed by genetic studies [27].

Besides their use in cell biological studies, monospecific inhibitors can also be used for kinase substrate identification. These inhibitors should significantly reduce the phosphorylation of the substrate proteins of the target kinase *in vivo*, which could be utilized to confirm those substrates identified in direct *in vitro* labeling experiments using orthogonal ATP analogs (Fig. 2). Therefore, the same approach provides two complementary methods for kinase substrate identification and validation.

It should be noted that a small number of kinases are not active when the gatekeeper residue is mutated to Gly or Ala. Such kinases display reduced stability or activity as purified proteins or in cellular assays (unpublished results). This problem can be addressed by introducing additional mutations to regain stability or activity, which was demonstrated by the work done on two isoforms of PKA, PKAα and PKAβ (G. S. McKnight, unpublished results). It was shown that PKAα tolerated the modification at the gatekeeper residue, while modified PKAβ showed a substantial loss of catalytic activity. This difference due to mutation of the gatekeeper residue was unexpected due to the high level of conservation between the two isoforms. Sequence alignment revealed that the differences between the two isoforms are all conservative except at position 47 (I47 in α and K47 in β). When Lys47 in PKAβ was substituted with Ile, as in PKAα, kinase activity was increased to a level near that of wild-type, based on a reporter assay. Thus, second-site-suppressor mutations can be rationally found based solely on sequence information with homologous kinases that are known to tolerate mutation of the gatekeeper residue to Ala or Gly. Currently, we are in the process of identifying positions important for stability or activity of kinases aided by the data we have on all the kinases in which the gatekeeper residue has been modified.

To summarize, a novel chemical genetic approach has been developed that possesses many advantages over purely genetic or chemical approaches for the study of protein kinase function. Most importantly, it is generalizable, with the potential of being applied to every protein kinase in the genome. We expect this method will be increasingly used in cell biology studies and will significantly accelerate the elucidation of various functions that kinases carry out in eukaryotic cells.

Acknowledgments

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SECTION B

Protein Dephosphorylation

Jack E. Dixon, Editor

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CHAPTER 99

Overview of Protein Dephosporylation

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There are two classes of enzymes that regulate signaling through the phosphorylation and dephosphorylation of proteins: protein kinases and protein phosphatases. This section of the *Handbook on Cell Signaling* will focus on the regulation, structure, and function of the protein phosphatases. Protein phosphatases are generally divided into two main groups based on substrate specificity. Protein phosphatases (PPs) specifically hydrolyze serine/threonine phosphoesters, while protein tyrosine phosphatases (PTPs) are phosphotyrosine specific. A subfamily of PTPs, the dual-specificity phosphatases, are capable of efficient hydrolysis of both phosphotyrosine and phosphoserine/threonine. Although both PPs and PTPs catalyze phosphoester hydrolysis, they employ different catalytic mechanisms.

The PPs comprise a large family of metallo-protein phosphatases whose functions within the cell are extremely diverse and highly regulated. Amino acid sequence comparisons within the catalytic domains revealed that two main gene families exist. PP1, PP2A, and calcineurin (PP2B) are members of the same gene family, whereas PP2C shares no sequence homology to PP1, PP2A, or PP2B and thus represents a distinct gene family. This section of the Handbook addresses the regulation, structure, and function, as well as the importance, of PP inhibitors in understanding the roles of PPs.

PTPs also play important roles in cellular regulation. All PTPs are characterized by the active-site sequence motif, HCxxGxxRS(T), within a catalytic domain of approximately 200 to 300 residues. The receptor-like PTPs often have two of these catalytic motifs, while intracellular PTPs have a single catalytic domain. Unlike the PPs, the PTPs do not require metal ions for catalysis. Outside of the catalytic domain, the amino acid sequences generally show limited identity. X-ray structures of intracellular receptor-like and dual-specificity phosphatases have led to a detailed understanding of the biology and catalytic mechanism of this family of proteins. In addition to structure, this section of the Handbook also focuses on important receptor phosphatase regulation and the functions of PTPs in disease states. Two important families of PTPs (PTEN and myotubularin), which do not function on protein substrates, are reviewed in the lipid second messengers section of the Handbook. Finally, a chapter is devoted to a family of "Styx/dead" PTPs (catalytically dead) that appear to be of biological importance.

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CHAPTER 100

Protein Serine/Threonine Phosphatases and the PPP Family

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Current Classification of Protein Serine/Threonine Phosphatases

Protein serine/threonine phosphatases are enzymes that reverse the actions of protein kinases by cleaving phosphate from serine and threonine residues in proteins. They are structurally and functionally distinct from the acid and alkaline phosphatases and are also separate from the family of protein phosphatases encompassing the tyrosine and dual specificity (tyrosine and serine/threonine) phosphatases.

The current classification of protein serine/threonine phosphatases is based upon the primary structures of their catalytic subunits. The amino acid sequences of the catalytic subunits fall into two main groups that have been termed the PPP family, of which the prototypic member is Ppp1c (PP1), and the PPM family, of which the prototypic member is Ppm1c (PP2C). The protein serine/threonine phosphatase that dephosphorylates the carboxy-terminal domain (CTD) of RNA polymerase II possesses a distinct amino acid sequence and thus represents the founding member of a third family, FCP (Table 1).

Background

The discovery that protein phosphatases regulate cellular functions originated from studies on the dephosphorylation of a single serine residue in glycogen phosphorylase [1,2]. Many different high-molecular-weight protein phosphatase activities dephosphorylating a variety of protein substrates were subsequently identified. A classification based on sensitivities to inhibitor proteins and activation by metal ions proposed that most known eukaryotic cytosolic phosphatase activities could be accounted for by four distinct types of protein phosphatase catalytic subunits: PP1, PP2A, PP2B, and PP2C [3]. Type 1 phosphatases (PP1 and its complexes) dephosphorylate the β subunit of phosphorylase kinase and are potently inhibited by inhibitor-1 and inhibitor-2. Type 2 protein phosphatases preferentially dephosphorylate the α subunit of phosphorylase kinase and are insensitive to the inhibitor proteins. PP2A is unaffected by metal ions, PP2B (found to be identical with a major calcium binding protein, calcineurin, isolated from brain [4]) is dependent on calcium ions and calmodulin, and PP2C is dependent on magnesium ions for activity. The primary structures for mammalian PP1 (GenBank Acc. Nos. X07798, X14832, X61639, M27071, M27073, D90163-D90166), PP2A (M16968, X06087, Y00763, M20192, M20193, J03804, X14159, X14087) and PP2B/calcineurin (J04134, M29551, D90035, D90036) deduced from the complementary DNA demonstrated that these catalytic subunits belong to the same family [5]. In contrast, analysis of cDNA encoding PP2C (JO4503, S87757, S87759, S90449) and pyruvate dehydrogenase phosphatase (L18966) revealed an unrelated amino acid sequence [6]. These studies therefore identified two distinct gene families encoding the protein serine/threonine phosphatases: the PPP family, which includes PP1, PP2A, and PP2B, and the PPM (Mg^{2+}-dependent protein phosphatase) family, for which PP2C is the founding member.

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Table I Families of Protein Serine/Threonine Phosphatases

Family	PPP	PPM	FCP
Signature motifs	–GDxHG– –GDxVDRG– –GNHE–	–ED– –DG– –DG–	–Lx(I/V/L)xLxxx(L/I)(V/I)H– –RPxxxxF–
Active site	Bimetal (Fe^{3+} + Zn^{2+} in native PPP3)	Bimetal (both Mn^{2+} in expressed Ppm1)	Not determined
Catalytic mechanism	Metal-ion-catalyzed dephosphorylation	Metal-ion-catalyzed dephosphorylation	Not determined
Other characteristics	Some active in absence of metal ions, others activated by Ca^{2+}; some members inhibited by naturally occurring toxins	Dependent on Mg^{2+} or Mn^{2+} for activity where tested; some members are also activated by Ca^{2+}	Dependent on Mg^{2+}, Mn^{2+} or Ca^{2+} for activity
Human genes	≥13	≥9	≥5
Common names of some members	Ppp1c/protein phosphatase 1/ PP1 Ppp2c/protein phosphatase 2A/ PP2A Ppp3c/protein phosphatase 2B/ PP2B calcineurin/ Ca^{2+}-calmodulin-regulated protein phosphatase	Ppm1/protein phosphatase 2C Ppm2/pyruvate dehydrogenase phosphatase *S. cerevisiae* PTC *A. thaliana* KAPP and ABII *B. subtilus* SpoIIE	RNA polymerase II CTD phosphatase
Evolution	Present in eukayotes and some prokaryotes	Present in eukaryotes and some prokaryotes	Present in lower and higher eukaryotes
Proteins with a similar domain that are not known to have protein Ser/Thr phosphatase activity	Diadenosine tetraphosphatase (*E. coli*) and related phosphatases (Purple acid phosphatases have weak sequence similarities to PPPs)	N-terminal domain of adenyl cyclase (*S. cerevisiae*) TAK-1 binding protein	Not determined

Note: The nomenclature for the human genes and encoded proteins of the PPP and PPM families proposed by a committee at the FASEB Conference on Protein Phosphatases in 1992 is used in Tables1 and 2 [28]. Other terms and synonyms in current usage for the proteins are also included. The PPP family are reviewed in [21,29,30] and the FCP family described in [31,32]. For the PPM (PP2C) family (see section by Russell).

Evolution and Conserved Features of the PPP Family

Members of the PPP family of protein phosphatases are widely distributed in all eukaryotic phyla (Fig. 1). Although initially believed to be absent from prokaryotes, a truncated PPP domain with protein serine phosphatase activity was detected in bacteriophages [7,8], cyanobacteria, and other (but not all) eubacteria [9–11], and an entire PPP domain was discovered in archeabacteria [12] (Fig. 2). These studies indicated that PPPs were present before the divergence of prokaryotes and eukaryotes, although acquisition of some PPPs by horizontal gene transfer to prokaryotes cannot be entirely ruled out.

The PPP catalytic domain, which spans ≈270 amino acids in eukaryotes, possesses the signature motifs –GDxHG–, –GDx(V/I)DRG–, and –GNHE–, which are virtually invariant among prokaryotic and eukaryotic protein phosphatases and are located in the amino terminal half of the molecules (Fig. 2). These invariant residues play crucial roles in binding divalent metal ions (probably Fe^{2+} and Zn^{2+} in the native enzymes), which are located at the catalytic center. They also interact with the phosphate group of the substrate and are considered to be the essential motifs for the phosphomonoesterase activity. The carboxy-terminal half of the eukaryotic catalytic domain contains four other motifs (–HGG–, –WxD–, –RG–, and –RxH–) that are virtually invariant among eukaryotes and archeabacteria [10,13] and are mainly involved in further interactions with one of the metal ions (see Chapter 100 for the crystal structures of Ppp1 and Ppp3). The –SAxNY– motif, invariant in eukaryotes but absent from prokaryotes, including archaebacteria, is in a flexible loop region that has been implicated in the binding of toxins and inhibitor-2 to the eukaryotic PPPs.

Catalytic Activities of the PPP Family Members

PPPs dephosphorylate phosphoserine and phosphothreonine residues in proteins *in vivo* and *in vitro*. Mammalian PP1, PP2A, and PP2C have also been shown to dephosphorylate histidine residues in proteins *in vitro* [14]. Mammalian PPPs expressed in *Escherichia coli* display properties that are slightly different from the native enzymes, including dependence or partial dependence on divalent metal ions such as Mn^{2+} or Mg^{2+} [15]. Bacterially expressed mammalian Ppp1c also exhibits protein-phosphotyrosine activity, in addition to serine/threonine phosphatase activities, but this disappears when the enzyme is refolded in the presence

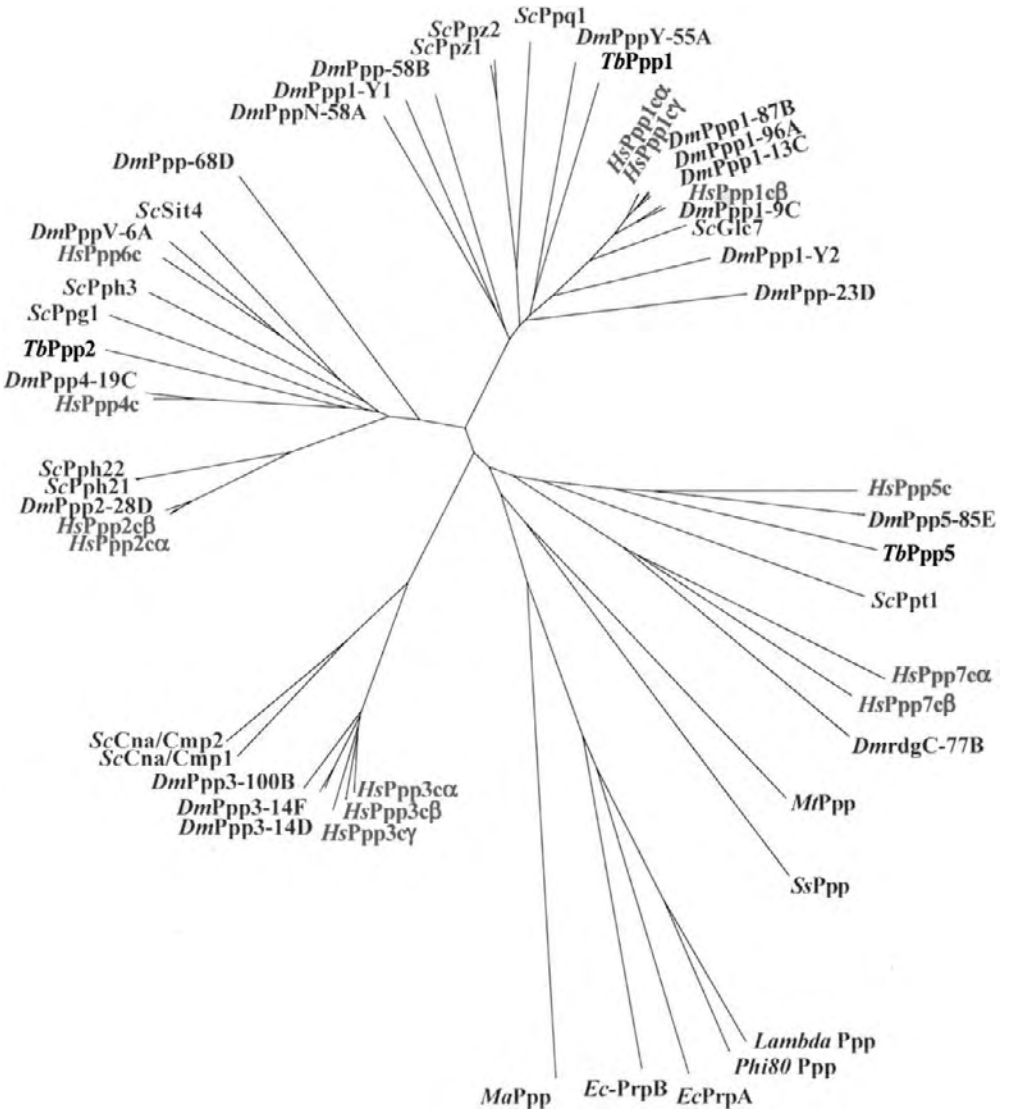


Figure 1 Phylogenetic tree, depicting the relationships between *Homo sapiens*, *Drosophila melanogaster*, budding yeast *Saccharomyces cerevisiae*, and prokaryotic protein serine/threonine phosphatases in the PPP family. Prokaryotes include archaebacteria *Methanosarcina thermophila* and *Sulfolobus solfataricus*, eubacteria *Escherichia coli* and *Microcystis aeruginosa* (cyanobacterium), and bacteriophages Lambda and Phi80. The chromosomal location is included for the *Drosophila* PPPs. The unrooted tree is derived from multiple alignment in CLUSTALW (http://www.clustalw.genome.ad.jp/) of the phosphatase catalytic domain (starting ≈30 amino acids prior to the invariant GDXHG motif and terminating ≈30 amino acids following the conserved SAXNY motif [13]). Note that the eubacterial and bacteriophage catalytic domains are only homologous to the N-terminal half of the eukaryotic and archeabacterial catalytic domains. Ppp1c and Ppp2c show approximately 40% sequence identity, isoforms of Ppp1c show >85% sequence identity, and novel *Drosophila* phosphatases in the Ppp1 subfamily show <65% sequence identity. Similar values occur in the PPP2A subfamily. Genbank accession numbers for human PPP sequences are Ppp1cα (J04759/M63960), Ppp1cβ (X80910), PPpp1cγ (X74008), Ppp2cα (J03804), Ppp2cβ (X12656), Ppp4c(X70218), Ppp6c (X92972), Ppp3cα (L14778), Ppp3cβ (M29551), Ppp3cγ (S46622), Ppp5c (X89416), Ppp7cα (X97867), and Ppp7cβ (AF023456). *Drosophila* PPP sequences are Ppp1-13C (X69974), Ppp1-87B (X15583), Ppp1-96A (X56438), Ppp1-9C (X56439), Ppp1Y2 (AF427494), PppY-55A (Y07510), Ppp-23D (AE003581), Ppp-58B (AE003456), Ppp1Y1 (AF427493), PppN-58A (Y17355), Ppp2-28D (X78577), Ppp4-19C (Y14213), PppV-6A (X75980), Ppp-68D (AY058490), Ppp3- 100B(M97012), Ppp3-14F (AE003502), Ppp3-14D (X77768), Ppp5-85E (AJ271781), and rdgC-77B(M89628). *S. cerevisiae* PPP sequences are Glc7 (M27070), Ppz1 (X74135), Ppz2 (X74136), Ppq1 (X75485), Pph21 (X56261), Pph22 (X56262), Pph3 (X58858), Sit4 (M24395), Ppg1 (M94269), Can/Cmp1 (M64839), Can/Cmp2 (M64840), and Ppt1 (X89417); *Trypanosoma brucei* Ppp1(X52746), *T. brucei* Ppp2 (M74168), *T. brucei* Ppp5 (AF305085); *M. thermophila* Ppp (U96772); *S. solfataricus* Ppp(U35278); *M. aeruginosa* Ppp (U80886); *Escherichia coli* PrpA(U51991), *E. coli* PrpB (U29579); lambda Ppp (J02459); Phi80 Ppp [7]. (A color version of this figure can be viewed in the CD edition of *Handbook of Cell Signaling, v. 1.*)

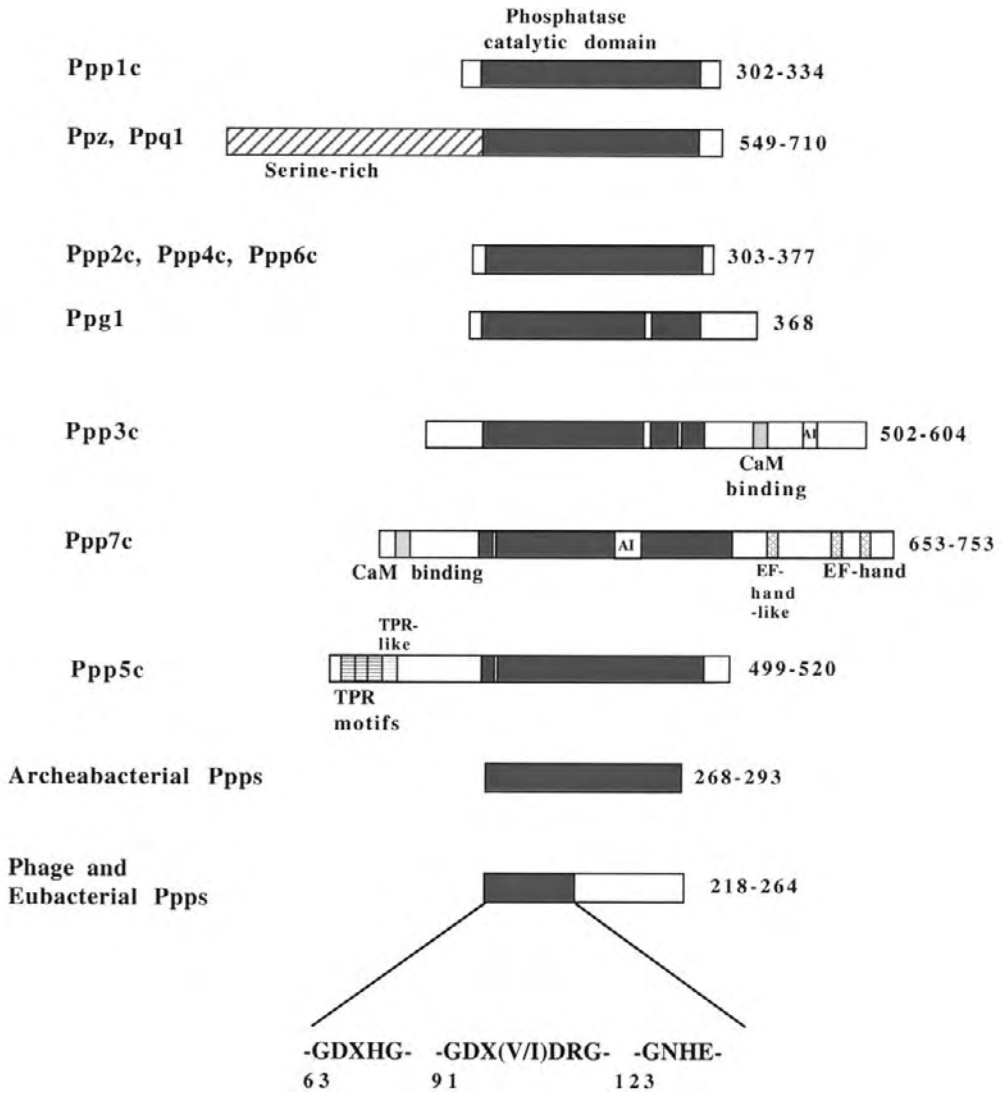


Figure 2 Domain organization of protein phosphatases in the PPP family. Bacteriophage and eubacteria Ppps show homology to the eukaryotic and archeabacterial protein phosphatases in the amino-terminal half of the catalytic domain, but the carboxy-terminal halves show little similarity. Autoinhibitory regions (AIs), Ca^{2+}-binding EF hands, tetratricopeptide repeats (TPRs), and calmodulin (CaM) binding sites are indicated. The numbers of amino acids in different phosphatases with each type of structure are given on the right. The three invariant amino acid motifs found in all PPP family members are shown at the bottom of the figure (numbers refer to the amino acid position in $PP1\alpha_1/PP1\gamma_1$).

of inhibitor-2, indicating that it is unlikely to be a property of the native enzyme *in vivo* [16]. Ppp2 (PP2A) exhibits some protein tyrosine phosphatase activity in the presence of the protein PTPA [17]. Bacterially expressed phage lambda Ppp exhibits protein-serine, -threonine, -histidine, and -tyrosine phosphatase activities [18], while native cyanobacterial PPP exhibits protein-serine, -tyrosine, -histidine, and -lysine phosphatase activities *in vitro* [11]. Thus, the activities of some Ppps may be wider than dephosphorylation of protein-bound phosphoserine and phosphothreonine residues. The discovery that the diadenosine tetraphosphatases have sequence similarities to the PPP family provides evidence that the essential PPP signature motifs for phosphomonoesterase activity have been utilized for the hydrolysis of biomolecules other than protein-bound phosphoesters [13].

PPPs dephosphorylate serine and threonine residues that lie in unrelated amino acid sequences, suggesting that, in contrast to many protein kinases, specificity is not determined primarily by the linear sequence of amino acids of either side of the phosphorylated residue. Rather, these observations suggest that higher order structures and/or interaction with regulatory subunits are involved [19].

Eukaryotic PPP Subfamilies

In eukaryotes, the PPP family was found to include four different subfamilies by comparison of the amino acid sequences of the catalytic domains: PPP1, PPP2, PPP3, and PPP5/PPP7, although the PPP5 and PPP7 groups are only

slightly more closely related to each other than to other phosphatase subfamilies (Fig. 1). The PPP2 subfamily includes recognizable subgroups PPP2, PPP4, and PPP6. Prokaryotic Ppps are highly divergent from eukaryotic Ppps but show the most similarities to members of the PP5/PPP7 subfamily.

Virtually all eukaryotes possess members of the PPP subgroups 1 to 7, an exception being Ppp3c, which does not appear to have been identified in plants. In addition, some protozoan species may not have representatives of all family members. Twelve PPP family members are known in *Saccharomyces cerevisiae* and 13 in the human species [20–22]. Somewhat surprisingly, sequencing of the human genome does not appear to have uncovered any more readily identifiable PPP members, indicating that there has not been a significant increase in the number of PPP catalytic subunits from yeast to humans. Interestingly, *Drosophila* possesses 19 PPP family members. Two are located on the Y chromosome (Ppp1-Y1 and Ppp1-Y2) and at least two others are related to male-specific functions (PppY-55A and Ppp N-58A, all of which appear to lack homologs in *Homo sapiens*. Some Ppps in *S. cerevisiae* (Ppq1, Ppz1, Ppz2, Ppg1) also have no homologs in humans. Thus, it is possible that gene duplication within different eukaryotes may be a mechanism used to produce regulation by Ser/Thr dephosphorylation of processes specific to particular organisms. In plants, at least 8 Ppp1c isoforms have been produced by gene duplication [23]. Most mammalian PPP members in the Ppp1-6 subgroups are present in all tissues that have been examined, but Ppp7c is restricted to retina and brain. The functions regulated by PPPs are extremely diverse (Table 2).

Domain and Subunit Structure of PPP Family Members

Many protein serine/threonine phosphatases in the PPP family are high-molecular-weight complexes containing one or more regulatory subunits. A large number of different regulatory subunits bind in a mutually exclusive manner to the Ppp1c catalytic subunit [24,25] or the Ppp2(PP2A) core complex of the catalytic subunit and a regulatory subunit [17]. These interactions allow a single PPP catalytic subunit to participate in many different cellular functions. Details of these PPP regulatory subunits and the functions they confer on their catalytic subunit are discussed in other chapters.

Although Ppp1c (PP1) and Ppp2c (PP2A) possess only very short amino- and carboxy-terminal regions outside the catalytic region, other PPP family members may possess fused amino- and carboxy-terminal domains that, at least in some cases, impart distinct properties to the catalytic domain. Thus, Ppp3c (PP2B, calcineurin) has a carboxy-terminal domain that allows the enzyme to be activated by Ca^{2+}/calmodulin, as well as an autoinhibitory pseudosubstrate domain [26]. Ppp7c has EF hand sequences that confer Ca^{2+} sensitivity and a calmodulin-binding motif that is distinct from that of Ppp3c [22]. PP5 has an inhibitory amino terminal domain containing three tetratricopeptide repeats (TPRs) that are likely to allow interaction with other proteins [21,27].

The amino acid sequences of Ppp1c and Ppp2c have been extremely conserved throughout the evolution of multicellular eukaryotes, and these enzymes are among the most slowly evolving proteins known. This may be because they interact with a wide variety of regulatory proteins and also because of

Table II Structures and Properties of the Human PPP Catalytic Subunits

Human gene name	Chromosomal location	Protein name	Number of amino acids[a]	Fused regulatory domain	Regulatory subunits	Subunit structure of complexes	Activators/ *inhibitors*	Functions regulated
PPP1CA	11q13	Ppp1cα/PP1α	α_1 330; α_2 341	No	>50 different regulatory subunits	Mainly heterodimeric, some heterotrimeric species	*Inhibitor-1* *Inhibitor-2* *Okadaic acid* *Microcystin* *Tautomycin*	Many, diverse; determined by variable regulatory subunit (see Chapter 102 references [24] and [25])
PPP1CB	2p23	Ppp1cβ/ PP1β/PP1δ	β 327					
PPP1CC	12q24	Ppp1cγ/PP1γ PP1cγ	$\gamma_1$323; γ_2 337					
PPP2CA	5q23-q31	Ppp2cα/PP2-Aα PP2Acα	α 309	No	Core regulatory PPPR1(A) + ≥14 different PPP2R2 (B) regulatory subunits OR α 4	Mainly heterotrimeric, some heterodimeric species	*Okadaic acid* *Microcystin*	Many, diverse; determined by variable regulatory subunit (see chapter reference [17])
PPP2CB	8p12-p11	Ppp2cβ/ PP2Aβ PP2Acβ	β 309					
PPP4C	16p 12-p11	Ppp4c/PP4/ PPX	307	No	PPP4R1, PPP4R2 α4	Heterodimeric + higher molecular mass structures	*Okadaic acid* *Microcystin*	Organization of microtubules at centrosomes [33–35]; signaling pathways (?) [36,37]

(continues)

(continued)

Human gene name	Chromosomal location	Protein name	Number of amino acids[a]	Fused regulatory domain	Regulatory subunits	Subunit structure of complexes	Activators/ *inhibitors*	Functions regulated
PPP6C	9q34	Ppp6c/PP6	305	No	α 4 (TAP42, SAP190, SAP185, SAP155, SAP4, in. *S cerevisiae*)	(Heterodimeric in *S. cerevisiae*)	(*Okadaic acid* in *S. cerevisiae*)	G1/S transition, cell shape regulation, translation initiation in *S. cerevisiae*) [38–41]
PPP3CA	4q21-q24	Ppp3cα/ PP2Bα/ CNAα/ CALNA	α_1 521; α_2 511	Calmodulin binding + autoinhi-	19 kDa Ca^{2+} binding PPP3R1(B1)	Heterodimer that interacts with	Ca^{2+}/calmodulin *FKBP12-FK506*	T-cell signaling; neurotransmitter release;
PPP3CB	10q21-q22	Ppp3cβ/PP2Bβ/ CNAβ/ CALNB	β_1 514/515; β_2 524/525; β_3 515	bitory regulatory domains	subunit	calmodulin	*Cyclosporin/ cyclophilin*	neuroreceptor-coupled Ca^{2+} channels (see Chapter 105)
PPP3CC	8p21	Ppp3cγ/PP2Bγ/ CNAγ	γ 502		19 kDa Ca^{2+}- –binding PPP3R2 (B2) subunit	Heterodimer? —		Unknown, specific to testis
PPP5C	19q13	Ppp5c/PP5/ PPT	499	TPR domain	None reported	Monomeric	*Okadaic acid microcystin*	Poorly understood; regulation of cell growth and multiple signaling pathways (?) [21,42–44]
PPP7 CA[b]	Xp22	Ppp7cα/ PPEF1	α 653	Calmodulin binding + Ca^{2+}	None reported	Monomeric? Interacts with calmodulin	Ca^{2+}/ calmodulin	Specific to retina and certain brain regions; dephosphorylation of rhodopsin in *Drosophila* [22,45,46]
PPP7-CB[b]	4q21	Ppp7cβ/ PPEF2	β_1 598; β_2 753	-binding EF hand domains				

[a]Two different values for the number of amino acids refers to splice variants; Ppp1cα X70484/S57501; Ppp1cγ, X74008; Ppp3cα, L14778; Ppp3cβ, M29551 (McPartlin *et al.*, 1991); Ppp7cβ, AF023456/AF023457.

[b]The names PPP7CA (AF027977) and PPP7CB are used here for genes referred to as PPEF1 and PPEF2 in some databases.

their many crucial roles in cellular processes, such as the cell cycle. The structural conservation of these phosphatases may explain why they have become targets of so many structurally distinct toxins.

Medical Importance of the PPP Family

The discovery that eukaryotic Ppp1 and Ppp2 are potently inhibited by naturally occurring toxins and tumor promoters, such as okadaic acid and microcystin, brought this group of enzymes to the attention of the general public, and the importance of protein serine/threonine phosphatases to the study of medicine was highlighted when it was discovered that Ppp3c (PP2B /calcinceurin) was the target of the widely used immunosuppressive drugs cyclosporin and FK506 (see Chapter 101). In addition, DNA tumor viruses (SV40 and polyoma virus) and HIV-1 have been shown to compromise the function of PP2A by producing proteins that compete with specific regulatory subunits [17]. Somewhat similarly, the dsRNA virus herpes simplex produces PP1-binding proteins that recruit Ppp1c from host cell complexes in order to enhance its own replication and evade host cell defense mechanisms [25].

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CHAPTER 101

The Structure and Topology of Protein Serine/Threonine Phosphatases

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Introduction

Structural studies of the two families of protein phosphatases responsible for dephosphorylating serine and threonine residues have revealed that, although these families are unrelated in sequence, the architecture of their catalytic domains is remarkably similar and distinct from the protein tyrosine phosphatases. The diversity of structure within the PPP and PPM families is generated by regulatory subunits and domains that function to modulate protein specificity and to localize the phosphatase to particular subcellular locations [1].

Protein Serine/Threonine Phosphatases of the PPP Family

The protein Ser/Thr phosphatases PP1, PP2A, and PP2B of the PPP family, together with PP2C of the PPM family, account for the majority of the protein serine/threonine phosphatase activity *in vivo*. While PP1, PP2A, and PP2B share a common catalytic domain of 280 residues, these enzymes are divergent within their noncatalytic N and C termini and are distinguished by their associated regulatory subunits to form a diverse variety of holoenzymes. Major members of the PPP family are encoded by numerous isoforms that share a high degree of sequence similarity, especially within their catalytic domains. Greater sequence diversity occurs within the extreme N and C termini of the proteins. Although these isoforms have similar substrate specificities and interact with the same regulatory subunits *in vitro*, the phenotype of a functional loss is isoform specific, indicating they perform distinct functions *in vivo*.

Overall Structure and Catalytic Mechanism

Structural analyses of members of the PPP family have begun to reveal the molecular basis for catalysis, inhibition by toxins, and aspects of their regulatory mechanisms. Crystal structures are available for (1) various isoforms of PP1 in complex with natural toxins [2,3], the phosphate mimic tungstate [4], and a peptide of the RVxF targeting motif [5]; (2) PP2B in the auto-inhibited state [6] and as a complex with FK506/FKBP [6,7]; (3) the PR65/A-subunit of PP2A [8]; and (4) a regulatory TPR domain of PP5 [9]. The catalytic domains of the PP1 catalytic subunit (PP1c) and PP2B share a common architecture consisting of a central β-sandwich of two mixed β-sheets surrounded on one side by seven α-helices and on the other by a subdomain comprised of three α-helices and a three-stranded β-sheet (Fig. 1A). The interface of the three β-sheets at the top of the β-sandwich creates a shallow catalytic site channel. Conserved amino acid residues present on loops emanating from the β-strands of this central β-sandwich are responsible for coordinating a pair of

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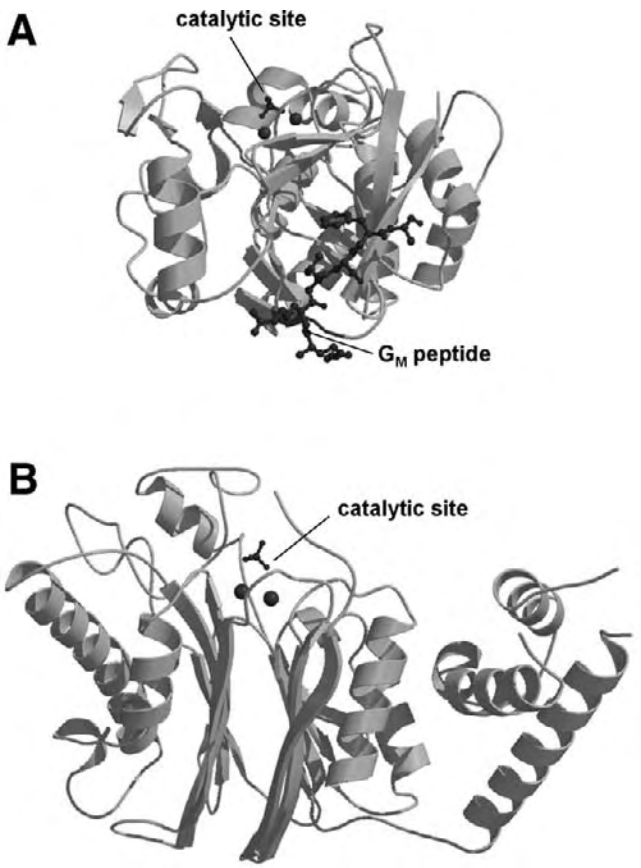


Figure 1 Ribbon diagrams showing an overview of the structures of protein Ser/Thr phosphatase catalytic domains. (A) The catalytic subunit of human protein phosphatase 1γ in complex with the RVxF motif PP1 binding peptide. The catalytic site is indicated by the two metal ions and bound sulfate ion. (From Egloff, M. P. *et al.*, *EMBO J.*, 16, 1876–1887, 1997. With permission.) (B) Human PP2Cα the catalytic site is indicated by the two metal ions and bound phosphate ion. (From Das, A. K. *et al.*, *EMBO J.*, 15, 6798–6809, 1996. With permission.)

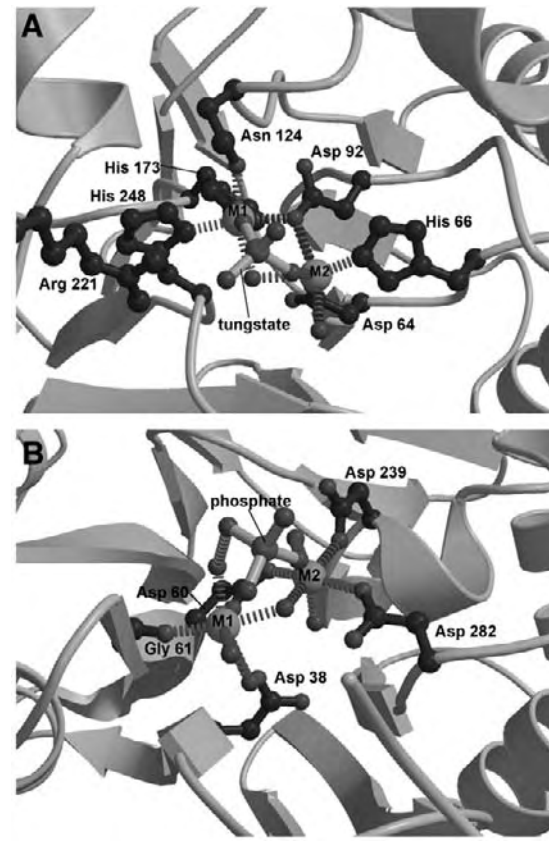


Figure 2 Comparison of the catalytic sites of the protein Ser/Thr phosphatases, showing the common binuclear metal center, coordinating the phosphate of the phosphorylated protein substrate: (A) human PP1γ (for clarity Arg 96 is not shown) and (B) human PP2Cα.

metal ions to form a binuclear metal centre (Fig. 2A). Crystallographic data on PP1c and PP2B provided the first compelling insight regarding the role of metal ions in the catalytic reaction of the PPP family. The identity of the two metal ions is slightly controversial. Proton-induced X-ray emission spectroscopy performed on PP1c crystals produced from the protein expressed in *Escherichia coli* indicated that the metal ions were Fe^{2+} (or Fe^{3+}) and Mn^{2+} [4], whereas atomic absorption spectroscopy of bovine brain PP2B indicated a stoichiometric ratio of Zn^{2+} and iron [10]. There have also been conflicting reports concerning the iron oxidation states, with both Fe^{2+} and Fe^{3+} being observed. Native PP2B is most likely to contain Fe^{2+}, explaining the time-dependent inactivation of PP2B that results from the oxidation of the Fe–Zn center [11]. Oxidation of the binuclear metal center and phosphatase inactivation may represent a mechanism for PP2B regulation by redox potential during oxidative stress or as a result of reactive oxygen species generation following receptor tyrosine kinase activation, in a process reminiscent of the inactivation of protein tyrosine phosphatases by oxidation of the catalytic site Cys residue by hydrogen peroxide and perhaps analogous to a possible PP2C regulatory mechanism, discussed later [10].

The structure of PP1c with tungstate and PP2B with phosphate indicated that two oxygen atoms of the oxyanion-substrate coordinate the metal ions (Fig. 2A) [4,7]. Two water molecules, one of which is a metal-bridging water molecule, contribute to the octahedral hexa-coordination of the metal ions. The metal coordinating residues (aspartates, histidines, and asparagines) are invariant among all PPP family members. These residues, together with Arg and His residues that interact with the phosphate group of the phosphorylated residue, occur within five conserved sequence motifs found in other enzymes, including the purple acid phosphatase, whose common function is to catalyze phosphoryl transfer reactions to water [12]. These observations suggest that PPP and purple acid phosphatases evolved by divergent evolution from an ancestral metallophosphoesterase. Consistent with roles in catalysis, mutation of these residues either eliminates or profoundly reduces catalytic activity.

PPPs catalyze dephosphorylation in a single step with a metal-activated water molecule or hydroxide ion. The most convincing evidence for this notion is that the purple acid phosphatase, which is generally related in structure to the PPPs at both the tertiary level and at the catalytic site [13], promote dephosphorylation with inversion of configuration

of the oxygen geometry of the phosphate ion [14]. This indicates that a phosphoryl-enzyme intermediate would not occur. The two metal-bound water molecules are within van der Waals distance of the phosphorous atom of the phosphate bound to the catalytic site, and one of them is likely to be metal-activated nucleophile.

Interactions with Regulatory Subunits

PP1 and PP2A are responsible for regulating diverse cellular functions by dephosphorylating multiple and varied protein substrates. This seemingly paradoxical situation was resolved by the discovery that distinct forms of PP1 and PP2A holoenzymes occur *in vivo*, where essentially the same catalytic subunit is complexed to different targeting and regulatory subunits. For PP1 it has been shown that targeting subunits confer substrate specificity by directing particular PP1 holoenzymes to a subcellular location and by enhancing or suppressing activity toward different substrates. The control of PP1 holoenzyme structure and activity by the combinatorial selection of different targeting/regulatory subunits has recently been the subject of numerous reviews [15,16] and will not be discussed at length here, although the contrasting structural mechanism by which the conserved PP1 and PP2A catalytic subunits are able to form diverse holoenzyme structures will be discussed.

In the case of PP1, it is known that the binding of targeting subunits to its catalytic subunit is mutually exclusive, suggesting that there are one or more common or overlapping binding sites, recognized by all PP1-binding subunits. It is therefore a little surprising that PP1-binding subunits are highly diverse structurally and share little to no overall sequence similarities. The key to understanding this paradox came from the crystal structure of PP1c in complex with a short, 13-residue peptide derived from a region of the PP1-glycogen targeting subunit (G_M) responsible for PP1c interactions (Fig. 1A). This structure showed that the peptide associated with the phosphatase via two hydrophobic residues (Val and Phe), which engage a hydrophobic groove on the protein surface formed from the interface of the two β-sheets of the central β-sandwich and remote from the catalytic site [5]. Two basic residues of the peptide immediately N-terminal to the Val residue form salt-bridge interactions with Asp and Glu residues at one end of the peptide binding channel. Alanine substitutions of either the Val or Phe residues of the peptide abolish PP1–peptide interactions. Analysis of other PP1-binding subunit sequences revealed the presence of the identical or related sequence motif RRVxF, found to mediate the interactions between the G_M peptide and PP1c [5]. The role of the degenerative RVxF motif in mediating PP1-regulatory subunit interactions is now supported by numerous experimental observations. First, for various regulatory subunits, mutation of either hydrophobic residue of the motif in native proteins weakens or eliminates their association with PP1c. Second, peptides corresponding to the RVxF motif competitively disrupt the interactions of regulatory subunits with PP1c. Third, the use of a common or overlapping PP1c binding site explains why the interactions of regulatory subunits is mutually exclusive. The number of PP1 holoenzyme structures that could be generated in this manner is potentially infinite, and to date over a hundred PP1 regulatory subunits have been characterized.

The residues of PP1 that interact with the RVxF peptide are conserved in all isoforms of PP1 in all eukaryotic species, although not within PP2A and PP2B, explaining why PP1-binding subunits are unique to PP1. The mechanism of combinatorial control of PP2A holoenzyme structure is different from that of PP1 and is mediated by a scaffolding subunit termed the PR65/A subunit, which simultaneously associates with the PP2A catalytic subunit and a variable regulatory B subunit. The ability to recognize a variety of regulatory subunits (perhaps over 50) is conferred by the architecture of the PR65/A subunit, which consists of 15 tandem repeats of a 39-amino-acid sequence termed the HEAT motif and related in structure to ARM repeats. These repeats assemble to create an extended molecule ideally suited for mediating protein–protein interactions [8]. Combinatorial generation of variable PP2A holoenzymes is achieved by the ability of different combinations of HEAT motifs to select different regulatory B subunits [8].

Interactions of Natural Toxins with PP1

PP1 and PP2A are specifically and potently inhibited by a variety of naturally occurring toxins such as okadaic acid, a diarrhetic shellfish poison and powerful tumor promoter, and microcystin, a liver toxin produced by blue-green algae [17]. Whereas PP2B is only poorly inhibited by the toxins that affect PP1 and PP2A, it is known to be the immunosuppressive target of FK506 and cyclosporin in association with their major cellular binding proteins, the *cis-trans* peptidyl prolyl isomerase FKBP12 and cyclophilin, respectively [18]. The mechanism of inhibition of PP1 by okadaic acid and microcystin LR have been defined by structures of PP1 in complex with these inhibitors. Both inhibitors, although structurally different, bind to a similar region of the phosphatase, occupying the catalytic channel to directly block phosphatase–substrate interactions. Regions of PP1 that contact the toxins include the hydrophobic groove and the β12/β13 loop, the latter undergoing conformational changes to optimize contacts with microcystin [2,3]. Both toxins disrupt substrate–phosphatase interactions by competing for sites on the protein that coordinate the phosphate group of the substrate. For example, carboxylate and carbonyl groups of microcystin interact with two of the metal-bound water molecules [2], whereas okadaic acid contacts the two phosphate-coordinating arginine residues (Arg-96 and Arg-221) [3]. A similar mechanism of phosphatase inhibition by steric hindrance of substrate binding is observed in the structure of the full-length PP2B holoenzyme, for which the autoinhibitory domain lies over the substrate binding channel of the catalytic domain in such a way that a Glu side chain accepts a hydrogen bond from two of the metal-bound water molecules [6].

Protein Serine/Threonine Phosphatases of the PPM Family

Protein phosphatases of the PPM family are present in both eukaryotes and prokaryotes for which the defining member is PP2C. Biochemically, the PPM family was distinguished from the PPP family by its requirement for divalent metal ions (Mg^{2+}) for catalytic activity, although it is now known from crystal structures that both PPP and PPM phosphatases catalyze dephosphorylation reactions by means of a binuclear divalent metal center. Within the PPM family, the PP2C domain occurs in numerous structural contexts that reflect structural diversity [19]. For example, the PP2C domain of the *Arabidopsis* ABI1 gene is fused with EF hand motifs, whereas in KAPP-1, a kinase interaction domain associated with a phosphorylated receptor precedes the phosphatase domain. Other less closely related examples include the Ca^{2+} stimulated mitochondrial pyruvate dehydrogenase phosphatase, which contains a catalytic subunit sharing 22% sequence identity with that of mammalian PP2C, and the SpoIIE phosphatase of *Bacillus subtilus*, which has ten membrane-spanning regions preceding the PP2C-like catalytic domain. A surprising homolog is a 300-residue region of yeast adenylyl cyclase present immediately N-terminus to the cyclase catalytic domain that shares sequence similarity with PP2C. This domain may function to mediate Ras-GTP activation of adenylyl cyclase activity and is not known to possess protein phosphatase activity. In eukaryotes, the various isoforms of PP2C have been implicated in diverse functions such as regulation of cell-cycle progression mediated by dephosphorylation of CDKs [20], to regulation of RNA splicing [21], control of p53 activity [22], and regulation of stress response pathways in yeast [23].

The sequences of protein phosphatases of the PPM family share no similarity with those of the PPP family, and the natural toxins that inhibit the PPP family have no affect on PPM family phosphatases. It therefore came as a surprise when the crystal structure of human PP2Cα revealed a striking similarity in tertiary structure and catalytic site architecture to the PPP protein phosphatases (Fig. 1B) [19]. Mammalian PP2C consists of two domains; an N-terminal catalytic domain common to all members of the PP2C family is fused to a 90-residue C-terminal domain, unique to the mammalian PP2Cs [19]. The catalytic domain is dominated by a central, buried β-sandwich of 11 β-strands formed by the association of two antiparallel β-sheets, both of which are flanked by a pair of antiparallel α-helices inserted between the two central β-strands, with four additional α-helices. The C-terminal domain is formed from three antiparallel α-helices remote from the catalytic site, suggesting a role in defining substrate specificity rather than catalysis.

At the catalytic site of PP2C, two Mn^{2+} ions, separated by 4 Å, form a binuclear metal center and are coordinated by four invariant aspartate residues and a nonconserved Glu residue (Fig. 2B) [19]. These residues are situated at the top of the central β-sandwich that forms a shallow channel suitable for the dephosphorylation of phosphoserine- and phosphothreonine-containing proteins. Six water molecules coordinate the two metal ions. One of these water molecules bridges the two metal ions and four form hydrogen bonds to a phosphate ion at the catalytic site. Dephosphorylation is probably catalyzed by a metal-activated water molecule that acts as nucleophile in a mechanism similar to that proposed for the PPP family. A recent kinetic analysis of PP2Cα by Denu and colleagues [24] indicated that Mn^{2+} and Fe^{2+} are the most effective divalent metal ions in promoting dephosphorylation reactions, suggesting that at least one of these ions must be present at the catalytic site. In contrast, Zn^{2+} and Ca^{2+} competitively inhibit PP2C Mn^{2+}-dependent activity. An Fe^{2+}-containing catalytic site would be analogous to the PPP protein phosphatases and possibly explains the H_2O_2-mediated inactivation of PP2C [25] consequent on the redox sensitivity of Fe^{2+} and its oxidation to the inactive Fe^{3+} valence state [24]. The finding that an ionizable group with a pKa of 7.0 has to be deprotonated for catalysis was fully consistent with the notion from the crystal structure that a metal activated water molecule acts as a nucleophile [19,24]. Fluoride has long been used as an inhibitor of both PPP and PPM phosphatases, and the rationale for this inhibition is now clear from the catalytic mechanism of serine/threonine phosphatases revealed by the crystal structures. By substituting for the metal-bound nucleophilic water molecule, fluoride prevents metal-activated nucleophilic attack on the phospho-protein substrate. Substitution of Ala for the Asp residues of the catalytic site in yeast and plant PP2C homologs and in the related phosphatase SpoIIE from *B. subtilus* abolishes catalytic activity, supporting a role for the metal ions in catalysis [26,27].

Conclusions

Although the PPP and PPM phosphatases share a similar tertiary structure, characterized by a central β-sandwich and flanking α-helices, with related catalytic site architectures and mechanisms, two observations suggest that these protein families have evolved from distinct ancestors. First, the secondary structure topology of the PPP and PPM families are unrelated and there is no simple rearrangement of chain connectivities that would allow the PPP topology to be transformed into the PPM topology. Second, the conserved sequence motifs of the PPP family required for metal binding and catalysis, typical of some metallophosphoesterases, are distinct from the conserved sequence motifs of the PPM family. The comparison of the PPP and PPM families of Ser/Thr protein phosphatases provides interesting contrasts with the protein phosphatases of the $C(X)_5R$-motif superfamily composed of three distinct gene families: (1) the conventional protein tyrosine phosphatases (PTPs), including dual-specificity phosphatases and PTEN lipid phosphatases; (2) low-molecular-weight protein tyrosine phosphatases (lmPTPs); and (3) Cdc25 dual-specificity phosphatases [28]. These proteins

share a similar overall tertiary structure composed of a central β-sheet surrounded on both sides by α-helices which results in an identical catalytic site architecture in all three families characterized by the nucleophilic Cys-residue located within a phosphate binding cradle. What distinguishes the three protein families are differences in their secondary structure topology; however, the topologies of the PTP and lmPTP families are related by a simple permutation of secondary structure connectivity, suggesting that these families may have evolved from the same ancestor and diverged as a result of exon shuffling events. In recent years, much has been learned of the structural details of Ser/Thr protein phosphatases relating to their overall folds, catalytic mechanisms, and interactions with regulatory subunits and toxins. However, still elusive is the structure of a Ser/Thr protein phosphatase in complex with a phosphoprotein substrate that would explain the basis for the selectivity for Ser/Thr residues and reveal the mechanism of substrate selectivity by regulatory subunits. It is hoped that future studies of Ser/Thr phosphatase will address these questions.

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CHAPTER 102

Naturally Occurring Inhibitors of Protein Serine/Threonine Phosphatases

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Introduction

A variety of natural products (Fig. 1) operate in diverse ecological contexts as mating lures and defense toxins. Humans encounter them as killers, water and food contaminants, a diarhetic shellfish poison, an aphrodisiac, horse dope, suspect tumor promoters, valuable research reagents, and cures for mild afflictions and cancer, while microcystin was branded a potential bio-weapon in the recent U.K. Antiterrorism, Crime and Securities Act. The distinctive effects and associations of each toxin are attributable to its site of production, cell permeability, stability, abundance, and potency, for every one of these chemicals exerts its biological effects by binding tightly to active sites of protein serine/threonine phosphatases in the eukaryotic PPP family [1]. Considering how much effort and money are invested by pharmaceutical companies to find even one useful inhibitor of many signaling enzymes, the number and variety of very potent protein phosphatase inhibitors found in Nature is remarkable.

Effects of Inhibitors in Cell-Based Experiments

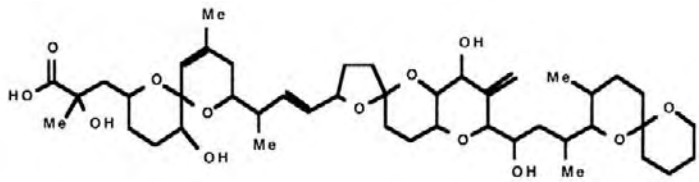
The myriad biological effects of these cell-permeable inhibitors have provided many compelling demonstrations that reversible phosphorylation of serine/threonine residues is an all-pervasive mechanism of cellular control. When protein phosphatases are inhibited, phosphate is trapped in phosphorylated substrates and accumulates if the relevant protein kinases are at least slightly active. Thus, use of protein phosphatase inhibitors in combination with protein kinase inhibitors and other effectors has provided clues about many signaling pathways. By way of example, protein phosphatase inhibitors cause sustained backward swimming of *Paramecium* in response to depolarizing stimuli (but only in the presence of external Ca^{2+} [2]), activate some and block other antifungal defense responses in plants [3], and promote apoptosis of mammalian cells [4]. Interestingly, fibroblasts that are resistant to okadaic acid-induced apoptosis have been selected from a population of cells expressing a human cDNA library which provides a novel approach to identifying those components of the apoptotic machinery for which phosphorylation is deregulated by the inhibitor [4].

Of course, the major limitation in using protein phosphatase inhibitors inside cells is that they inhibit many closely related enzymes, albeit with different relative potency (see Fig. 1), making dissection of cellular functions for each individual protein phosphatase difficult or impossible. The best we can do is to provisionally assign roles for PP1 when tautomycin has more potent effects than okadaic acid [5], or implicate PP2A, PP4, or PP5 when fostriecin is most effective [6].

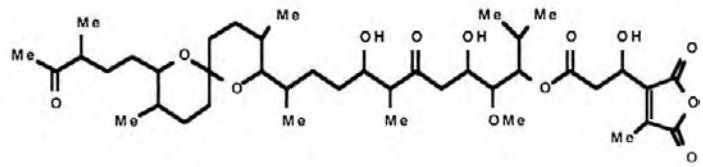
One way around the problem of discriminating among homologous family members might be to replace the natural

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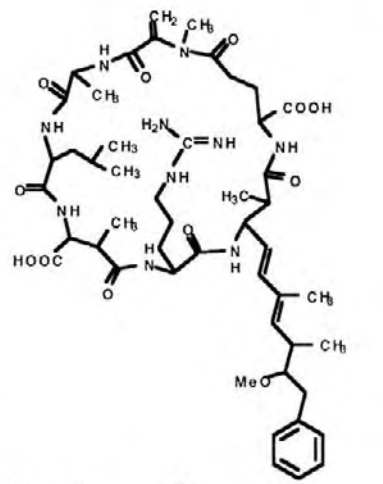
Okadaic acid
Diarrhetic shellfish poison, produced by dinoflagellates. Tumour promoter.
IC50's: PP2A=PP4<PP1.



Tautomycin
Produced by species of *Streptomyces*.
IC50's: PP1>> PP2A.

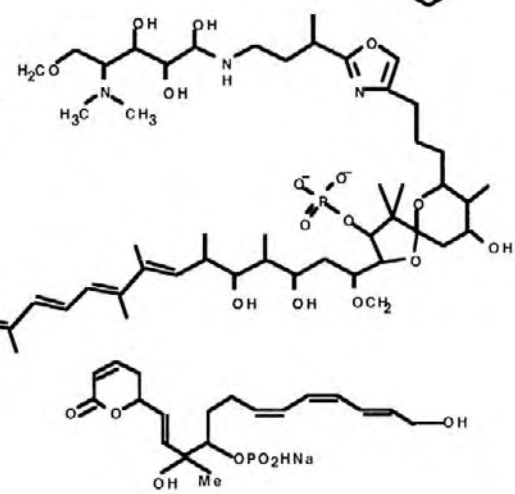


Microcystin-LR
Hepatotoxic cyclic heptapeptide from species of freshwater cyanobacteria. Suspect tumour promoter.
IC50's: PP2A [= or slightly <] PP1.



The nodularins are hepatotoxic pentapeptides from species of *Nodularia* cyanobacteria.
IC50's and toxicity of nodularins are similar to microcystins.

Calyculin A
Isolated from the marine sponge *Discodermia calyx*.
IC50's: PP1=PP2A.



Fostriecin
Produced by *Streptomyces pulveraceus*.
Has shown promise in treating cancers.
IC50's: PP2A=PP4<<<PP1.
Inhibits topoisomerase II at higher concentrations.

Cantharidin
Transferred as a mating 'gift' from male to female blister beetles. Horses killed by beetles in hay. Causes blistering and irritation, regarded by some as an aphrodisiac 'Spanish Fly' but has killed humans. Wart treatment. IC50's: PP2A<PP1.

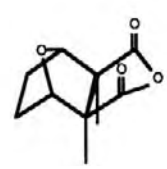


Figure 1 Naturally occurring inhibitors of PP1, PP2A, and related enzymes in the PPP family. None of these toxins is an effective inhibitor of PP2B (the rapamycin and FK506 drugs that target PP2B are not considered in this chapter). Representatives of each toxin are shown, although variants exist in Nature; for example, more than 60 congeners of microcystin exist. The toxins generally bind to the enzyme with nanomolar and subnanomolar affinities, except for cantharidin, which operates in the micromolar range.

enzymes in cells with forms that are more or less toxin sensitive. Another would be to use the natural toxins as "leads" for the synthesis of more specific inhibitors.

As well as soothing the frustrations of cell biologists, there are also medical reasons to seek specific inhibitors. Fostriecin is reported to confer an antitumor effect [7] at concentrations that probably inhibit PP2A, PP4, and PP1 in cells. In contrast, okadaic acid and microcystin are potent tumor promoters [8]. Which actions underpin the pro- and antitumor properties of these toxins? Can more specific cancer therapies with fewer side effects than fostriecin be designed?

The Toxins Bind to the Active Sites of Protein Phosphatases

The microcystins, nodularins, okadaic acid, and tautomycin adopt similar tadpole shapes in solution, and crystal structures show that okadaic acid and microcystin-LR bind to common residues in three distinct regions of the PP1 active site [9,10]. Carboxylates of both toxins interact directly with the hydrated metal ions in the substrate-phosphate binding center, while their rigid hydrophobic tails tuck into a hydrophobic groove that runs out from the active site. The third major contact is with the C-terminal β12–β13 loop, which protrudes over the catalytic center. When microcystin-LR binds, the β12–β13 loop is pulled closer into the active site, and the dehydroalanine residue of microcystin-LR forms a Michael adduct with Cys-273 in the β12–β13 loop of PP1γ [9,11,12]. This covalent bond is not essential, because neither reducing the dehydroalanine nor mutating Cys-273 has much impact on the inhibitory potency [12]. However, when the adjacent Tyr-272 is mutated or the enzyme truncated at the Ala-268 of the $_{267}$SAPYNYC$_{273}$ motif of the β12–β13 loop, the sensitivity to okadaic acid and microcystin-LR is lowered dramatically, although the phosphorylase phosphatase activity of the enzyme is unchanged [13,14].

Molecular modeling, binding kinetics, enzyme mutagenesis, and toxin modification studies suggest that the other inhibitors share the same binding site as okadaic acid and microcystins but depend on contacts with the common residues to different extents. Thus, unlike okadaic and microcystin-LR, fostriecin binds equally well to intact PP1C and the enzyme with an incomplete β12–β13 loop [13,14]. Paradoxically, however, when yeast PP2ACα was mutated within its predicted β12–β13 loop, binding to both fostriecin and okadaic acid was impaired [6]. Perhaps fostriecin forms a bridge between the catalytic center and the β12–β13 loop in PP2A but cannot find complementary contacts in the β12–β13 loop of PP1 [6]. This scenario might explain the much higher potency of fostriecin for PP2A and PP4, compared with PP1.

Dissecting further details of contacts at the catalytic center and β12–β13 overhanging loop should explain the distinct potencies of the toxins for the different protein phosphatases. Why is PP2B so toxin resistant? Does the unsaturated lactone in fostriecin bind covalently to the cysteine in the β12–β13 loop of PP2A (analogous to microcystin) [6]? How do cantharidides and calyculin A bind? A consensus is being reached that calyculin A binds in a position similar to okadaic acid, with its phosphate group slotting into the substrate-phosphate binding pocket. However, phosphate docking cannot be a major binding determinant because a dephospho-calyculin A is also a potent PP1 inhibitor [15].

Clearly, binding at the active site limits possibilities for changing the toxin sensitivity of enzymes without accompanying changes in catalytic properties. Expressing PP2A with a mutated β12–β13 loop has been useful in implicating this enzyme in effects of fostriecin in yeast [6]. However, this loop may also mediate allosteric regulation of the protein phosphatase catalytic subunits by their regulatory subunits. More immediate possibilities for altering inhibitory specificity may come from redesigning the toxins.

Chemical Synthesis of Protein Phosphatase Inhibitors

These toxins have been a challenge to synthetic chemists, requiring innovative strategies to form multiple bonds and control many chiral centers. Nevertheless, total syntheses of okadaic acid, tautomycin, calyculins, cantharidides, microcystin, and fostriecin, as well as several fragments and analogs, have been successful [16–19]. Analogs of cantharidin, microcystin, and tautomycin with improved selectivity for either PP1 or PP2A compared to the parent compounds have been made [20,21]. These results give rise to hopes of the rational design of even more selective inhibitors.

Another goal is to improve the chemical stability of fostriecin [19]. Phase I clinical trials of fostriecin as a cancer treatment were halted before reaching therapeutic or maximum-tolerated toxic doses, reportedly due to problems of poor drug stability in storage and *in vivo* [7].

Microcystin Affinity Chromatography and Affinity Tagging

Microcystins have unique attributes that enhance their utility. First, the dehydroalanine of microcystins can be linked by simple Michael addition to small, reactive thiols carrying an amine group that can, in turn, be linked to N-hydroxysuccinamide-activated Sepharose, biotins, or other compounds [22,23]. Recall that similar Michael chemistry is used in Nature to link the dehydroalanine to a conserved cysteine in the β12–β13 loop of the protein phosphatases [9,11,12]. The covalent link is not essential for potent inhibition however [12], which means that the synthesized adducts can be used to affinity-purify protein phosphatases and their regulatory subunits. Active native forms of PP1 can be eluted from microcystin–Sepharose using chaotropic salts, while denaturing buffers are needed to remove PP2AC from the column [22]. Perhaps electroelution would work?

The covalent link to the enzymes means that microcystin can also be used as an enzyme affinity tag. Microcystin–protein phosphatase complexes can be detected after

SDS-PAGE or column chromatography by using a radiolabeled microcystin or antimicrocystin antibodies [22,24,25] (Diplexcito *et al.*, unpublished data). We suggest that microcystin tagging may have untapped potential for probing cellular regulation of protein phosphatases.

Avoiding the Menace of Toxins in the Real World Outside the Laboratory

Our enthusiasm for using the toxins in biomedical research was tempered by news of a most tragic case of microcystin poisoning in 1996. More than 100 dialysis patients in Caruara, Brazil, were infused with microcystic water and most died of liver failure [26]. The scale of tumor promotion and liver damage worldwide is more difficult to assess. However, microcystin levels above the WHO limit (1 μg/liter) and suspected human and animal poisonings are often reported [8].

How can researchers who understand toxin–phosphatase interactions help? One need is for better toxin tests. Identifying microcystins is a trivial matter in a research laboratory, but a test intended for wider use must be very robust and preferably give a visual signal. Several laboratories are working to design both simple dipstick tests and methods to destroy microcystins. As more and more cellular effects of microcystins are documented, microcystin-specific biomarkers may emerge to track whether this toxin is at the root of many cases of chronic liver damage.

Perhaps more challenging than the science, though, are issues of communication and politics. In contrast to the rapid enactment of legislation to prevent malevolent use of microcystin by terrorists, several nations are currently moving toward the development of health guidance levels for microcystins in drinking water. Maybe the new reputation of microcystin as a potential danger to national security will motivate systematic action to ensure that no one ever has to imbibe this toxin in drinking water from natural sources.

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CHAPTER 103

Protein Phosphatase 1 Binding Proteins

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Introduction

The vast majority of cellular activities occur within microenvironments such as those of the nucleus, membrane, cytoskeleton, ribosome, mitochondria, glycogen particles, and other organelles. Because protein phosphorylation is a primary regulatory mechanism, the key interconverting enzymes, protein kinases and phosphatases, must also be localized. The paradigm for regulation by targeting was established for the striated muscle glycogen-associated phosphatase PP1G [1], in which the regulatory subunit R_{GL}/G_M directs the enzyme to the glycogen particle, in proximity to its substrates. This basic concept was expanded to include not only other forms of protein phosphatase 1 (PP1), but also other protein phosphatases and protein kinases [2]. Targeting is often achieved by interaction of domains in protein kinase and phosphatase regulatory subunits with various cellular structures. This implies that control of phosphorylation depends not simply on the activity of protein kinases and phosphatases, but also on their location in the cell and the partners with which they associate. By this mechanism, broad specificity kinases and phosphatases can acquire selectivity toward a specific subset of substrates. Further specificity is achieved if associated subunits participate in the recognition of substrate. In recent years, targeting/anchoring/scaffolding proteins such as A-kinase anchoring proteins (AKAPs) have been identified that tether both protein kinases and protein phosphatases to distinct intracellular locales [2].

Completion of the genome sequences of various organisms predicts that 2 to 3% of the genome encodes protein kinases and phosphatases. The protein kinases (over 500 in the human genome) outnumber the phosphatases. This is especially true for the serine/threonine protein phosphatases. It is estimated that ~300 serine/threonine kinases are encoded by the human genome but only some two dozen catalytic subunits of serine/threonine protein phosphatases. The question of how such a limited number of protein phosphatases can dephosphorylate the myriad of cellular phosphoproteins in a specific and regulated manner appears to be satisfied by the existence of a multitude of regulatory/targeting subunits. In this review, discussion is limited to mammalian PP1-binding subunits and how they can account for the pleiotropic functions of the enzyme in the cell. The reader is also referred to other recent reviews on this topic that include a more extensive citation list than permitted in this chapter [3–5].

Protein Phosphatase 1 (PP1)

Protein phosphatase 1 belongs to the PPP family of phosphatases and is involved in the regulation of a wide variety of cellular processes ranging from intermediary metabolism to apoptosis. In mammals, three genes code for four or five highly conserved (~90%) isoforms of catalytic subunits of PP1 (PP1c), PP1cα_1 and α_2, PP1cδ (also called PP1cβ) and PP1cγ_1 and γ_2, the subscripts indicating forms generated by alternative splicing. In the cell, PP1c does not exist as a free monomer but is present in oligomeric holoenzyme forms consisting of a catalytic subunit complexed with one or two regulatory and/or targeting subunits. Studies *in vitro* have failed to reveal either specificity of interaction between PP1c isoforms and various regulatory components or to

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demonstrate isoform-related substrate specificity. Evidence, however, is accumulating that some PP1c isoforms may be involved in distinct cellular processes *in vivo* [6].

PP1 Regulatory or Targeting Subunits

Protein phosphatase 1 enzymes acquire specificity of function by their association with targeting/regulatory proteins that direct the enzyme to distinct subcellular structures or compartments in proximity to physiological substrates, confer substrate specificity and/or modulate enzyme activity. Over 40 PP1-associated proteins are currently known. Most interact with PP1c through multiple sites, a shared site recognizable in many of the subunits, and other sites unique to the individual proteins. The common docking site on PP1c is formed by a hydrophobic channel situated opposite the catalytic site and is flanked by an acidic region. This domain accommodates any of the variant forms of the binding motif [R/K][V/I/L]X[F/W/Y] (often given as RVXF) found in a large number of PP1c-bound proteins. However, the presence of the tetrapetide does not necessarily define a PP1c-binding protein, as this sequence is found in more than 10% of proteins. Furthermore, its presence is not an absolute requirement for association with PP1c. The initial proposal of a mutually exclusive association of polypeptides harboring this motif with PP1c has been weakened by the recent findings that at last two polypeptides containing the motif can simultaneously associate with PP1c [7], supporting the notion that additional sites participate in the binding.

Based on analysis of eukaryotic genome sequences, Ceulemans and coworkers [5] have traced the evolution of 13 families of PP1 regulatory/targeting proteins and have suggested the existence of nine additional isoforms not previously recognized. Table 1 presents a classification of over 40 PP1c-binding proteins, and Fig. 1 shows a schematic diagram of the structure of representative members of the different groups. Some of the binding subunits function as inhibitors/modulators of activity and do not contain domains for targeting to specific locations. Others function as targeting subunits to direct the phosphatase to specific subcellular structures or substrates and have no known regulatory role. Yet other PP1c-binding proteins may perform both a targeting and a regulatory function.

PP1 Inhibitors or Modulators

The proteins in this group inhibit or modulate the activity of PP1 but do not contain targeting domains. In fact, PP1 was originally defined by its sensitivity to heat-stable protein inhibitor 1 (I-1) and 2 (I-2). I-1 and its brain homolog DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of apparent M_r 32,000), I-2 , inhibitor-3/HCGV (I-3), and the G substrate inhibit the free PP1c, whereas the PKC-phosphorylated inhibitor protein CPI-17 and its homologs PHI and KEPI are able to inhibit holoenzymes containing targeting subunits such as the glycogen- and myosin-associated phosphatases [8,9]. Furthermore, I-1, DARPP-32, I-2, and I-3 all contain a variant of the PP1c-binding consensus RVXF motif (Fig. 1). The ^{8}KIQF^{12} sequence (homologous to RVXF) in I-1 and DARPP-32 is essential for inhibition, whereas the equivalent sequence ^{144}KLHY^{147} in I-2 is dispensable [10]. However, the N-terminal ^{12}IKGI^{15} residues in I-2 are required for inhibitory activity. This sequence occupies a unique site on PP1c, located adjacent to the hydrophobic groove [11]. Three additional PP1c-interacting sites have been identified in I-2 (Fig. 1) [10], establishing the paradigm that high-affinity binding may be achieved by multiple contacts. The activity of the majority of the inhibitory/modulatory subunits is controlled by phosphorylation. I-1 and DARPP-32 become potent inhibitors after phosphorylation by the cAMP-dependent protein kinase (PKA) at a conserved threonine residue, whereas phosphorylation by cyclin-dependent kinase 5 (Cdk5) prevents phosphorylation by PKA, rendering I-1 and DARPP-32 less effective inhibitors. The inhibitory activity of CPI-17, PHI, and G-substrate is also enhanced by phosphorylation. I-2 does not require phosphorylation and is a complex modulator of PP1 activity. Its stable interaction with PP1c at five distinct sites forms the inactive ATP-Mg^{2+}-dependent holoenzyme that is activated by phosphorylation at T72 by glycogen synthase kinase 3 (GSK3), mitogen-activated protein kinase (MAPK), or Cdc2. Reactivation does not cause dissociation, arguing against a proposed chaperone role for I-2. The importance of these inhibitor proteins in the control of the phosphatase activity is highlighted by the phenotype of the DARPP-32 and I-1 knockout mice. DARPP-32 disruption impairs dopamine signaling, and the animals show decreased learning and reduced responses to substances of abuse [12]. I-1 knockout mice lack long-term potentiation at the perforant path–dentate cell synapses and have an impaired cardiac β-adrenergic response that is less severe than that caused by the over expression of PP1c [13].

Glycogen Targeting Subunits

Four glycogen-targeting subunits have been characterized and three more putative forms, encoded by *PPP1R3E*, *F*, and *G*, have been identified in the human genome based on homology to PP1c-binding and targeting motifs [5]. Whether or not they represent *bona fide* PP1 glycogen-targeting components remains to be determined. R_{GL}, also called G_M, was the first glycogen-binding subunit of PP1c identified and is exclusively expressed in striated muscle [14,15]. The N-terminal region contains binding sites for PP1c, glycogen, and possibly glycogen synthase (GS), whereas a hydrophobic region in the C-terminus anchors the protein to membranes. Interaction with PP1c most likely involves multiple contacts, one of which is the ^{65}RVSF^{68} sequence.

It has been proposed that muscle PP1G/R_{GL} plays a major role in insulin and epinephrine control of glycogen metabolism via phosphorylation of the targeting subunit [1]. Insulin would cause phosphorylation of R_{GL} at S48 and activation toward glycogen synthase, whereas epinephrine would

Table I Mammalian Regulatory/Targeting Subunits of Protein Phosphatase 1

Regulatory/targeting subunit	Gene name[a]	Established or putative cellular function controlled and/or effect on PP1c
Inhibitors/modulators		
Inhibitor-1	*PPP1R1A*	PKA-mediated PP1c inhibition
DARPP-32	*PPP1R1B*	Neurotransmitter signaling
Inhibitor-2	*PPP1R2*	Modulation of PP1c
Inhibitor-3	*PPP1R11*	Inhibition of PP1c
CPI-17	*PPP1R14A*	Smooth muscle contraction
PHI	*PPPP1R14B*	Inhibition of PP1 holoenzymes
KEPI		Morphine signaling
G substrate		Inhibition of PP1c
Glycogen targeting		
R_{GL}/G_M	*PPP1R3A*	Muscle glycogen metabolism
G_L	*PPP1R3B*	Liver glycogen metabolism
PTG/R5	*PPP1R3C*	Glycogen metabolism
R6	*PPP1R3D*	Glycogen metabolism
Myosin targeting		
Mypt1/M110	*PPP1R12A*	Smooth muscle contraction, cell
Mypt2	*PPP1R12B*	Shape and motility
p85	*PPP1R12C*	Skeletal muscle contraction
		Actin cytoskeleton
Nuclear targeting		
NIPP1	*PPP1R8*	Pre-mRNA splicing
Sds22	*PPP1R7*	Exit from mitosis
PNUTS	*PPP1R10*	Transcription/RNA maturation
AKAP350		Centrosome and Golgi function
Nck2		Centrosome separation
PSF1		Pre-mRNA spicing
HCF		Transcription
Membrane/cytoskeleton targeting		
Neurabin I	*PPP1R9A*	Neurite outgrowth
Neurabin II/spinophilin	*PPP1R9B*	Dendritic spine formation
Neurofilament-L		Neuronal morphology
AKAP220		Peroxisome/cytoskeletal activities
Yotiao		NMDA/ion channel activity
AKAP149		Nucleus reassembly
Endoplasmic reticulum/ribosome targeting		
GADD34	*PPP1R15A*	Stress responses
GRP78		Chaperone/protein folding
L5		Protein synthesis
RIPP1		Protein synthesis
Others		
Rb		Cell cycle
53BP2	*PPP1R13A*	Cell cycle/apoptosis
Hox11		Cell cycle
Bcl2		Apoptosis
PFK	*PPP1R16B*	Glycolysis
Ryanodine receptor	*PPP1R16A*	Calcium channel activity
BH-protocadherin-c		Cell adhesion
NKCC1		Ion transport
PRIP-1		$Ins(1,4,5)P_3$ signaling
PP1bp80		Unknown
TIMAP		TGFβ signaling
Mypt3		TGFβ signaling
I_1^{PP2A} I_2^{PP2A}		Stimulation of PP1c/substrate specificity

[a]The human genome nomenclature recently redefined by Ceulemans *et al.* [5] for the PP1c-binding proteins is indicated.

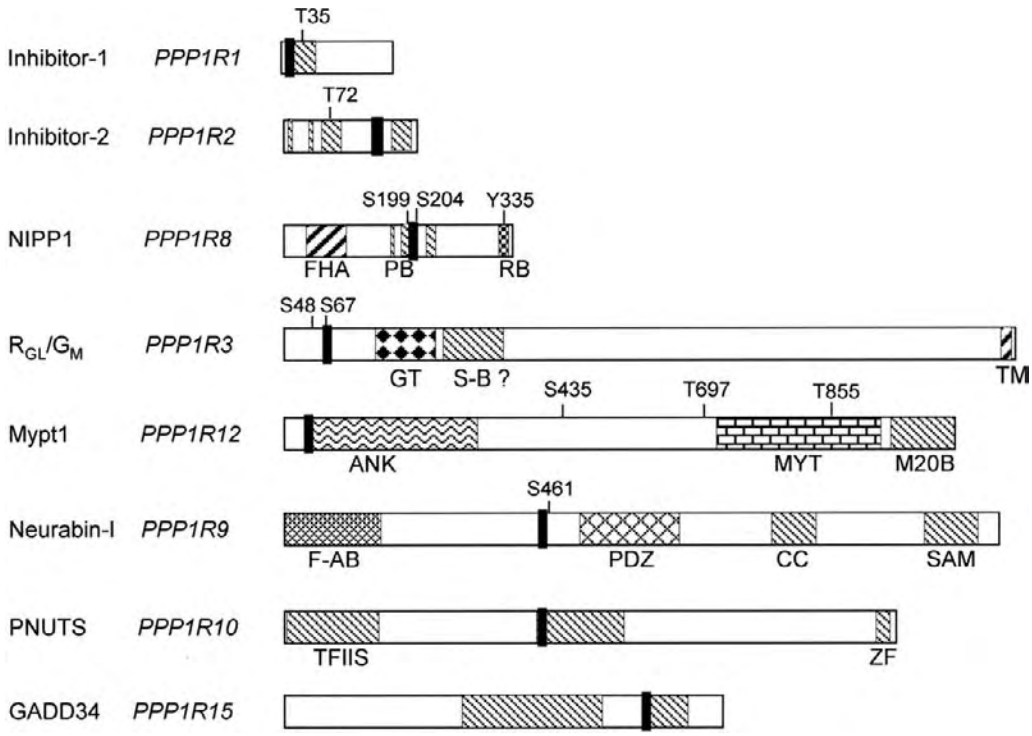


Figure 1 Domain structure of representative members of PP1c-binding proteins. Phosphorylation sites and various domains are indicated. The filled black box indicates the common PP1c-binding motif. Targeting domains are indicated by various patterns and abbreviations: FHA, forkhead associated; PB, polybasic; RB, RNA-binding; GT, glycogen targeting; S-B?, putative substrate-binding; TM, transmembrane; ANK, ankyrin repeats; MYT, myosin targeting; M20B, M20-binding; F-AB, F-actin binding; CC, coiled-coil; SAM, sterile alpha motif; TFIIS, transcriptional factor IIS; ZF, zinc finger.

induce phosphorylation at S67, mediated by PKA, and cause dissociation of PP1c. The released PP1c would be less active toward glycogen-associated and perhaps membrane-bound substrates. Furthermore, activation of PKA would lead to phosphorylation of I-1, which then becomes a potent inhibitor of the released PP1c. However, recent studies have shown that R_{GL} is not phosphorylated at S48 in response to insulin [16,17], and disruption of the R_{GL} and I-1 genes has shown that neither is required for either insulin or epinephrine control of glycogen metabolism [17–19]. R_{GL}, though, is essential for control of GS by exercise and muscle contraction [20]. Direct phosphorylation of GS by GSK-3 and PKA, the protein kinases regulated by insulin and epinephrine, respectively cannot account for the effects on glycogen metabolism, as changes in phosphorylation at the GS sites recognized by these individual kinases are insufficient to account for the alterations of activity caused by the two hormones.

Of the other three glycogen-targeting subunits, G_L was thought to be liver specific, but a recent report describes G_L in human, but not in rodent, skeletal muscle [21]. PTG expression is higher in skeletal muscle, liver, heart, and fat, whereas R6 is more ubiquitously expressed. G_L, PTG, and R6 share homology to the N-terminal region of R_{GL} and lack the extended C-terminal tail and the membrane-binding domain [22,23]. All three contain the PP1c-binding motif and the glycogen-binding domain, but the two phosphorylation sites of R_{GL} are not conserved. PTG interacts with glycogen metabolizing enzymes but, unlike the liver-specific $PP1G/G_L$ complex, PP1G/PTG is not controlled allosterically by phosphorylase *a*. Expression of the G_L subunit is downregulated in diabetic rats. Overexpression of PTG in Chinese hamster ovary cells increases the basal and insulin-stimulated GS activity, but neither insulin nor forskolin induce detectable PTG phosphorylation [23]. A mechanism has been proposed whereby PTG would affect PP1 activity by relieving inhibition by DARPP-32. However, neither I-1 nor DARPP-32 is required for insulin activation of glycogen synthase [19]. Thus, the mechanisms for control of PTG- and R6-containing phosphatases are largely unknown. Homozygous disruption of PTG results in embryonic lethality. Heterozygous PTG knockout mice retain activation of GS by insulin in skeletal muscles but appear to develop impaired glucose disposal with aging [24].

Myosin Targeting Subunits

Three subunits target PP1c to myosin [25]. The best characterized, Mypt1/M110, interacts with myosin type II and is involved in control of smooth muscle contraction, cell shape, and migration. The myosin phosphatase complex is a heterotrimer containing also a smaller polypeptide, M20 (~20 kD), that does not bind to PP1c but interacts with Mypt1 and myosin. In addition to a targeting function, Mypt1 confers substrate specificity, enhancing phosphatase activity

toward the regulatory myosin light chains while decreasing it toward phosphorylase. Two other actin-binding proteins, adducin and moesin, are also associated with and regulated by Mypt1 in non-muscle cells, supporting a role in actin cytoskeleton organization. The RVXF motif in the N-terminal region of Mypt1 is followed by seven or eight ankyrin repeats that may be involved in interaction with PP1c and/or myosin. The C-terminus harbors domains that bind to myosin and M20. Phosphorylation by a RhoA-activated kinase (ROCK), ZIP-like kinase, or the myotonic dystrophy protein kinse (DMPK) at T697 inhibits phosphatase activity, leading to increased light-chain phosphorylation and contraction in smooth muscle in the absence of changes in intracellular calcium levels. In contrast, phosphorylation at S435 during mitosis increases myosin light-chain phosphatase activity. Another feature of this phosphatase is that it is potently and specifically inhibited by CPI-17 [25], providing an additional mechanism for enhancement of myosin phosphorylation and smooth-muscle contraction. CPI-17 does not contain an RVXF motif. Phosphorylation by PKC causes a conformational change that appears to expose a region that may interact with PP1c.

Of the other two members of the family, Mypt2 is expressed in skeletal muscle, heart, and brain, whereas p85 is more widely distributed and appears to be required for assembly of the actin cytoskeleton. Both share structural similarity with Mypt1, including the PP1c-binding motif and the N-terminal ankyrin repeats. Interestingly, the small M20 subunit appears to be generated by alternative splicing of *PPP1R12B*, the gene that codes for Mypt2. A newly identified protein was named Mypt3, due to the presence of ankyrin repeats in addition to the RVXF motif [26]. However, based on the absence of a myosin-binding domain and on gene structural similarity, Ceulemans and coworkers [5] have reclassified it as a member of the TIMAP family, which may be involved in TGFβ signaling (Table 1).

Nuclear Targeting Subunits

Protein phosphatase 1 is abundantly expressed in the nucleus, where it is complexed with a variety of regulatory subunits to control such processes as cell-cycle progression and division, transcription, and pre-mRNA splicing. Sds22, a protein required for exit from mitosis, and HCF-1 (human factor C1) have no discernable RVXF motif [27]. Disruption of the PP1c hydrophobic docking channel does not impair Sds22 binding, indicating that interaction involves other sites. Multiple contacts are also established between PP1c and NIPP1, nuclear inhibitor of PP1 (Fig. 1) [28]. Binding of the RVXF motif is not inhibitory by itself. A polybasic sequence preceding the common motif as well as a C-terminal region are required for high potency inhibition. Similar to I-2, phosphorylation is not required for inhibitory activity, and the action of two protein kinases, PKA and casein kinase II, relieves the inhibition without causing dissociation of the NIPP1/PP1c complex. NIPP1 is localized to nuclear "speckles" where it interacts with splicing factors through its N-terminal forkhead-associated domain. The C-terminus binds RNA, supporting a role of NIPP1 in pre-mRNA splicing [29]. The splicing-factor-associated protein PSF1 also binds PP1c, perhaps allowing control of SF1, which inhibits early spliceosome formation once phosphorylated. Two proteins, AKAP350 and Nek2 (NIMA-related protein kinase 2), may localize PP1c to the centrosome [30,31]. Nek2 has been implicated in centrosome separation and, together with its substrate C-Nap1, is a PP1 substrate. PNUTS/p99 is another putative nuclear targeting subunit of PP1c [32]. An N-terminal sequence related to domains present in other transcriptional factors, TFIIS, elongin A, and CRSP70, and the presence of a zinc finger motif in the C-terminus may account for its chromatin association and a potential role in transcription.

Membrane or Cytoskeleton Targeting Subunits

A number of proteins associated with membrane and cytoskeletal structures have been shown to bind PP1c. Neurabin I and neurabin II (also known as spinophilin) are actin cross-linking proteins enriched in postsynaptic densities and dendritic spines [33,34]. Recent studies have shown that both neurabins and neuofilment-L display binding selectivity for the PP1c α and γ1 isoforms [6]. Neurabins contain an N-terminal F-actin-associating domain that accounts for localization at the actin cytoskeleton, C-terminal coiled-coil and SAM (sterile alpha motif) modules that mediate homo- and heterodimerization, and a central PDZ protein-binding domain that may link PP1c to transmembrane proteins (Fig. 1). Phosphorylation of neurabin I by PKA at S461, immediately C-terminal to the RVXF sequence, reduces PP1c-binding, and a S461E mutation decreases inhibitory potency, suggesting regulation by cAMP signaling. Spinophilin knockout mice have provided evidence for a role in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptor channel activity and in dendritic spine development [35].

A defining feature of the AKAPs is the localization of PKA to different intracellular structures, including cytoskeleton, mitochondria, nucleus, membrane, and vesicles [2]. Several AKAPs also interact with protein phosphatases, resulting in colocalization of enzymes that potentially exert opposite effects [30]. The AKAP Yotiao, an NMDA-receptor-associated protein, binds PP1c. Although Yotiao contains the RVXF motif, this sequence is not essential for interaction. The tethered PP1c is active and may negatively regulate receptor activity. Recently, it has been shown that Yotiao, complexed with PKA and PP1c, also associates with cardiac potassium channels. Mutations that disrupt the interaction correlate with inherited cardiac arrhythmias [36]. AKAP149, an integral protein of the endoplasmic reticulum/nuclear envelop network, recruits PP1c to the lamina of the nuclear membrane where it may function to dephosphorylate B-type lamins for reassembly of the nuclear envelop at the end of mitosis. AKAP220 binds to and inhibits PP1c through multiple contacts and recruits the phosphatase to vesicles.

Endoplasmic Reticulum/Ribosome Targeting Subunits

Biochemical and genetic studies have implicated PP1 in the control of protein synthesis. Several components of the translational machinery are controlled by phosphorylation. In response to various stress stimuli, such as ultraviolet irradiation, viral infection, and nutrient deprivation, the eIF2α subunit becomes phosphorylated and translation initiation is inhibited. GADD34, a growth arrest and DNA-damage-induced gene, has been implicated in growth arrest and apoptosis induced by endoplasmic reticulum (ER) stress signals and has been shown to be associated with reticular structures. In response to stress signals, protein synthesis is shut off through phosphorylation of eIF2α. GADD34 forms a complex with PP1c that specifically promotes the dephosphorylation of eIF2α. The stress-dependent expression of GADD34 implies that it may provide for a negative feedback mechanism to evade or promote recovery from the translational inhibition. GADD34 interacts with PP1c through its C-terminus, a region homologous to the herpes virus ICP34.5 protein domain that also interacts with PP1c and that redirects the phosphatase to dephosphorylate eIF-2, enabling continued protein synthesis in virally infected cells. Interestingly, I-1 binds both GADD34 and PP1c via different domains [7]. The C-terminus of I-1 is essential for interaction with a central region of GADD34, whereas the N-terminus binds PP1c, raising the possibility for formation of a heterotrimeric complex containing two PP1 regulatory components, each of which harbors a canonical docking site. Similarly, hSNF5/INI1, a member of the hSWI/SNF chromatin remodeling complex, binds to free GADD34 and PP1c as well as to the GADD34/PP1c complex to form a stable heterotrimer [37]. These findings indicate that association of different regulatory subunits with PP1c may not necessarily be mutually exclusive even if each contains an RVXF sequence. Another ER protein that binds PP1c is the glucose-regulated protein chaperone GRP78 [38], which is involved in protein translocation and folding and is induced by ER stress. Although the role of this phosphatase in the ER is not clearly understood, it may be part of a general mechanism to overcome the inhibition of translation in response to cellular stress conditions.

Other PP1c-binding proteins that may be involved in control of translation are the large ribosomal protein L5 and RP111. L5 [39] is located at the interphase between the small and large ribosomal subunits where translation initiation takes place and is therefore positioned for potential control of this step. In addition, the phosphorylated S6 (small ribosomal subunit) protein promotes the preferential recruitment of polypirimidine-track-containing mRNAs. The phosphatase that dephosphorylates S6 is a type 1 enzyme.

Other PP1c-Binding Proteins

Although much has been learned about localization and function of PP1, the precise roles of most PP1c-binding proteins are not completely understood. Not all physically target the enzyme to subcellular compartments. Some of the reported binding proteins may simply be substrates. Included would be the retinoblastoma protein pRb, phosphofructokinase (PFK), the ryanodine receptor, and the Na-K-Cl co-transporter NKCC1, all of which are controlled by phosphorylation and some of which do not contain any recognizable PP1c docking site. The 53BP2 [40] interacts with p53 and Bcl2 and may specifically direct the phosphatase activity toward proteins whose phosphorylation state is critical for the control of apoptosis. The ability of PRIP-1 to bind inosito1,4,5-trisphosphate [41] may allow recruitment of PP1c to membranes in response to stimuli that generate the phospholipid. The two heat-stable inhibitors of PP2A, I_1^{PP2A} and I_2^{PP2A} have recently been shown to enhance *in vitro* PP1c activity toward specific substrates [42]. These polypeptides also do not contain a recognizable PP1c-binding site. However, whether or not they can function as activators of PP1 *in vivo* remains to be determined.

Conclusions

All forms of PP1 holoenzymes contain a similar, highly conserved catalytic subunit but differ in the associated regulatory subunits. The large number of associating proteins provides compelling evidence that the distinctive features of different PP1 holoenzymes reside in the regulatory components. Thus, functionally distinct forms are generated by combination of a similar catalytic component with different regulatory subunits that are responsible for targeting the enzyme to specific cellular locales, for conferring substrate specificity, or for controlling the enzyme activity. The large number of targeting/regulatory subunits of PP1 can thus account for the pleiotropic function of the type 1 phosphatase in the cell.

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CHAPTER 104

Role of PP2A in Cancer and Signal Transduction

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Introduction

PP2A is the most abundant serine/threonine-specific phosphatase in mammals, representing approximately 0.3% of the total cellular protein [1]. Over the last decade, PP2A has been recognized as a major player in many biological processes, including differentiation, development and morphogenesis, organ function, and growth control. As a consequence of its role in growth control, PP2A is implicated in the development of cancer. On the cellular level, PP2A acts in numerous signaling pathways by counteracting protein kinases. It can function in the nucleus, in the cytosol, or at the plasma membrane. The great versatility of PP2A is based on the large number of regulatory subunits, which, in combination with two isoforms of the catalytic subunit, could in theory give rise to over 70 different forms of PP2A. Furthermore, PP2A interacts with an ever-increasing number of other cellular proteins (approximately 40 at the latest count) as well as viral proteins. We are only beginning to appreciate the complexity and importance of PP2A. In this review, current knowledge regarding the structure of PP2A and its presumed role in cancer and signaling are reviewed. Because of space limitations, the review is inevitably incomplete and the reader is referred to excellent recent reviews for more detailed discussion of various aspects of PP2A [2–7].

Structure of PP2A

PP2A exists in cells as two major forms: holoenzyme and core enzyme [8,9]. The core enzyme consists of a 36-kDa catalytic C subunit and a 65-kDa regulatory A subunit. Holoenzymes are composed of a core enzyme to which one of several regulatory B subunits is bound (Fig. 1). It has been proposed that the core enzyme is an artifact of enzyme isolation and does not exist in cells. This is highly unlikely, however, as under the gentlest conditions of enzyme purification the core enzyme represents one-third to one-half of total cellular PP2A [8,9]. Recently, it has been suggested that the core enzyme is unstable unless associated with a regulatory B subunit [10]. It remains to be seen, however, whether in these experiments the absence of core enzyme is a consequence of eliminating all B subunits, which is not physiological, or whether it has resulted from toxic experimental conditions. The A and C subunits exist as two isoforms: Aα and Aβ and Cα and Cβ, respectively. Thus, four core enzymes, AαCα, AβCα, AαCβ, and AβCβ, might exist in cells that could have distinct substrate specificities and distinct abilities to interact with regulatory subunits or other cellular proteins. Therefore, the question of whether core enzymes are a physiological reality is an important one.

The B subunits fall into four families, designated B, B′ (also called B56), B″, and B‴ which appear unrelated by sequence alignment. The B family has four members: Bα, Bβ, Bγ, and Bδ. The B′ family consists of five genes encoding B′α, B′β, B′γ, B′δ, and B′ε. Including isoforms and splice variants, there are a total of at least eight B′ subunits. The B″ family has four members, designated PR48, PR59, PR72, and PR130. The latter two are splice variants of the same gene. The B‴ family has two members: striatin and SG2NA. The existence of so many regulatory subunits suggests that PP2A is a highly regulated phosphatase and that its various forms fulfill

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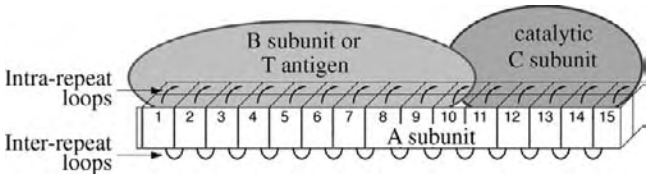


Figure 1 Model of PP2A.

numerous distinct functions. Another class of proteins able to associate with PP2A core enzyme are tumor (T) antigens encoded by polyoma viruses. Their binding domains on the A subunit overlap with those of B subunits. T antigens play a key role in neoplastic transformation by polyoma viruses, and their association with PP2A is essential for transformation [11].

Subunit Interaction

To elucidate the function of PP2A, it is important to understand how its subunits interact with each other and what controls the interaction. A model of the core and holoenzymes and of complexes of the core enzyme with T antigens is shown in Fig. 1 [12,13]. The A subunit consists of 15 nonidentical repeats [14–16]. Each repeat is composed of two α-helices connected by a loop (intra-repeat loop). Adjacent repeats are connected by inter-repeat loops. Collectively, the repeats form an extended, hook-shaped molecule that is stabilized by hydrophobic interactions. The intra-repeat loops are involved in binding B and C subunits as well as T antigens. The B subunits bind to repeats 1 to 10 and the C subunits to repeats 11 to 15 of the A subunit. SV40 small T binds to repeats 3 to 6, and polyoma virus small T and middle T bind to repeats 2 to 8 [12,13].

Determining how the different B subunit family members and T antigens, although largely unrelated by sequence, bind to overlapping regions of the A subunit has been approached by site-directed mutagenesis, showing that some Aα mutations in loops 1 to 10 affect binding of all B subunits (B, B′, and B″) whereas others affect binding of specific B subunits. Thus, the binding sites on the A subunit for different types of B subunits are composed of both common and distinct amino acids [17]. Interestingly, a recent study revealed that all members of the B, B′, and B″ families, but not B‴, share two evolutionary conserved domains, 103 and 58 residues in length, which are involved in binding to Aα [18]. The limited homology of these domains escaped detection when the complete sequences of B, B′, and B″ were aligned. Another study identified two adjacent arginine residues in Bγ (R165 and R166) which form an essential salt bridge to a pair of glutamic acid residues (E100 and E101) in the intra-repeat loop of repeat 3 of Aα [19].

Carboxymethylation of Leu 309 at the C terminus of the catalytic subunit is important for recruiting B subunits to the core enzyme [20]. In addition, phosphorylation at tyrosine 307 affects holoenzyme formation as well as phosphatase activity [9].

Association of PP2A with Cellular Proteins

PP2A associates with numerous cellular proteins involved in growth regulation or apoptosis. These include several protein kinases, Rb-related protein p107, heat shock protein HSF2, translation termination factor eRF1, homeobox protein HOX11, myeloid-leukemia-associated protein SET, caspase 3, Bcl2, and cyclin G [2,6]. The mechanisms by which these interactions may affect growth regulation or apoptosis are largely unknown. PP2A also forms complexes with the tumor suppressor proteins adenomatous polyposis coli (APC) [21] and axin [22,23], two key players in the Wnt signaling pathway, as discussed in detail later [24–26].

Alteration or Inhibition of PP2A Is Essential in Human Cancer Development

When it was proposed over 10 years ago that PP2A might be a tumor suppressor [27], based on findings that okadaic acid acts as a potent tumor promoter as well as a strong inhibitor of PP2A, most regulatory B subunits had not yet been discovered and our view of PP2A was much simpler. Now it is clear that PP2A exists in numerous forms, some of which might suppress growth (see later discussion), while others might be growth stimulatory [28,29]. Strong support for the idea that PP2A is involved in growth control comes from the discovery that SV40 small T antigen and polyoma virus small T and middle T antigen form stable complexes with PP2A (Fig. 1) [2,11]. Of particular interest are two recent publications on the role of SV40 small T in the transformation of primary human cells. Yu *et al.* [30] reported that the transformation of primary human diploid fibroblasts and of mesothelial cells in culture depends on both SV40 large T and small T. Transformation of human cells does not occur without SV40 small T, and small T cannot be replaced by oncogenic Ras, in contrast to primary rodent cells, which can be transformed by the combination of SV40 large T and oncogenic Ras. Expression of telomerase is not required for transformation of human cells but only for growth beyond the point of senescence [30]. Similar results were reported by Hahn *et al.* [31] using human fibroblasts and human embryonic kidney cells. However, these authors found that Ras *is* required for transformation in addition to SV40 large T and small T, and transformation does not occur in the absence of either Ras or small T. This difference in Ras requirement between these two studies could be due to the time at which the transformation assay was scored (one group scored earlier than the other), the amount of small T expressed (one group used retroviral vectors, the other transfection), or the difference in cell type (K. Rundell, personal communication). The importance of both reports lies in the recognition that inhibition or alteration of PP2A by SV40 small T is required for transformation of primary human diploid fibroblasts and epithelial cells. In addition, transformation requires inactivation of p53 and pRb by complex formation with SV40 large T. Expression of telomerase is essential for the purpose of immortalization.

Further evidence for the importance of SV40 small T in transformation comes from experiments showing that human mesothelial cells expressing SV40 large T *and* small T are easier to transform by carcinogens than cells expressing large T only [32]. The potency of small T was also demonstrated in a transgenic mouse model in which only small T is expressed in breast epithelial tissue under the control of the acidic milk protein promoter [33]. Cyclin D1 is constitutively overexpressed in the small-T-expressing mammary epithelial cells, and mammary gland differentiation is inhibited. Importantly, 10% of the transgenic animals develop breast tumors. In contrast, transgenic mice expressing only large T in breast epithelium develop breast cancer at a lower rate and after a longer latency period [33].

Mutation of Aα and Aβ Isoforms in Human Cancer

An independent line of evidence for PP2A playing a role in human cancer development comes from recent findings that PP2A is mutated in a variety of human malignancies, including cancer of the lung, breast, colon, and skin. Wang *et al.* [34] discovered that the gene encoding the Aβ subunit of PP2A is mutated or deleted in 15% of primary lung and colon cancers. Takagi *et al.* [35] described mutations in the Aβ subunit gene in four colon cancer cases. Furthermore, Calin *et al.* [36] reported that *both* the Aα and Aβ subunit isoforms are genetically altered in a variety of primary human cancers. These findings lend strong support to the idea that PP2A is a tumor suppressor.

We investigated many of the cancer-associated Aα and Aβ mutants described by Wang *et al.* and Calin *et al.* Based on the location of the mutated amino acids in intra-repeat loops or nearby, we suspected that the mutant A subunits might be defective in binding B and/or C subunits. Indeed, we found that all Aα and most Aβ subunit mutants are defective in binding B or both B and C subunits [37,38]. Most importantly, the Aα subunit mutants Glu64 → Asp (E64D) and Glu64 → Gly (E64G) found in lung and breast cancer, respectively, were specifically defective in binding the B′α1 subunit, a member of the B′ family, whereas binding of Bα and B″ (PR72) was normal, as was Cα and Cβ subunit binding. The finding that the most specific Aα mutants affect binding of B′ subunits only suggests that B′ subunits or B′-containing holoenzymes play a crucial role in the presumed tumor suppressor function of PP2A as described below. It is important to note that reduced expression of the Aα subunit in human gliomas occurs in the absence of mutations in the Aα and Aβ subunit genes [39].

Differences between Aα and Aβ Subunits

The discovery that Aβ is mutated in human cancer has drawn attention to this subunit, which is 86% identical to Aα. Previously, the main focus had been on Aα, and all biochemical studies of PP2A have been carried out with enzyme preparations containing Aα. Now there is growing evidence that Aα and Aβ have different properties in regard to expression, binding of B and C subunits, and function [38,40]. Therefore, it seems possible that they play different roles in growth control.

PP2A and Wnt Signaling

During embryogenesis, the Wnt signaling pathway regulates cell proliferation and development [26]. An inappropriate activation of the Wnt pathway has been found in a wide variety of human cancers [24], where it promotes the growth of cancer cells by inducing cyclin D and c-*myc*. Currently, three genetic changes that cause an increase in β-catenin levels and activation of the Wnt signaling pathway are known. First, some mutations in the β-catenin gene alter specific N-terminal serine or threonine residues, thereby preventing β-catenin phosphorylation by GSK3β and degradation by the ubiquitin/proteosome pathway. Second, mutations in the APC gene that cause loss of APC binding to axin prevent recruitment of β-catenin into the β-catenin-destabilizing complex. Therefore, β-catenin escapes from degradation. Third, axin mutations in the β-catenin binding site destroy the ability of axin to recruit β-catenin into the destabilizing complex, also resulting in β-catenin accumulation. In addition, mutating PP2A may represents a fourth mechanism to activate the Wnt pathway as suggested by Seeling *et al.* [21], who reported that regulatory B′ subunits bind to APC (Fig. 2, site Z). Furthermore, they found that overexpression of B′ in 293 cells dramatically decreases the level of β-catenin. Importantly, B′ inhibits β-catenin-dependent transcription by the transcription factor LEF. Seeling *et al.* proposed that PP2A may act as a tumor suppressor by downregulating Wnt signaling through dephosphorylation and activation of GSK3β. Cancer-associated Aα or Aβ mutations might prevent B′-mediated recruitment of PP2A into the β-catenin-destabilizing complex, resulting in downregulation of GSK3β and upregulation of Wnt signaling [37]. Yamamoto *et al.* [23] discovered that B′ also binds to axin. The site of interaction (site X) is different from the binding sites for GSK3β, β-catenin, APC, and dishevelled (Dvl) (Fig. 2). They also found that B′ suppresses β-catenin- and Tcf-dependent transcription [21]. Axin associates not only with the B′ subunit but also with the catalytic C subunit [22]. The C subunit binding region (site Y) is separate from the B′ binding region. It has been suggested that binding to site Y may stimulate Wnt signaling [41]. Ratcliffe *et al.* [42] studied the role of PP2A in early *Xenopus* embryos. They discovered that the B′ subunit strongly inhibits secondary axis formation and Wnt signaling. The C subunit, on the other hand, appears to stimulate Wnt signaling [23,42].

The precise mechanism by which PP2A affects Wnt signaling remains to be elucidated. Willert *et al.* [43] suggested that PP2A dephosphorylates axin, resulting in release of β-catenin from the β-catenin-destabilizing complex [43]; however, according to this model, PP2A would actually

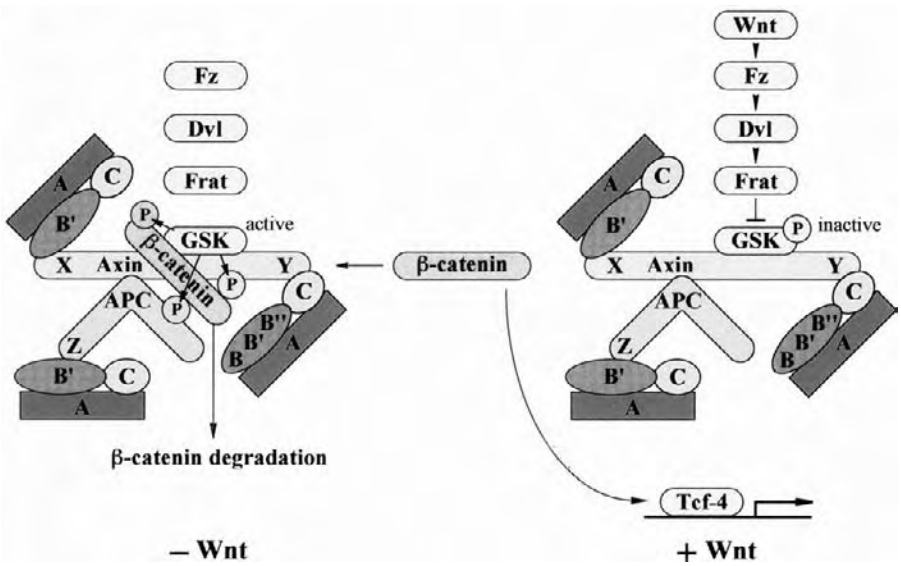


Figure 2 Multiple binding sites of PP2A in the β-catenin degradation complex.

stimulate Wnt signaling. Yamamoto *et al.* [23] discussed whether PP2A controls APC-mediated nuclear export of β-catenin or acts on the transcription factor Tcf. In order to resolve these questions, it is important to identify the substrates for each PP2A molecule bound to the different sites on the β-catenin-destabilizing complex. At present, GSK3β, axin, β-catenin, and APC all have to be considered as potential substrates. While published reports on the role of PP2A in Wnt signaling are conflicting, there is general agreement that the B′ subunit inhibits the Wnt pathway. All findings are consistent with the idea that the B′ subunit, or the B′-containing holoenzyme, is a tumor suppressor that functions by downregulating the Wnt pathway. It is important to note that CKIδ and CKIε, which phosphorylate several components of the β-catenin degradation complex, are regulators of the Wnt signaling pathway[44,45].

PP2A and the MAP Kinase Pathway

Growth factors and cell adhesion to the extracellular matrix stimulate growth through the Ras → Raf → MEK → ERK signaling pathway by inducing cyclin D and activating G1-phase cyclin-dependent kinases [46]. Evidence that PP2A negatively controls the MAPK → ERK pathway and inhibits growth comes from experiments with SV40 small T, which inhibits PP2A [47,48], thereby preventing dephosphorylation and inactivation of MEK and ERK [49]. A recent report suggests that this direct inhibitory effect of PP2A on MEK and ERK is mediated by a B-containing holoenzyme, while a B′-containing holoenzyme has antiapoptotic activity [10]. Sontag *et al.* [50] provided further evidence that PP2A inhibits growth using SV40 small T as a tool to inhibit PP2A in cells. They demonstrated that inhibition of PP2A by small T induces growth through activation of PI3K, which acts upstream of protein PKCζ and MEK: ST (PP2A) →→ PI3K → PKCζ → MEK → ERK. They proposed that small T stimulates PKCζ-dependent but not serum-dependent growth; therefore, its effect can only be measured in serum-starved cells. It is difficult to determine the contribution of the PKCζ → MEK pathway to cell growth as compared to the growth-factor-dependent pathway involving Ras and Raf. The substrate of PP2A acting upstream of PI3K remains to be determined. While these reports emphasize the growth inhibitory role of PP2A, Kubicek *et al.* [51] showed that PP2A is a positive regulator of the MAP kinase pathway. The PP2A core enzyme binds to Raf-1 and dephosphorylates Ser 259. This causes activation of Raf-1, not by affecting its specific activity but by facilitating its association with the plasma membrane. Taken together, PP2A plays opposing roles at different sites in the MAPK → ERK pathway. Whether PP2A acts at all sites simultaneously or whether its positive and negative effects occur in different cells or in response to different environmental stimuli remains to be investigated.

Summary

PP2A plays a critical role in growth control and cancer. Importantly, loss or alteration of PP2A activity is an essential step in the development of human cancer, consistent with the idea that PP2A functions as a tumor suppressor. However, PP2A has many, sometimes seemingly conflicting, functions that are poorly understood. On the one hand, it suppresses cell growth, but on the other it is required for cell-cycle progression. Also, it positively and negatively regulates the MAPK/ERK and Wnt signaling pathways. Other important functions of PP2A are its inhibitory role in the interleukin-3-stimulated JAK2-STAT5 signaling pathway [52] and its involvement in NF-κB signaling [50,53], protein kinase B/Akt signaling [54], and integrin-mediated regulation

of Akt and GSK3β [55]. Furthermore, the catalytic subunit of PP2A binds to the alpha-4 protein, a homolog of yeast TAP42 involved in translational control [9,56].

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CHAPTER 105

Serine/Threonine Phosphatase Inhibitor Proteins

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Introduction

Reversible protein phosphorylation is the most widely used mechanism for regulating the physiology of eukaryotic cells. Current estimates indicate that as much as 10% of the human genome is utilized in the control of protein phosphorylation. Up to 1000 human genes encode protein kinases; however, significantly fewer genes encode phosphatase catalytic subunits. This finding promoted the viewpoint that selectivity in hormone signaling is principally derived from activation of protein kinases, with the phosphatases playing a more pleotropic role in the control of protein phosphorylation. A number of protein kinases respond to changes in intracellular second messengers but only one serine/threonine phosphatase, calcineurin (or PP2B), is activated by the second messenger calcium. This observation fostered the opinion that most phosphatases are unregulated, and it was suggested that hormone-induced increases in protein kinase activity must be sufficiently high as to overcome the opposing actions of phosphatases. It also meant that hormone signals are severely dampened and/or slowed by the constitutive activity of phosphatases. However, it was found that phosphatase inhibitors allow cells to lower this barrier and accelerate or even amplify the kinase signals. The inherent appeal of this mechanism prompted an active search for hormone-regulated phosphatase inhibitors. Work over the last two decades has identified numerous gene products that regulate protein serine/threonine phosphatases, firmly dismissing the idea of unregulated serine/threonine phosphatases.

Protein Phosphatase 1 (PP1) Inhibitors

Huang and Glinsmann [1] and Lee and co-workers [2] first noted changes in phosphorylase phosphatase activity in extracts of rabbit skeletal muscle stimulated by adrenaline. Later studies [3] revealed two inhibitory activities, inhibitor-1 (I-1) and inhibitor-2 (I-2), which potently inhibit the skeletal muscle phosphorylase phosphatase. I-1 is an effective inhibitor only after it is phosphorylated by cAMP-dependent protein kinase (PKA) and is most likely responsible for hormone-induced reduction in phosphorylase phosphatase activity in the muscle extracts. In contrast, I-2 was found to be a constitutive inhibitor. The availability of these inhibitor proteins made it apparent that not all muscle phosphorylase phosphatase activity is eliminated by these proteins, opening the way for separation of muscle serine/threonine phosphatase activity into two general groups. Type 1 phosphatase (PP1) was defined as phosphorylase phosphatase activity that is potently inhibited by I-1 and I-2, and type 2 phosphatases are insensitive to low concentrations of these inhibitors [4]. Subsequent studies further separated type 2 phosphatases based on subunit structure and substrate specificity. Protein phosphatase-2A (PP2A) dephosphorylates phosphorylase *a*, while protein phosphatases 2B and 2C have been observed to have little activity against this substrate and are much more effective against other phosphoproteins. PP2A, PP2B, and PP2C are all insensitive to I-1 and I-2.

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I-1, DARPP-32, and Other Phosphorylation-Dependent Phosphatase Inhibitors

Protein phosphatase 1 (PPI) is responsible for most of the serine/threonine phosphatase activity in skeletal muscle and was the first phosphatase catalytic subunit to be purified to homogeneity. Thus, much of our current knowledge of phosphatase regulation is derived from studies of PP1. I-1 and its structural homolog, DARPP-32 (dopamine and cAMP-regulated phosphoprotein of apparent M_r 32,000) are both PKA-activated PP1 inhibitors. It has been speculated that phosphorylation of I-1 (and DARPP-32) is among the earliest events that follow PKA activation. This results in PP1 inhibition and greatly enhances the phosphorylation of other substrates by PKA. PP1 inhibition also promotes protein phosphorylation by other protein kinases, thus I-1 and DARPP-32 broaden the cAMP signals and impose cAMP regulation on proteins that are not directly phosphorylated by PKA. The most compelling evidence for the importance of cAMP-mediated phosphatase inhibition in amplifying hormone signals comes from the disruption of mouse genes encoding I-1 and DARPP-32. The DARPP-32 mutant mouse, in particular, was impaired in nearly all aspects of dopamine signaling. The importance of this finding was recognized by the 2000 Nobel Prize for Medicine or Physiology being awarded to Paul Greengard of Rockefeller University. A more complex phenotype was seen in the I-1 null mouse, in part reflecting the presence of multiple I-1 genes. Recent studies also show that I-1 associates with PP1 complexes that contain other regulators, such as the protein product of growth arrest and the DNA-damage-inducible gene, GADD34. This suggests that I-1 functions may be directed or restricted by other PP1 regulators, and this cooperation is necessary for the function of I-1 in regulating protein translation.

Both I-1 and DARPP-32 are the most extensively studied phosphatase inhibitors. A considerable amount of data points to a critical role for PKA phosphorylation in the function of these proteins as PP1 inhibitors. Deletion analyses have also highlighted an N-terminal tetrapeptide sequence conserved in I-1 and DARPP-32 as also being essential for PP1 inhibition. Crystallization of a PP1 catalytic subunit with a peptide containing a homologous sequence has demonstrated its docking at a unique site on the PP1 catalytic subunit [5]. It is now clear that the PP1-docking motif (KIXF) is also conserved in many other PP1 regulators. Virtually all PP1-binding proteins containing this motif inhibited the *in vitro* dephosphorylation of phosphorylase *a* by PP1. Some of these proteins, such as the glycogen-binding and the myosin-binding subunits, promote PP1-mediated dephosphorylation of other substrates—glycogen synthase and myosin, respectively. They also associate with subcellular structures, such as glycogen and myofibrils, defining them as PP1-targeting subunits. Unless a substrate or a location can be defined for a KIXF-containing PP1-binding protein, it is impossible to predict whether this putative regulator acts a PP1 inhibitor or targeting subunit.

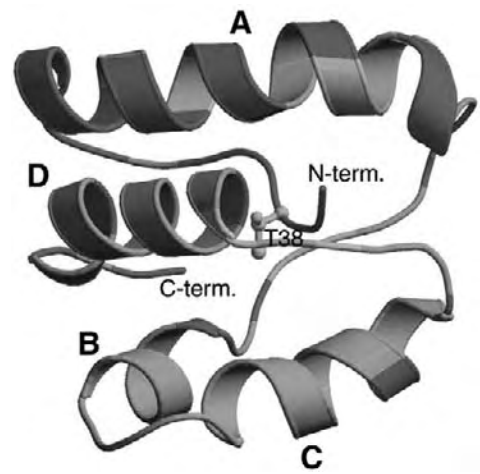


Figure 1 Three-dimensional structure of the PKC-activated PP1 inhibitor, CPI-17. NMR structures of both unphosphorylated and PKC-phosphorylated CPI-17 were solved. Overlap of the two structures shows a bundle of four helices, labeled A, B, C and D. Regions that undergo little significant modification (blue) following phosphorylation and those most significantly modified (red) are shown in color. The threonine-38 phosphorylated by PKC is shown in yellow.

Protein phosphatase 1 activity also responds to PKC activation, and several PKC-activated phosphatase inhibitors have been identified. The forerunner of this family of inhibitor proteins is CPI-17 (C-kinase-activated phosphatase inhibitor of apparent M_r 17,000). Several kinases (PKCα and δ, ROCK, PKN, and Zip-like kinase) promote CPI-17 phosphorylation and increase its activity as a PP1 inhibitor. While the precise mode of action of this inhibitor is unclear, CPI-17 does not contain a KIXF motif. This suggests that other molecular determinants can also specify PP1 selectivity. The three-dimensional structure of CPI-17 has recently been determined (Fig. 1). The most significant conformational change induced by PKC phosphorylation in the four-helix bundle that is CPI-17 is the rotation of helix A to create a new surface that may facilitate PP1 binding and inhibition [6]. CPI-17 and its structural homologs PHI-I, PHI-II, and KEPI most effectively target the PP1 holoenzyme containing the myosin-binding subunit. How the regulatory subunit and inhibitor collaborate to regulate myosin dephosphorylation is currently under investigation. The KEPI mRNA is elevated in brain in response to morphine. This suggests that G-protein-coupled receptors activate PKC signaling to promote KEPI expression and phosphorylation and lower PP1 activity to transduce hormone signals.

Latent Phosphatase Complexes Activated by Inhibitor Phosphorylation

I-2 is a complex regulator of PP1 activity, requiring as many as five distinct interactions with the PP1 catalytic subunit to inhibit (rapid and reversible suppression of PP1 activity) and inactivate (slower and more stable reduction in

activity) the enzyme. PP1 inactivation by I-2 can be reversed by glycogen synthase kinase 3 (GSK3), mitogen-activated protein kinase (MAPK), or Cdk5, which phosphorylate I-2. Thus, the PP1/I-2 complex may represent a latent cytosolic pool of serine/threonine phosphatase that is commissioned into action by growth factors and hormones. Over-expression of I-2 (and its budding yeast homolog, Glc8) has paradoxical effects on cell physiology, consistent with both activation and inhibition of PP1. The ability of I-2 to refold denatured PP1 *in vitro* has prompted the hypothesis that I-2 may be a PP1 chaperone that refolds the newly synthesized phosphatase catalytic subunit. Subsequent exchange or delivery of PP1 to various targeting subunits may account for the increase in PP1 activity.

NIPP-1 (nuclear inhibitor of PP1) is another phosphatase inhibitor protein that is stably associated with the nuclear pool of PP1 catalytic subunits. The activity of this phosphatase complex is increased by NIPP-1 phosphorylation by PKA or casein kinase II. Interestingly, the two protein kinases have additive effects on activation of the PP1/NIPP-1 complex and may integrate distinct hormone signals to control nuclear PP1 activity. NIPP-1 also demonstrates functions independent of PP1 binding. Recent studies showed that the NIPP-1 mRNA suppresses protein translation, while the NIPP-1 protein possesses endonuclease activity. Elevated NIPP-1 mRNA levels have been correlated with increased malignancy of rat hepatomas but it is uncertain which of the NIPP-1 functions contributes to carcinogenesis.

Inhibitors of Type-2 Serine/Threonine Phosphatases

A number of proteins bind type 2 phosphatases and inhibit their activity. Following a strategy similar to that used to isolate I-1 and I-2, two thermostable protein inhibitors of PP2A were isolated. I_1^{PP2A} and I_2^{PP2A} not only inhibit PP2A but also activate PP1 *in vitro*, suggesting that they function as molecular switches that reciprocally regulate the two major eukayotic serine/threonine phosphatases. Over-expression of both proteins increased c-*jun* expression and transcription of genes regulated by the AP-1 transcription factor in cultured cells, consistent with PP2A inhibition. Protein products of two DNA tumor viruses, SV40 and polyoma, also inhibit PP2A activity. Unlike I_1^{PP2A} and I_2^{PP2A}, SV40 small T and polyoma middle T do not directly associate with the PP2A catalytic subunit. Instead, the viral proteins displace B subunits from selected cellular PP2A heterotrimers composed of A, B, and C subunits. This suggests that cellular proteins such as the many B-subunits can also modulate PP2A activity in cells, inhibiting some functions while activating others.

In contrast to PP1 and PP2A, PP2B or calcineurin shows a much more restricted panel of substrates, at least *in vitro*. A number of cellular proteins sharing a conserved PP2B-binding sequence, first identified in the transcription factor NFAT (nuclear factor of activated T cells), a PP2B substrate. These include CAIN, MCIP, and the product of the disease gene associated with Down's syndrome, the thyroid hormone-inducible protein ZAKI-4. Over-expression of these proteins suppresses PP2B functions consistent with their actions as PP2B inhibitors. PP2B also binds to immunophilins that in turn bind the immunosuppressive drugs cyclosporin and FK506 to inhibit calcineurin activity. Experimental evidence suggests that some immunophilins bind PP2B in the absence of drugs and may regulate its phosphatase activity.

Conclusions

Emerging evidence suggests that mammalian cells express a multitude of serine/threonine phosphatase inhibitors, many of which are not fully analyzed. Comparison of known serine/threonine phosphatase inhibitor proteins suggests that they utilize many different mechanisms to inhibit phosphatases. Moreover, the phosphatase inhibitors may be regulated by hormone-induced changes in expression, alternate splicing, or reversible phosphorylation. Finally, inhibitor proteins most likely collaborate with other phosphatase regulators to control unique phosphatase populations and integrate multiple physiological signals that regulate protein phosphorylation.

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CHAPTER 106

Calcineurin

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Introduction

Calcineurin (also called PP2B), a protein phosphatase under the control of Ca^{2+} and calmodulin (CaM), is ideally suited to play an important role in modulating cellular responses in response to the second messenger Ca^{2+}. The identification of calcineurin as the target of the immunosuppressive drugs cyclosporin A (CsA) and FK506, complexed with their respective binding proteins cyclophilin A (CypA) and FKBP12 (FK506 binding protein), revealed the key role of calcineurin in the Ca^{2+}-dependent steps of T-cell activation [1]. This discovery led to purification of the transcription factor NFAT (nuclear factor of activated T cells) and the first identification of a complete transduction pathway from the plasma membrane to the nucleus [2,3]. The specific inhibition of calcineurin by FK506 and CsA and the over-expression of the catalytic subunit of a CaM-independent derivative of calcineurin (calcineurin Aα, residues 1 to 392) have been widely used to identify the roles of calcineurin in the regulation of cellular processes as diverse as gene expression, ion homeostasis, muscle differentiation, embryogenesis, secretion, and neurological functions. It is no wonder that alteration of calcineurin activity has been implicated in the pathogenesis of such diseases as cardiac hypertrophy, congenital heart disease, and immunological and neurological disorders. For further information, the reader is referred to comprehensive reviews and references therein [2–5b].

Enzymatic Properties

The serine/threonine phosphatase activity of calcineurin is completely dependent on Ca^{2+} concentrations found in stimulated cells (0.5–1 μM). A 19-residue synthetic peptide containing the phosphorylation site of the RII subunit of cAMP-dependent protein kinase (PKA) is routinely used to measure the phosphatase activity of the purified enzyme. A small activation is observed upon addition of Ca^{2+} ($K_{act} = 0.5\ \mu M$), while addition of an equimolar amount of CaM results in a 50- to 100-fold increase of the V_{max} [4]. The cooperative Ca^{2+} dependence of the CaM stimulation (Hill coefficient of 2.5 to 3) allows calcineurin to respond to narrow Ca^{2+} thresholds following cell stimulation. Because of its high affinity for CaM ($K_{act} \leq 10^{-10}\ M$), the activation of calcineurin in response to a Ca^{2+} signal can precede the activation of most, if not all, CaM-regulated enzymes.

In crude extracts, calcineurin activity is distinguished from that of PP1, 2A, and 2C by (1) its Ca^{2+} and CaM dependence; (2) its resistance to the endogenous inhibitors (inhibitor-1, DARPP-32, inhibitor-2), okadaic acid, microcystin, and calyculin; and (3) its specific inhibition by FK506 (but not rapamycin) and CsA in the presence of saturating amounts of their respective binding proteins, FKBP12 and CypA [2]. The crude enzyme, with a specific activity 10 to 20 times that of the purified enzyme, is subject to a time- and Ca^{2+}/CaM-dependent inactivation that is prevented by superoxide dismutase and reversed by ascorbate. This observation suggested that *in vivo* calcineurin activity may also be modulated by reactive oxygen species [6]. This reversible inactivation provides a mechanism for the temporal regulation of the protein phosphorylation by CaM-dependent kinases and phosphatases [7]. Determination of calcineurin activity *in vivo* can only be achieved by monitoring the extent of dephosphorylation of endogenous substrates, such as the transcription factor NFAT, if they are present at detectable levels [2].

The substrate specificity of calcineurin depends not only on recognition of the sequence surrounding the phosphorylated residues but to the presence of docking domains. NFAT contains two such domains responsible for its Ca^{2+}-dependent and phosphorylation-independent anchoring to

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calcineurin [2,8]. The anchoring of NFAT allows its dephosphorylation, despite its low intracellular concentration, and the nuclear cotranslocation of calcineurin and NFAT.

Structure

Calcineurin is also characterized by a unique and highly conserved subunit structure. It is a heterodimer of a 58- to 64-kDa catalytic subunit, calcineurin A (CnA), tightly bound even in the presence of EGTA, ($K_d \leq 10^{-13}$ *M*), to a regulatory 19-kDa regulatory subunit, calcineurin B (CnB). CnB is an EF-hand Ca^{2+} binding protein of the CaM family. It binds 4 mol of Ca^{2+}, two with high affinity (K_d $^{-7}$ *M*) and two with moderate affinity, in the micromolar range [9,10]. The crystal structure of the recombinant α-isoform of human calcineurin (Fig. 1A) confirmed the domain organization of CnA predicted by limited proteolysis and site-directed mutagenesis [2,4]. The N-terminal two-thirds of the molecule contains the catalytic domain, the structure of which is similar to those of PP1 and PP2A. The active site contains an Fe^{3+}-Zn^{2+} dinuclear metal center [11]. Iron and zinc are bound to residues provided by the two faces of a β-sandwich. The last β-sheet extends into a five-turn amphipathic α-helix, the top polar face of which is covered by a 33-Å groove formed by the N- and C-terminal lobes and the C-terminal strand of CnB. With the exception of two short α-helices corresponding to the inhibitory domain blocking the catalytic center, the C-terminal regulatory domain (including the CaM binding domain), not visible in the electron density map, is flexible and sensitive to proteolytic attack in the absence of CaM. The catalytic and CnB binding domain (residues 1 to 392), associated with the fully liganded form of CnB, is resistant to proteolysis. It is fully activated in the absence of CaM but still requires the presence of less than 10^{-7} *M* Ca^{2+} [2].

The crystal structure of a proteolytic derivative of bovine calcineurin (residues 15 to 392) complexed with FKBP12–FK506 (Fig. 1B) is similar to that of the recombinant protein. Myristic acid, covalently linked to the N-terminal glycine of CnB, lies parallel to the hydrophobic face of the N-terminal helix of CnB. This perfectly conserved posttranslational modification of CnB is apparently not involved in membrane association or required for enzymatic activity. It may serve as a stabilizing structural element and is required for interaction with phospholipids [2,4,12]. The polar bottom face of the CnB binding helix of CnA and CnB forms the site of interaction with the FKBP–FK506 and CsA–CyP complexes. The key role of CnB in forming the drug binding site provides a molecular basis for the exquisite specificity of FK506 and CsA as calcineurin inhibitors.

Regulation

Role of CaM

The crystal structure of calcineurin helps to define the different roles and mechanisms of action of the two structurally similar Ca^{2+}-regulated proteins, CaM and CnB, in the regulation of calcineurin. The catalytic center blocked by the inhibitory domain and the flexible calmodulin binding domain, freely accessible for calmodulin binding, is consistent with the widely accepted mechanism of CaM stimulation of CaM-regulated enzymes. According to this mechanism, binding of CaM results in the displacement of the inhibitory domain and exposure of the catalytic center [4]. The requirement for Fe^{2+} (as opposed to Fe^{3+}) for calcineurin activity explains the redox sensitivity of calcineurin activity in crude tissue extracts [13,14]. Crude and ascorbate-activated purified calcineurin is an Fe^{2+}–Zn^{2+} enzyme with an optimum pH of 6.1 [14]. The Ca^{2+}/CaM-induced exposure of Fe^{2+} facilitates its oxidation, which is responsible for the inactivation of the enzyme. Partial depletion of iron and zinc as well as oxidation of the iron during the purification procedure are responsible for the low activity of the purified enzyme and its stimulation by 0.1-m*M* Mn^{2+} and 6-m*M* Mg^{2+} [14].

Role of CnB

CnB serves both a structural and a regulatory role. Ca^{2+}-independent binding of CnB to CnA, mediated by the high-affinity C-terminal sites, ensures the folding of active enzyme [9,10]. Ca^{2+} binding to the N-terminal sites induces a conformational change of the regulatory domain resulting in the exposure of the drug and CaM binding domains [1,15].

Endogenous Regulators

The presence of anchoring and inhibitory proteins, for which expression varies from tissue to tissue, adds another level of complexity to calcineurin regulation. The PKA scaffold protein, AKAP79, anchors calcineurin to specific sites of action but also inhibits its activity (see Volume II, Chapter 185). Calsarcin-1 and -2, which tether calcineurin to α-actinin at the z-line of the sarcomere in cardiac and skeletal muscle, respectively, have been proposed to couple calcineurin activity to muscle contraction [16]. Calsarcins interact with calcineurin close to its active site and inhibit its activity. What is not clear is how the Ca^{2+}-independent binding of AKAP79 (K_I = 200 n*M*) and the inhibition of calsarcin are reversed.

Cain/Cabin1, a 240-kDa nuclear protein of yet unknown function, has been identified as a noncompetitive inhibitor of calcineurin [17,18]. It is abundant in brain, kidney, liver, and testis, but is absent in muscle. *In vivo* binding of Cabin1 to calcineurin requires Ca^{2+} and PKC activity and is inhibited by FK506–FKBP, suggesting that it binds at the drug interacting site. A basic domain in the C terminus of Cabin1 has been identified as a calcineurin binding site [17].

A family of 22- to 24-kDa proteins identified in yeast (RCn1p) and mammalian cells (calsuppressins; MCIP1, 2, and 3) are believed to be feedback inhibitors of calcineurin [19,20]. The mammalian proteins are identical to proteins encoded in the DSCR1 (Down's syndrome critical region 1) gene on chromosome 21 (ZAKI-4, DSCRIL1, and DSCRIL). Their expression in heart and skeletal muscle is upregulated

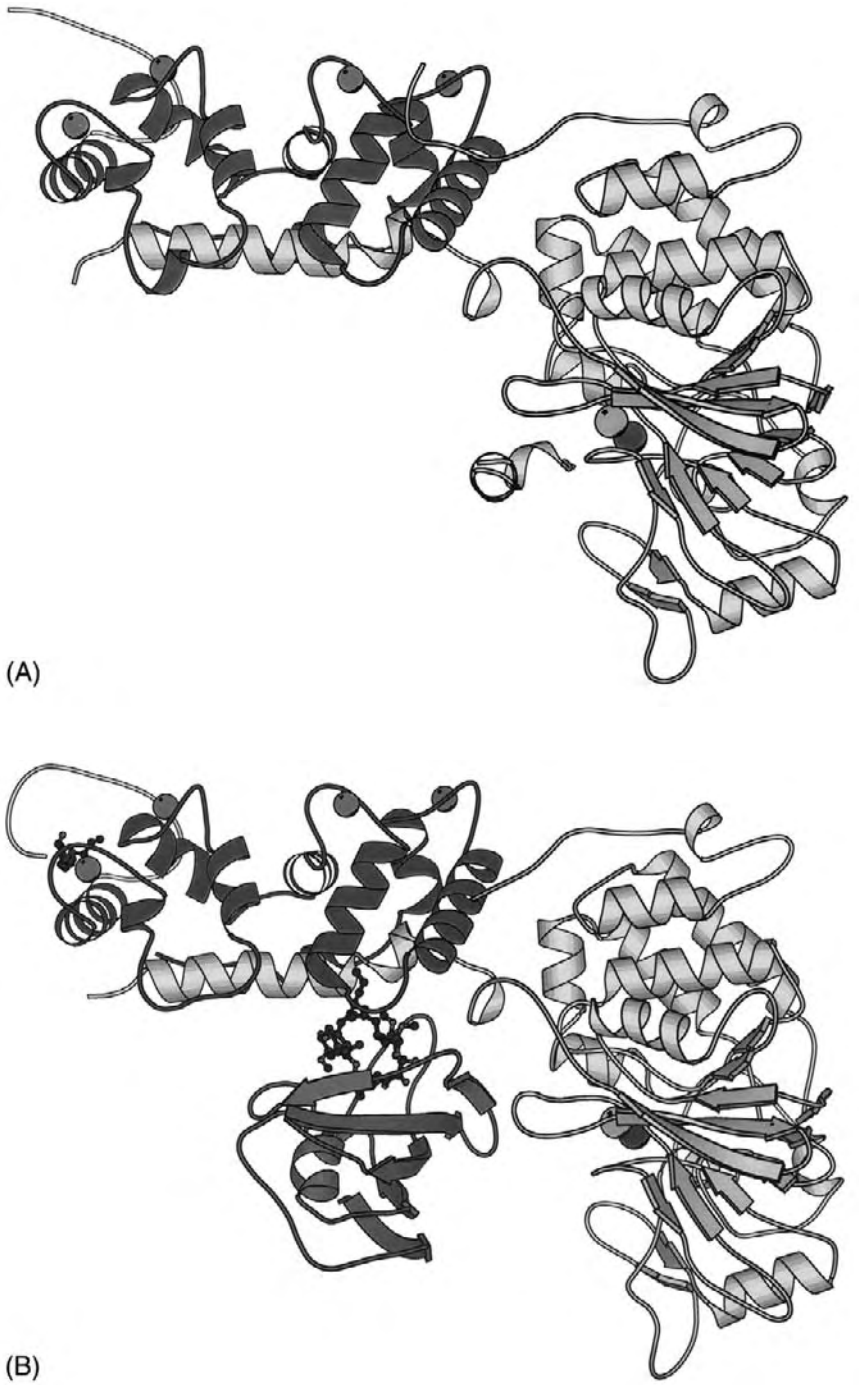


Figure 1 Ribbon representation of (A) the crystal structure of human recombinant α-calcineurin and (B) truncated calcineurin complexed with FKBP12–FK506. CnA is shown in light gray, CnB in dark gray, Iron and zinc are shown as light gray and black spheres, respectively. The four Ca^{2+} bound to CnB are shown as dark gray spheres, and FKBP12 is shown in dark gray. Myristic acid, covalently linked to the N-terminal glycine, and FK506 are shown in ball and stick representations (PDB code 1AUI [43] and 1TCO [44]).

by a calcineurin/NFAT-dependent mechanism. MCPs inhibit both calcineurin activity and expression. They do not compete with CaM or FK506/FKBP but interact with calcineurin in a Ca^{2+}-independent fashion through a highly conserved ISPPxSPP motif, similar to the SP motifs of NFAT; they inhibit calcineurin activity *in vitro* as well as NFAT activation *in vivo*.

Distribution and Isoforms

Although found predominantly in neural tissues, calcineurin is present in all eukaryotes and in all tissues examined. There are three mammalian isoforms (α, β, γ) of calcineurin. The human genes (PPP3CA, PPP3CB, PPP3CC) are located on human chromosomes 4, 10, and 8, respectively.

Additional isoforms, products of alternative splicing, have not been characterized at the protein level. An N-terminal polyproline motif is a conserved feature of the β isoform. A C-terminal nuclear localization signal and stretch of basic residues are responsible for the high pI (7.1) of the testis-specific γ isoform, whereas the neural α and the broadly distributed β isoform have pIs of 5.6 and 5.8, respectively [2,4]. Two mammalian isoforms of calcineurin B—CnB1 (associated with the α and β isoforms of CnA) and CnB2 (expressed only in testis)—are the products of two genes: PPP3R1 (located on chromosome 2) and PPP3R2 [2]. The α and β isoforms have been expressed in SF9 cells and bacteria where coexpression of the two subunits is required to yield a soluble recombinant β isoform [2].

Functions

T-Cell Activation

The T-cell Ca^{2+}/calcineurin/NFAT pathway, also shown to be applicable to other cell types [2,3–5], requires a sustained release of Ca^{2+} from IP_3-sensitive stores [21]. Four NFAT isoforms (NFAT1-4) share a conserved N-terminal regulatory domain composed of a serine-rich motif, a nuclear localization signal, and three SP motifs flanked on both sides by two calcineurin binding motifs [2,8]. The N-terminal motif, PxIxIT, missing in NFAT5, binds calcineurin ($K_d = 2.5\ \mu M$) at a site tentatively identified as residues 1 to 14 [22], and the C-terminal motif binds calcineurin ($K_d = 1.3\ \mu M$) at a site that may overlap with the drug binding domain [8]. Dephosphorylation of NFAT results in nuclear translocation of the calcineurin–NFAT complex and enhancement of DNA binding and transcriptional activity [2,3]. Nuclear export depends on NFAT rephosphorylation upon removal of Ca^{2+} or calcineurin inactivation (or dissociation?). Identifying the mechanisms and kinases involved in the process has been elusive. GSK3, casein kinase 1/MEKK1, and Jun N-terminal kinase (JNK) have all been implicated [2,3]. The specificity of these kinases for different isoforms of NFAT as well as the complexity due to cell background may be responsible for the failure to identify a single kinase responsible for this process [2,3].

Muscle Differentiation

Calcineurin-mediated dephosphorylation of two transcription factors, NFAT3 and MEF2, plays a critical role in the switch of muscle fiber subtype that follows the onset of innervation and nerve activity [5]. This contractile phenotype transition consists of an increased expression of slow fiber proteins (slow MHC-1, SERCA 2a) and decreased expression of the fast fiber proteins (MHC2a, SERCA1, creatine kinase, citrate synthase). It is achieved by NFAT as well as by MEF2D activation through calcineurin-mediated dephosphorylation and a Ca^{2+}-dependent phosphorylation of MEF2D at specific serines residues [23]. The previously reported role of calcineurin in muscle hypertrophy is controversial. No hypertrophy is observed in transgenic mice over-expressing calcineurin [24]; activation of the mTOR (phosphatidylinositol 3-kinase [PI3K]/AKT) pathway plays a major role in the insulin-like growth factor 1 (IGF-1)-induced hypertrophy of preformed myotubes [25]; and calcineurin inhibitors do not block the increase of fiber size induced by nerve stimulation in regenerating muscle [26].

Cardiac Hypertrophy

Calcineurin-mediated activation of NFAT3, which, in conjunction with the transcription factor GATA4, leads to the induction of fetal cardiac genes along with natriuretic factor, have been shown to play a major role cardiac hypertrophy [5]. Calcineurin can also induce cardiac hypertrophy acting synergistically with a Ca^{2+}-dependent kinase to activate MEF2D. Regardless of its cause (over-expression of CaM-independent calcineurin, pressure overload, induction by hypertrophic agonists), cardiac hypertrophy is prevented by over-expression of MCP1 and the inhibitory domains of Cabin1/Cain or AKAP79 [5,27,28]. Less reproducible inhibition by FK506 and CsA may be due to high levels of calcineurin or low levels of binding proteins [5]. Furthermore, the hypertrophic response to calcineurin activation is impaired in transgenic mice expressing a constitutive form of GSK3 [28], and transgenic mice lacking the β-isoform of calcineurin (the predominant isoform in heart) have impaired ability to develop cardiac hypertrophy in response to hypertrophic agonists [29]. The calcineurin-mediated activation of PKC by calcineurin [30,31] suggests that calcineurin, acting upstream of PKC, can also be implicated in cardiac hypertrophy through the activation of the PKC and JNK in parallel or downstream of MAP kinase pathways.

Cell Death and Differentiation

Emphasizing the general role of calcineurin in the regulation of gene expression is the broad tissue distribution of NFAT and the many genes for which expression is directly induced by NFAT—NFAT2; the cytokines IL-2, -3, -4; TNFα GM-CSF; IFNγ the chemokines IL-8 and MIP-1a; and the receptors FasL, CD40L, CTLA-4, NF-AT2, Oct2, Egr, NF-κB50p—as well as other genes activated by calcineurin, such as Elk-1 and BAD) [2,3]. Three calcineurin-mediated pathways—NFAT activation of NF-κB and FasL, synergistic induction of a member of the steroid/thyroid receptor family, Nur77, by NFAT and MEF2D; and dephosphorylation of BAD—perhaps explain the involvement of calcineurin in Ca^{2+}- and possibly H_2O_2-induced apoptosis [2,32,33]. The calcineurin/NFAT pathway is essential for the development of heart valves and the vascular developmental pattern during embryogenesis [34].

Ion Homeostasis

In yeast and fungi, calcineurin plays a major role in the regulation of ion homeostasis by a mechanism similar to the

NFAT-mediated transcriptional control in mammalian cells [35–37]. In addition to its regulation of the Na^+/K^+ pump in kidney, recent evidence indicates that the Ca^{2+}/calcineurin/NFAT pathway is also responsible for the regulation of expression of the inositol 1,4,5-triphosphate (IP3) receptors, the plasma membrane Ca^{2+} pumps, and the Ca^{2+} exchanger in mammalian cells [38]. It was also reported that the regulation of Ca^{2+} fluxes from the IP3 and ryanodine channels was mediated by an FKBP-mediated interaction of calcineurin with the receptors acting as endogenous analogs of FKBP, but recent evidence indicates that calcineurin does not interact with either one of these two receptors [39].

Neuronal Functions

Consistent with the high concentration of calcineurin in the brain (1% of total protein), the list of neuronal functions modulated by calcineurin is continuously expanding. A major role of calcineurin in brain is to trigger a protein phosphatase cascade initiated by the dephosphorylation of two endogenous inhibitors of PP-1 (inhibitor-1 and DARPP-32). It is sensitive to PP-1 as well as calcineurin inhibitors. The dephosphorylation of these inhibitors, which do not contain anchoring domains, is not inhibited by specific inhibitors of NFAT [2]. This cascade counteracts the stimulatory effects induced by cAMP- and Ca^{2+}-regulated kinase. It has been shown to explain the antagonistic effects of glutamate binding to the NMDA receptor and dopamine binding to the D1-like dopamine receptors in striatal neurons [40], as well as the complex regulation of synaptic plasticity that includes induction of long-term potentiation (LTP) and long-term memory [41,42] and the modulation of the activity of the transcription factor CREB [7]. Calcineurin plays an important role in cellular trafficking by dephosphorylating a family of proteins involved in endocytosis and in the release of neurotransmitters [2]. Dephosphorylation of other calcineurin substrates involved in the downregulation of receptor- and voltage-gated channels remains to be identified. Two other potentially important substrates of calcineurin are NO synthase and adenylate cyclase [2].

Conclusion

The importance of calcineurin in the regulation of cellular processes and its involvement in the pathogenesis of many diseases is now well established, but the role of other signaling molecules should not be underestimated. To fully assess the contribution of calcineurin in the transduction of so many diverse signals it is evident that we must understand how different pathways interact with each other.

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CHAPTER 107

Protein Serine/Threonine-Phosphatase 2C (PP2C)

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Introduction

In early biochemical studies of protein phosphatase activities in mammalian tissues, protein serine/threonine-phosphatase 2C (PP2C) was defined as a Mg^{2+}- or Mn^{2+}-dependent serine/threonine-specific activity that is insensitive to a phosphatase inhibitor, okadaic acid [1]. Subsequent isolation of PP2C genes from yeast to humans demonstrated that PP2C is an evolutionarily conserved phosphatase family that shares no apparent similarity in amino acid sequence with other phosphatase families. However, the crystal structure of the PP2C catalytic core shows significant resemblance to those of other serine/threonine phosphatases, such as PP1 [2]. PP2C functions as a monomer, and no regulatory subunit has been reported.

Eukaryotic organisms appear to have multiple PP2C genes; even the simplest eukaryotes, the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, have five PP2C genes in their genomes. In humans, there are at least four isoforms of the PP2C phosphatase: α, β, γ, and δ. Several genes encoding PP2C-like phosphatases have also been found in the genomes of other organisms such as the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*. *Arabidopsis thaliana*, the most popular plant model system, has more than 30 PP2C genes in its genome. Intriguingly, several Mg^{2+}-dependent phosphatases in prokaryotes also possess clear sequence similarities with eukaryotic PP2C enzymes.

PP2Cs and related protein phosphatases are implicated in numerous biological processes in different organisms. In this chapter, we will review various signal transduction mechanisms from bacteria to mammals for which the molecular functions of PP2C enzymes have been relatively well elucidated.

Regulation of the Stress-Activated MAP Kinase Cascades

One of the well-defined roles of PP2C in cell signaling is the downregulation of the stress-activated MAP kinase cascades in eukaryotes. A MAPK cascade is composed of three kinases: mitogen-activated protein kinase (MAPK), MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK). Extracellular stimuli are transmitted into the nucleus through sequential phosphorylation of these kinases; a signal-stimulated MAPKKK phosphorylates a specific MAPKK, which in turn phosphorylates the conserved threonine and tyrosine residues of its cognate MAPK [3]. Subsequently, an activated MAPK phosphorylates and activates nuclear transcription factors, inducing a set of genes for cellular responses. Consequently, dephosphorylation of any of the kinases in the cascade results in downregulation of the final outcome.

Studies in the budding yeast *S. cerevisiae* have shown that the multicopy expression of PP2C genes rescues the mutations that cause hyperactivation of the Hog1 osmosensing MAPK cascade [4]. In the fission yeast *S. pombe*, inactivation of the stress-activated MAPK Spc1 (also known as Sty1) suppresses the phenotypes of PP2C-deficient cells [5,6]. These genetic data suggested that PP2C phosphatases negatively regulate the MAPK pathways through dephosphorylation of one or more components. Subsequent biochemical studies demonstrated that the fission yeast PP2C enzymes

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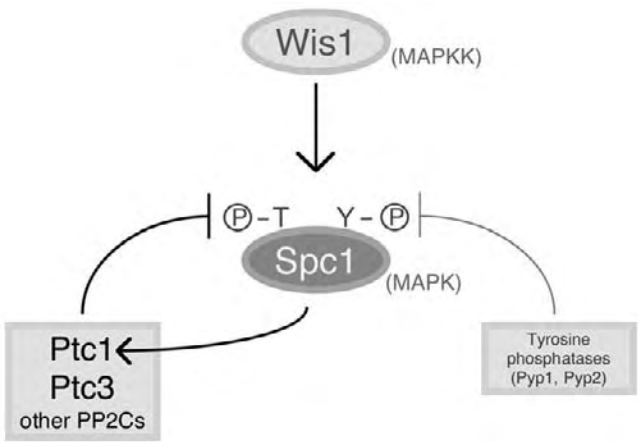


Figure 1 Downregulation of the Spc1 MAP kinase by PP2Cs. The Wis1 MAPKK is activated by extracellular stress phosphorylate Thr-171 and Tyr-173 residues in the activation loop of the Spc1 MAPK. Subsequently, activated Spc1 phosphorylates downstream transcription factors, which in turn induce stress response genes as well as *ptc1*$^+$ encoding a PP2C phosphatase. Ptc1 dephosphorylates Spc1 Thr-171 to inactivate the Spc1 pathway. Ptc3, the other PP2C dephosphorylating Spc1, is expressed constitutively. Phosphorylation of Spc1 Tyr-173 is negatively regulated by two tyrosine-specific phosphatases, Pyp1 and Pyp2.

Ptc1 and Ptc3 dephosphorylate the Spc1 MAPK at Thr-171 [7], for which phosphorylation is essential for Spc1 activity [8]. Interestingly, the *ptc1*$^+$ gene is transcriptionally induced by activation of the Spc1 pathway, while the *ptc3*$^+$ gene is expressed constitutively [9]. Thus, PP2C participates in suppressing the Spc1 MAPK under normal growth conditions and in a negative feedback regulation of Spc1 activated by stress (Fig. 1). In budding yeast, a PP2C enzyme, Ptc1, dephosphorylates Thr-173 of Hog1 MAPK, the equivalent of Spc1 Thr-171 in fission yeast [10].

PP2C regulation of stress MAPK cascades is also conserved in mammalian and plant systems. Mammalian cells have two stress-activated MAP kinases, p38 and JNK. PP2Cα inhibits both MAPK cascades by dephosphorylating p38 and its MAPKK, MKK6, as well as the SEK1 MAPKK upstream of JNK [11]. The p38 MAPK is also inactivated by a PP2Cδ isoform, Wip1 [12]. Wip1 is transcriptionally induced in a p53-dependent manner in response to a variety of stresses such as γ-irradiation, ultraviolet irradiation, and H_2O_2. Moreover, Wip1 induction depends on the p38 MAPK, which phosphorylates and stimulates p53 in response to ultraviolet irradiation. Thus, like the Spc1 MAPK and the Ptc1 phosphatase in fission yeast, the mammalian p38 MAPK and Wip1 PP2Cδ form a negative feedback loop. Recently, Wip1-deficient mice have been generated that exhibit defects in reproductive organs, immune function, and cell-cycle control [13]. In alfalfa plants, a stress-inducible PP2C, MP2C, was identified as a negative regulator of the stress-activated MAPK cascade [14].

Control of the CFTR Chloride Channel by PP2C

Cystic fibrosis is the most common autosomal lethal genetic disease among Caucasians, and the protein product of the disease-causing gene is known as the cystic fibrosis transmembrane conductance regulator (CFTR) [15]. CFTR is the known only member of the ATP-binding cassette (ABC) transporter family that forms an ion channel. It is located mostly in the apical membrane of epithelia, where it mediates transepithelial salt and liquid movement. CFTR consists of five domains: two transmembrane domains, two nucleotide-binding domains, and a large cytoplasmic domain called the regulatory domain. The regulatory domain has about 20 potential sites for phosphorylation by protein kinase A (PKA) and C (PKC). Phosphorylation of the regulatory domain by PKA is essential for the Cl^- channel activity of CFTR, which is also positively regulated by PKC.

PP2C has emerged as the most likely phosphatase that negatively regulates CFTR through dephosphorylation [16–18]. PP2C dephosphorylates CFTR *in vitro*, leading to reduction of the Cl^- channel activity. On the other hand, a PP1/PP2A inhibitor (okadaic acid) and a PP2B inhibitor (FK506) do not affect CFTR activity. Coexpression of PP2C with CFTR in epithelia decreases the Cl^- current and increases the rate of the CFTR channel inactivation. PP2C and CFTR form a stable complex *in vivo* that may facilitate inactivation of CFTR in the absence of cAMP stimulation.

Plant Hormone Abscisic Acid Signaling

As described above, the large number of PP2C genes found in the *Arabidopsis thaliana* genome may reflect the importance of PP2C in plant physiology. Among those are *ABI1* and *ABI2* encoding PP2C enzymes in the abscisic acid (ABA) signaling pathway [19,20]. ABA is a plant hormone important for the maintenance of seed dormancy, stomatal closure, and growth inhibition. The loss-of-function mutations in *abi1*/*abi2* genes cause hypersensitivity to ABA. On the other hand, dominant mutations that retain the ABI phosphatase activities are insensitive to ABA, indicating that ABI1 and ABI2 are likely to be negative regulators of ABA signaling. Interestingly, the amount of the *ABI1* and *ABI2* mRNA increases in response to ABA [21], suggesting that ABI PP2Cs are part of a negative-feedback loop in the ABA pathway. The target substrate of the ABI1 and ABI2 phosphatases remains unknown.

Fem-2: A Sex-Determining PP2C in Nematode

The mechanism to achieve sexual dimorphism in the nematode *Caenorhabditis elegans* has been a subject of extensive research. The determinant of sex in this organism is the ratio of X chromosomes to autosomes. Genetic studies have identified a large number of genes required for the induction of sexual dimorphism, including a PP2C gene, *fem-2* [22,23]. Fem-2 has a PP2C-like domain and an amino-terminal, non-catalytic extension, both of which are essential for Fem-2 function [24]. PP2C activity of Fem-2 has been demonstrated biochemically, and the *fem-2* gene can complement

the budding yeast PP2C mutant. Recently, human and rat phosphatases similar to Fem-2 have been isolated [25]. Interestingly, the rat Fem-2 homolog is identical to the previously identified phosphatase that specifically dephosphorylates Ca^{2+}/calmodulin-dependent protein kinases [26].

Stress-Responsive PP2Cs in *Bacillus subtilis*

Bacillus subtilis has five PP2C-like phosphatases, SpoIIE, PrpC, RsbU, RsbX, and RsbP, with the latter three involved in the regulation of the general stress-responsive σ^B factor. The σ^B transcription factor can be activated by energy stress (i.e., starvation of carbon, phosphate, or oxygen) or environmental stress (i.e., high salt, heat shock, or ethanol), leading to the induction of many stress-response genes [27]. Energy and environmental stresses are transmitted by distinct signaling cascades, which are linked by the RsbV anti-anti-σ factor (Fig. 2). Energy stress signaling is mediated by the RsbP phosphatase dephosphorylating RsbV to induce the general stress response through inhibition of the anti-σ factor RsbW [28]. Environmental stress signals are conveyed through dephosphorylation of RsbV by the RsbU phosphatase, which is activated by upstream regulators, including the RsbX phosphatase [29]. In the absence of stress, the kinase activity of the RsbW anti-σ factor represses the pathway via phosphorylation of RsbV.

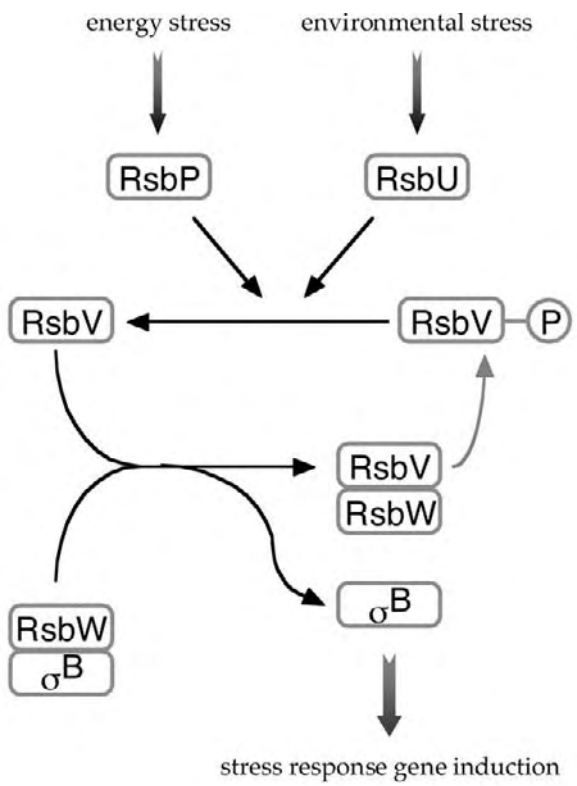


Figure 2 Stress signaling in *B. subtilis*. The anti-anti-σ factor RsbV is dephosphorylated by two PP2C-like phosphatases, RsbP and RsbU, which are activated upon energy or environmental stress, respectively. Dephosphorylated RsbV binds to the RsbW anti-σ factor to release σB. Free σB then induces a set of stress responsive genes. RsbW also has a protein kinase activity that phosphorylates and inactivates RsbV.

RsbP contains a PP2C-like catalytic domain as well as a PAS domain essential for its function *in vivo*. In bacteria, PAS domains with associated chromophores are found in a variety of signal transduction proteins, regulating the activity of a linked output domain in response to changes in the redox potential. The expression level of RsbP is not regulated in response to the energy stress [28], and it is possible that the stress signals modulate the phosphatase activity of RsbP through its PAS domain.

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CHAPTER 108

Overview of Protein Tyrosine Phosphatases

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Background

The phosphorylation of tyrosyl residues in proteins is of paramount importance to the control of such fundamental physiological functions as cell proliferation, differentiation, survival, metabolism, and motility. Initially, research in this area focused on the protein tyrosine kinases (PTKs), following their identification as receptors for growth factors and hormones and as the products of oncogenes. The first description of the existence of protein tyrosine phosphatases (PTPs) can be traced back to early studies of the PTKs. When cells expressing temperature-sensitive mutants of Src were shifted from the permissive temperature for PTK function to the nonpermissive temperature, a rapid decrease in the extent of tyrosine phosphorylation of cellular proteins was observed, reflecting the activity of potent PTPs [1]. However, a description of the nature and properties of these enzymes was harder to obtain. Originally, it was suggested that there would be a small number of PTPs that served essentially a housekeeping function, with the subtlety and sophistication of the regulation of signal transduction exerted at the level of the PTKs. Today, we know that this original concept was incorrect and that signal transduction is tightly regulated at the level of both protein phosphorylation and dephosphorylation. Unlike the protein kinases, which are all descended from a common ancestor, the phosphatases have evolved in separate families. Thus, the PTPs are structurally and functionally distinct from the family of Ser/Thr phosphatases [2]. This overview introduces the structural diversity of the PTP family and highlights some of the recent developments that have stimulated interest in these enzymes as critical regulators of cell function.

Structural Diversity within the PTP Family

The first PTP to be purified and characterized was termed PTP1B [3,4]. Following the determination of its amino acid sequence, its homology with CD45, a transmembrane receptor-like protein of hematopoietic cells, was established [5]. Shortly thereafter, it was demonstrated that CD45 possessed intrinsic PTP activity [6]. This observation was important because it established the existence of receptor-like PTPs with the potential to regulate signal transduction directly through ligand-controlled dephosphorylation of tyrosyl residues in proteins. This triggered great interest in the PTPs and, following application of PCR and low-stringency screening, a wide variety of these enzymes were identified in diverse organisms. The availability of the first draft of the human genome sequence, together with data on the whole genomes of various organisms, now offers the potential to define the composition of the PTP family and to explore evolutionary relationships. Current estimates suggest that the family of PTPs in humans will comprise a total of ~100 enzymes.

The PTPs are defined by the presence of a signature sequence motif, [I/V]HCXXGXXR[S/T]. This motif, which is referred to as the PTP loop, forms the base of the active site cleft. Within this motif, the Cys and Arg residues are invariant and essential for catalysis [7,8]. Due to the environment of the active site, in particular the presence of the invariant Arg residue, this Cys displays an unusually low pKα, which enhances its ability to execute a nucleophilic attack on the phosphate group of the substrate in the first step of the catalytic mechanism [9,10]. An invariant Asp residue (D181 in PTP1B), which is located in a conformationally flexible loop (the WPD loop), is also essential and

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functions as a general acid to protonate the phenolate leaving group of the substrate [11]. This first step in catalysis results in formation of a cysteinyl-phosphate intermediate. In the second step, this invariant Asp functions in combination with a Gln residue, equivalent to Q262 in PTP1B, to activate a water molecule and promote hydrolysis of the phosphoenzyme intermediate [12]. Although all members of the PTP family use this same basic catalytic mechanism, structural differences allow them to be subdivided into two broad categories, those enzymes that are specific for phosphotyrosyl residues in proteins, termed the classical PTPs, and the dual-specificity phosphatases (DSPs), which have the ability to recognize Ser/Thr, as well as Tyr residues.

The Classical PTPs

In the classical PTPs the signature motif is contained within a conserved catalytic domain of 280 residues, which is flanked on either the N- or C-terminal side by noncatalytic sequences that serve a regulatory function. Similarities in the catalytic domain sequence, which coincide with similarities in the structural and functional domains present in the regulatory segments, allow the PTPs to be grouped into 17 subtypes, including receptor-like and nontransmembrane categories (Fig. 1) [2].

The specificity of the enzymes for phosphotyrosyl residues is explained in part by the depth of the active site cleft. This is defined by a tyrosyl residue (Y46 in PTP1B), which forms one side of the cleft [13]. Thus, a pTyr residue in a substrate is of sufficient length to gain access to the nucleophilic Cys at the base of the active site cleft, whereas pSer and pThr residues would be too short to be dephosphorylated. Recently, a search of cDNA sequences in the GenBank database revealed the existence of 113 such PTPs in vertebrates, including 37 in humans [2]. Additional mining of the human genome sequence increased the number of classical PTPs to 38 and identified 12 pseudogenes (see Chapter 109

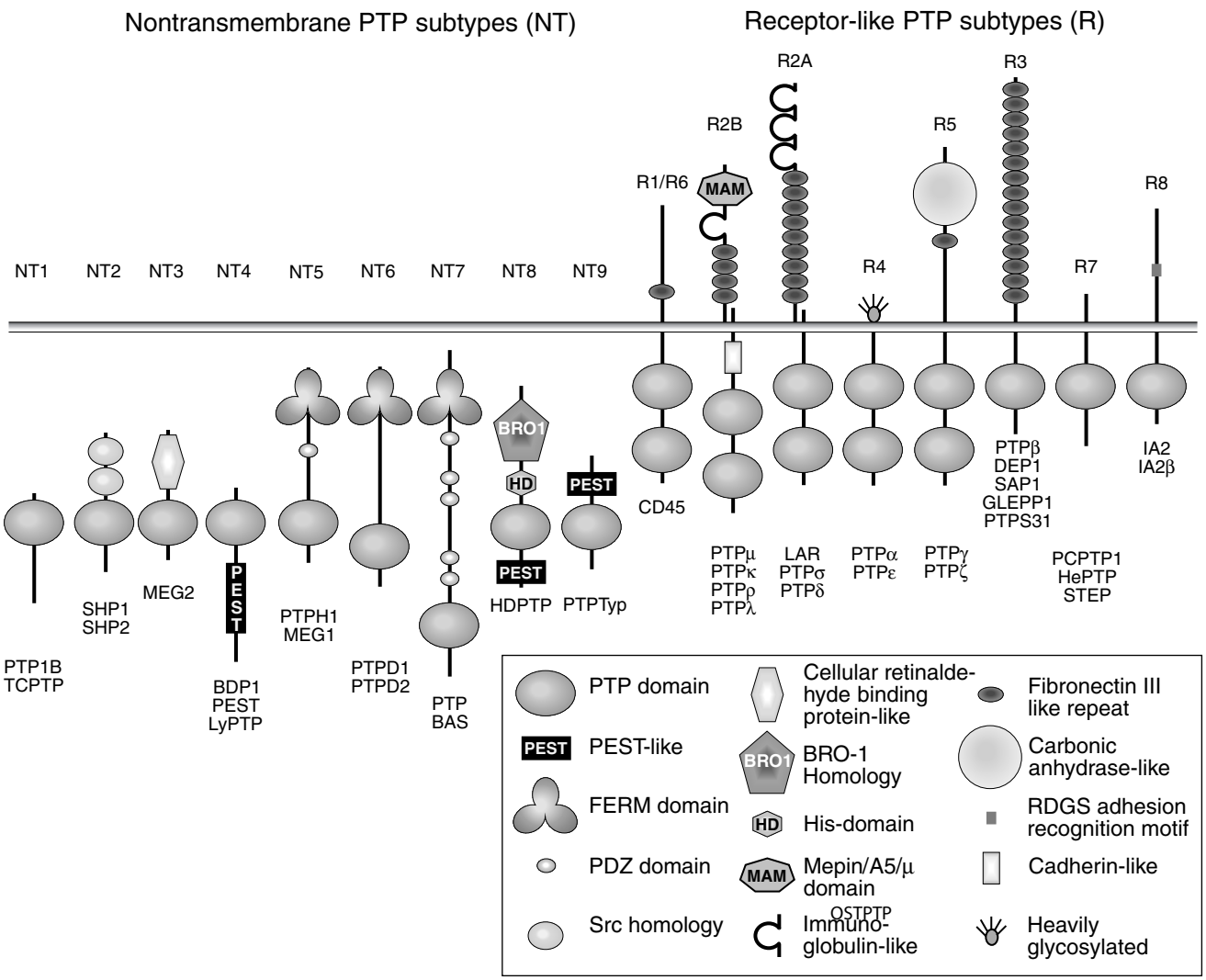


Figure 1 Schematic representation of PTP family members. The PTPs have been classified into nine non-transmembrane (NT) and eight receptor-like (R) subtypes based on sequence similarity. Only the human PTPs are listed, and a representative of each subtype is shown. (From Andersen, J. N. *et al.*, *Mol. Cell. Biol.* 21, 7117–7136, 2001. With permission.)

and Andersen *et al*. submitted). To provide a framework for a genome-wide analysis of the PTPs we have compiled a comprehensive online resource for sequence analysis of the pTyr-specific members of the PTP family. The website includes amino acid sequence alignments, phylogenetic classification of family members, and evaluation of amino acid conservation in three dimensions using X-ray crystal structures of PTP domains and low-resolution homology modeling. The PTP database is available online at http: //ptp.cshl.edu or http: //science.novonordisk.com/ptp. In the future, we plan to expand this resource to include pseudogenes, intron/exon organization, and splice variants as well as mutations, polymorphisms, and disease linkages.

The Dual Specificity Phosphatases (DSPs)

The DSPs display greater variation in structure than the classical PTPs and, although there is conservation in the fold of the catalytic domain, sequence similarity between the two groups is largely restricted to the signature motif. The DSPs are characterized by a more open active site cleft than the classical PTPs which allows them to accommodate different phosphorylated residues [14,15]. The first of these enzymes to be described was VH1, the product of an open reading frame from the pox virus *Vaccinia* that is essential for virion infectivity [16,17]. The study of the DSPs not only highlighted the structural diversity within the family but also provided some of the first illustrations of specificity and functional importance. Links between the DSPs and phosphorylation events that are critical for normal cell function were soon established. For example, the MKPs (MAP kinase phosphatases) comprise a group of DSPs that dephosphorylate particular members of the MAP kinase family [18] and the different cdc25 gene products regulate transition through the cell cycle by dephosphorylation of the cyclin-dependent kinases (Cdks) [19]. Although described as "dual specificity," certain DSPs within this group can display preference for one particular type of amino acid. For example, VHR preferentially dephosphorylates the tyrosyl residue of the activation loop of Erk MAP kinases [20] and KAP dephosphorylates T160 from the activation loop of Cdks [21,22]. Interestingly, DSPs can also recognize non-protein substrates. For example, PTEN, the product of the tumor suppressor gene on human chromosome 10q23, is specific for phosphate on the 3 position of the sugar ring of the phosphatidylinositol phospholipids PI(3,4,5)P3 and PI(3,4)P2 and, therefore, regulates PI 3-kinase-dependent signaling pathways [23,24]. In addition, myotubularin, the product of the gene that is mutated in X-linked myotubular myopathy, dephosphorylates phosphatidylinositol 3-phosphate (PI3P) [25,26].

The DSPs can be divided into three groups. The largest includes the VH1-like DSPs, and a summary of those that have been described in the literature to date is presented in Table 1. In addition, there are the myotubularins (MTMs) [27,28] and the cdc25s A, B, and C [19]. We have conducted exhaustive searches and phylogenetic analyses of the VH1-like DSPs from various genomes, revealing many novel enzymes and illustrating structure–function relationships within the group. Thus far, we have identified a total of 43 VH1-like DSPs in humans. A detailed description of this analysis is in preparation and will be added to the website listed above.

Table I Mammalian VH1-Like Dual-Specificity Phosphatases

Name	Aliases
MKP-1 [86]	3CH134 (mouse) [87], CL100 (human) [88], ERP [89], HVH1 [90], DUSP1
MKP-2 [91]	TYP 1 [92], HVH2 [93], DUSP4 [94]
HVH3 [95]	B23 [96], CPG21 [97], DUSP5
PAC-1 [98]	DUSP2 [99]
MKP-3 [100]	Pyst1 (human) [101], rVH6 (rat) [102], DUSP6 [94]
Pyst2 [103]	MKP-X [94], B59 [104], DUSP7 [94]
MKP-4 [105]	Pyst3, DUSP9
MKP-5 [106]	MKP5 [107], DUSP10 [108]
MKP-7 [109]	MKP-M [110]
HVH5 [111]	M3/6 (mouse) [112], DUSP8
MKP6 [113]	DUSP14, MKP-L
VHR [114]	DUSP3
TMDP [115]	DUSP13
JSP1 [116]	LMW-DSP2 [117], VHX [118], JKAP [119], MKPX
SKRP1 [120]	LDP-2 [121]
hSSH-1 [122]	
hSSH-2 [122]	
hSSH-3 [122]	
hYVH-1 [123]	GKAP (rat) [124], DUSP12
PTEN [77]	MMAC-1 [78], TEP [125]
TPTE [126]	
TPIP [127]	
PIR1 [128]	DUSP11
PRL1 (rat) [129]	PTP(CAAX1) [130], PTPIVA1 [131], OV-1 [132]
PRL2 (mouse) [133]	PTP(CAAX2) [130], PTPIVA2 [131]
PRL3 (mouse) [133]	PTP(CAAX3), PTPIVA3
hCdc14A [134]	
hCdc14B [134]	
KAP-1 [135]	Cdi1 [136]
Laforin [137]	EPM2 [138]
HCE1 [139]	mRNA Capping Enzyme
STYX [140]	
MK-STYX [141]	STYX 2

A list of mammalian DPSs is provided, with a primary name assigned in the left column and alternatives found in the literature provided on the right. Appropriate references for each name are provided.

Regulation of PTP Function

It was apparent from early in the study of these enzymes that members of the PTP family have the potential to represent a formidable barrier to PTK function [4], suggesting

that mechanisms must exist to attenuate their activity and permit a tyrosine phosphorylation-dependent signaling response. The fact that PTPs may not only antagonize PTK function, but also act in concert with PTKs to promote signaling, as in the activation of Src family PTKs by CD45 [29], introduces further levels of complexity. Obviously, for receptor-like PTPs there is the potential for regulation of activity in response to ligands. With the exception of homophilic binding interactions between the extracellular segments of certain receptor-like PTPs (RPTPs) [30], however, the identity of such ligands has proven elusive. Many RPTPs display structural features of cell adhesion molecules and have been implicated in the regulation of phenomena associated with cell–cell contact, such as neuronal pathfinding during development [31]. More recently, both soluble (e.g., the interaction of pleiotrophin with PTPζ/β [32]) and surface-bound (e.g., interaction of heparin sulfate proteoglycans with PTPσ [33]) ligands for RPTPs have been described. Nevertheless, the effects of ligand binding on RPTP activity remain to be fully characterized. Structural analyses of PTPα led to a proposal, which gained wide acceptance in the field, that ligand-regulated dimerization of RPTPs may inhibit activity due to occlusion of the PTP active site in the dimer [34]. Interestingly, biological data consistent with such a model have been presented [35]. Nevertheless, a direct demonstration that dimerization regulates activity has yet to be provided and further structural analyses of other RPTPs [36,37] suggest that this model, if correct, may not apply broadly across the family. There have now been suggestions that ligand binding may activate certain PTPs, such as following engagement of the extracellular segment of DEP-1 by components of the extracellular matrix [38], but the mechanisms underlying such effects remain to be explored.

An important aspect of regulation of the nontransmembrane PTPs is that of subcellular targeting, which has been referred to as the "Zip Code" model [39]. Structural motifs within the noncatalytic segments of these PTPs target the enzymes to defined subcellular locations. Thus, the physiological functions of these PTPs are restricted by the nature of the substrates to which they have access at these defined locations. For example, the SH2 domain-containing PTPs (SHP1 and 2) are recruited into signaling complexes at the plasma membrane via binding of the SH2 domains to specific pTyr sequence motifs [40,41]. The enzyme TC-PTP occurs in two alternatively spliced forms that share the same catalytic domain, but have different C termini that target the enzyme either to the endoplasmic reticulum (ER) or the nucleus [42]. Recent elegant studies using fluorescence imaging techniques have shown that PTP1B, which is targeted to the cytoplasmic face of membranes of the ER, functions in a "dephosphorylation compartment" acting upon RPTKs that have been downregulated by endocytosis [43]. Nevertheless it is important to note that PTP function and specificity are not solely regulated by control of location. PTPs have the ability to display intrinsic specificity for particular substrates which is determined by features of both the active site of the phosphatase and the structure surrounding the phosphorylation site in the target. Furthermore, in addition to targeting, the noncatalytic segments of the PTPs can regulate activity directly. For example, the C-terminal segment of TC-PTP contains an inhibitory sequence, the effects of which can be overcome *in vitro* by proteolytic removal of the segment or its engagement with antibodies [44]. Presumably, the interaction of TC-PTP with physiological regulatory proteins modulates activity *in vivo*. In addition, in the absence of an appropriate ligand, the N-SH2 domain of the SHPs binds to and occludes the active site, thereby inactivating the enzyme [45]. Thus, interaction of specific pTyr sequences with the SH2 domains of the SHPs both targets the enzyme to particular substrates and directly activates the enzyme.

Many PTPs are phosphoproteins *in vivo*. Such phosphorylation events may create docking sites that promote protein–protein interactions, such as in the association of Grb2 with SHPs [46] or 14-3-3 with PTPH1 [47] and cdc25 [48]. Phosphorylation may also modulate activity directly, such as in the phosphorylation of PTP-PEST by PKC and PKA, which inhibits PTP activity and thus may underlie a mechanism for cross-talk between Ser/Thr- and Tyr-phosphorylation-dependent signaling pathways [49]. Thus, inhibition of PTP function by phosphorylation of Ser/Thr residues could indirectly promote Tyr phosphorylation of other cellular proteins. PTPs are also susceptible to proteolysis. This has been implicated in the generation of isoform diversity, such as in the production of cytosolic forms of RPTPε [50], as well as in generating forms of a PTP in which regulatory constraints are removed, such as following calpain-induced cleavage of PTP1B [51].

Oxidation of PTPs in Tyrosine Phosphorylation-Dependent Signaling

Reactive oxygen species (ROS) are produced in response to a wide variety of cellular stimuli [52]. A substantial body of data emphasizes the importance of ROS production as a mechanism for fine-tuning tyrosine-phosphorylation-dependent signaling through the transient oxidation and inactivation of members of the PTP family [53]. In the unique environment of the PTP active site, the invariant Cys residue of the signature motif, which displays an unusually low pKα, is present predominantly as the thiolate anion [9,10]. This not only enhances its nucleophilic properties but also renders it susceptible to oxidation. Oxidation can yield a stable, single-oxidized sulfenic acid modification of the Cys (Cys–SOH), which inhibits activity because the oxidized Cys can no longer function as a nucleophile. This modification is reversible and thus can form the basis for a mechanism of reversible regulation of PTP activity. Glutathionylation of the sulfenic acid form of PTPs has been reported [54] which may not only promote reduction back to the active state but also prevent further, irreversible oxidation by the addition of two (sulfinic acid) or three (sulfonic acid)

oxygens to the active site Cys. Treatment of both PTPs and DSPs with H_2O_2 *in vitro* results in oxidation and inactivation [55]. More importantly, oxidation and inactivation of PTPs have now been demonstrated in response to physiological stimuli. For example, PTP1B is oxidized and inactivated in response to growth factors, such as epidermal growth factor (EGF) [56], and hormones, such as insulin [57].

In order to examine this issue further, we developed a modified "in-gel" PTP assay to visualize the oxidation of PTPs in response to a stimulus in a cellular context. We observed the reversible oxidation of multiple PTPs in response to treatment of Rat 1 cells with H_2O_2 and demonstrated that this oxidation was required for the mitogenic effects of H_2O_2 [53]. We also demonstrated that stimulation of Rat 1 fibroblasts with platelet-derived growth factor (PDGF) led to the production of reactive oxygen species, which induced the rapid and reversible oxidation of the PTP SHP2 [53]. Ligand-induced autophosphorylation of the PDGF receptor (PDGFR) generates docking sites for various signaling proteins, including SHP2. We showed that mutant forms of the PDGFR that were unable to bind to SHP2 displayed enhanced autophosphorylation and enhanced activation of MAP kinase. Thus, SHP2 appears to recognize the PDGFR as a substrate and functions as an inhibitor of PDGFR signaling. Interestingly, it was only the population of SHP2 that was bound to the PDGFR that was susceptible to reversible oxidation and inhibition. We propose that PDGF stimulation induces localized production of ROS, leading to the rapid oxidation of the pool of SHP2 that has been recruited into a complex with the PDGFR. This augments autophosphorylation of the receptor and initiation of the signaling response.

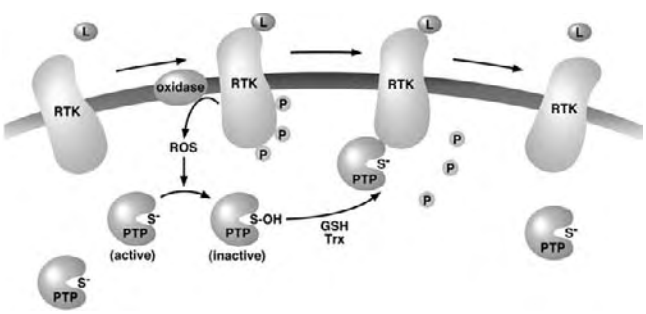


Figure 2 Regulation of protein tyrosine phosphatase (PTP) activity by reversible oxidation. Ligand-dependent activation of a receptor protein tyrosine kinase (RTK) triggers the activity of a Rac-dependent NADPH oxidase leading to production of reactive oxygen species (ROS). ROS oxidize the active site Cys residue of members of the PTP family, converting it from a thiolate ion (the active form) to sulfinic acid. Oxidation results in inhibition of PTP activity, thereby promoting tyrosine phosphorylation. However, due to the action of glutathione or thioredoxin, oxidation of the PTPs is transient. Restoration of PTP activity following reduction back to the thiolate form of the active site Cys residue terminates the tyrosine-phosphorylation-dependent signal. A variety of growth factors, hormones, and cytokines induce ROS production and stimulate tyrosine phosphorylation. We are developing methods to identify the PTPs that become oxidized in response to a physiological stimulus as a way of establishing links between particular PTPs and the regulation of defined signaling pathways.

The transient nature of the oxidation ensures reduction and reactivation of the pool of SHP2, which promotes dephosphorylation of the PDGFR and termination of the signal. These data illustrate how ligand-induced production of ROS may augment tyrosine-phosphorylation-dependent signaling in general through inactivation of PTPs (Fig. 2).

The production of ROS is observed in response to a wide variety of stimuli, including growth factors, hormones, cytokines, and activators of G-protein-coupled receptors, leading to PTK activation. The operating principle is that the stimulus enhances tyrosine phosphorylation directly by activation of a PTK or indirectly by inactivation of a PTP. Thus, one function of ROS produced following agonist stimulation is transient inactivation of the critical PTP that provides the inhibitory constraint upon the system, thus facilitating initiation of the signaling response to that stimulus. We propose that stimulus-induced oxidation may be used as a means of "tagging" and identifying those PTPs that are integral to the regulation of the signaling events triggered by that stimulus. It is hoped that this will provide further insights into the physiological function of members of the PTP family.

Substrate Specificity of PTPs

Studies of protein and peptide dephosphorylation *in vitro* have illustrated the importance of residues flanking the site of phosphorylation in a substrate for optimal PTP activity [58]. Now the issue of substrate specificity in a cellular context has been explored in a variety of experimental approaches, which illustrate that, contrary to initial expectations, the PTPs exhibit exquisite substrate specificity *in vivo*. For example, the function of several PTPs has been investigated through the generation of knockout mice. Interestingly, in several cases, ablation of closely related PTPs has yielded dramatically different phenotypes. This is illustrated by comparisons of the phenotypes generated by knockouts of PTP1B [59] and TC-PTP [60], SHP-1 [40], and SHP-2 [41] or within the LAR group of RPTPs [61–66]. These distinct phenotypes are consistent with exquisite specificity in substrate recognition and function.

The crystal structure of PTP1B in a complex with a phosphotyrosyl peptide substrate revealed that a profound conformational change accompanied substrate binding. The WPD loop that forms one side of the active site cleft closes around the side chain of the pTyr residue and positions the invariant Asp residue for its catalytic function [13]. This imagery of the jaws of the active site closing around the substrate stimulated an analysis by site-directed mutagenesis, in which we generated a form of PTP1B that maintains a high affinity for substrate but does not catalyze dephosphorylation effectively [67]. Thus, we converted an extremely active enzyme into a "substrate trap." Furthermore, the residue that is mutated to generate the substrate-trapping mutant is the invariant catalytic acid (Asp181 in PTP1B) that is conserved in all members of the PTP family. This has afforded us a unique approach to identification of physiological substrates of PTPs in general.

Following expression, the mutant PTP binds to its physiological substrates in the cell but, because it is unable to dephosphorylate the target efficiently, the mutant and substrate become locked in a stable, dead-end complex. Potential substrates can be identified by immunoblotting lysates of cells expressing the mutant PTP with antibodies to pTyr to reveal proteins for which the phosphorylation state is altered as a consequence of expression of the mutant. In addition, the complex between the mutant PTP and the pTyr substrate can be isolated by immunoprecipitation and associated proteins identified by immunoblotting or, on a larger scale, by primary sequence determination. We have used this method to demonstrate specificity of PTP-PEST for p130cas [68] and PTPH1 for VCP [69], as well as differences in specificity of the spliced variants of TC-PTP (see Chapter 110) [70].

Specificity in substrate recognition is now further emphasized by the demonstration of examples in which a PTP not only recognizes a specific target substrate but also shows preference for particular phosphorylation sites within that target. A dramatic example is the specificity of PTEN for the 3 position in the inositol ring of phosphatidylinositol phospholipids [23,24]. Furthermore, there are similar examples with protein substrates, such as the preferential recognition of Tyr 239 in Shc by TC45 [70]. The mechanisms underlying such substrate specificity are now being defined at the molecular level. For example, an X-ray crystal structure of a complex between PTP1B and the activation loop of the insulin receptor as a substrate led to the definition of a consensus sequence motif of D/E-pY-pY-R/K for optimal substrate recognition [71,72]. In several cases, interactions between the PTP and substrate at sites remote from the active center have been shown to be important. This is illustrated by PTPs such as PTP-SL and STEP [73] and DSPs such as MKP-3 [74,75], which dephosphorylate Erk MAP kinases. In addition, the highly specific nature of the interaction between PTP-PEST and p130cas appears to result from a combination of two distinct substrate recognition mechanisms; the catalytic domain of PTP-PEST contributes specificity to the interaction with p130cas, whereas the SH3 domain-mediated association of p130cas and PTP-PEST dramatically increases the efficiency of the interaction [76].

PTPs and Human Disease

The importance of PTPs to the control of signal transduction has been further reinforced by numerous examples in which the disruption of normal PTP expression or function has been implicated in human disease. A summary of developments in this area is presented in Table 2. In light of the large number of PTKs that have been shown to play a role in oncogenesis, it was anticipated that many of the PTPs may be the products of tumor suppressor genes. Although PTPs have been linked to inhibition of cell proliferation, it was not until the demonstration that PTEN was encoded by the locus at human chromosome 10q23, which is mutated in a large number of tumors, that the first tumor suppressor PTP was identified [77,78]. Even then, PTEN is unusual within the PTP family, displaying specificity for phosphatidylinositol phospholipids [23,24]. More recently, the RPTP DEP-1 has been identified as a tumor suppressor associated with cancers of colon, breast, and lung [79]. Although PTP1B has been implicated in the dephosphorylation of several growth factor receptor PTKs, mice in which the gene for PTP1B has been ablated display disruption of signaling in response to insulin and leptin but no increased incidence of tumors [59,80–82]. Recent studies have shown that disruption of the *PTP1B* gene does lead to hyperphosphorylation of the EGF and PDGF receptors, but with only minimal changes in signaling [83]. Thus, it would appear that mechanisms exist within the cell to compensate for disruption in PTP expression. Such mechanisms may apply broadly across the PTP family, explaining why so few tumor suppressor PTPs have been identified.

The current environment of fear regarding the potential for acts of bioterrorism has also drawn attention to the PTP family of enzymes. The prototypic DSP, encoded by the VH1 open reading frame of *Vaccinia*, is essential for normal virion infectivity [16,17], and homologs of this enzyme are also known to be essential for the viability of other poxviruses [71]. *Variola* virus, the cause of small pox, is closely related to *Vaccinia*, suggesting that its VH1-like DSP is an essential element of small pox infections. PTPs have also been implicated in bacterial infections, and, in this context, the function of the PTP Yop of *Yersinia*, the causative agent of bubonic plague, is of interest. Progress has been made in defining the function of this enzyme, which has been shown to target p130cas at focal adhesions in infected host cells [84]. The importance of these PTPs in infection suggests that development of inhibitors of the enzymes may offer strategies to counter the threat posed by these organisms. Furthermore, the ability of a variety of infectious agents to usurp normal tyrosine phosphorylation-dependent signaling pathways suggests that drugs designed to inhibit members of the PTP family may yield new classes of anti-infectives.

Recent progress in establishing links between PTPs and human diseases, together with developments in understanding the function of several of these enzymes, has raised awareness of the PTPs in the pharmaceutical industry. The appreciation that PTPs have the ability to display specificity for substrates *in vivo* and, therefore, to exert effects that would be restricted to specific signaling pathways suggests that PTP-directed drugs would induce defined, rather than global, changes in cellular tyrosine phosphorylation. A spectacular example of the potential importance of PTPs in the development of novel therapeutic strategies was provided by the phenotype of the PTP1B knockout mouse. The mice show no obvious deleterious effects; however, they display enhanced sensitivity to insulin and a resistance to obesity induced by a high-fat diet, which is accompanied by increased basal metabolic rate and total energy expenditure [59,80]. These effects have been defined in terms of a regulatory function for PTP1B in signaling through the insulin and leptin receptors [59,80–82]. Therefore, an inhibitor of PTP1B

Table II Linkages Between Protein Tyrosine Phosphatases and Disease

PTP1B	Diabetes and obesity	Phenotype of PTP1B$^{-/-}$ mice. QTL at chromosome 20q12–13 (containing *PTP1B* gene) linked to NIDDM and obesity. Polymorphisms in PTP1B segregate with diabetes.
DEP1	Tumor suppressor	Positional candidate for the colon cancer susceptibility locus Scc1 (QTL in mice). Frequently deleted in human cancers of colon, breast and lung.
CD45	Autoimmunity	Links to SCID and HIV-1 infection and possible links to multiple sclerosis. Antibody to CD45 prevents transplant rejection. Lupus-like phenotype of CD45-E613R mutant mice.
SHP1	Inflammation	Autoimmune/proinflammatory phenotype in motheaten mice. Factor in infection of glial cells by TMAV encephalomyelitis virus. Downregulated in human T cell malignancies and patients with Sezary syndrome.
SHP2	Noonan syndrome Stomach ulcers	Developmental disorder affecting 1 : 2500 newborns. Target of *Helicobacter pylori*.
FAP-1	Apoptosis	Upregulated in human cancers; inhibits CD95-mediated apoptosis.
IA2	Type I diabetes	Major autoantigen in Type I diabetes; role in glucose-stimulated insulin secretion.
YopH	Infectious disease	Virulence determinant of *Yersinia*.
PTEN	Tumor suppressor	Mutated in human cancers; Cowden disease.
PRL-3	Metastasis	Upregulated in metastases of colon cancer.
MTM1	X-linked myotubular myopathy	Mutations reduce phosphatidylinositol phosphatase activity.
MTMR2	Charcot-Marie-Tooth disease type 4B	Neuropathy associated with truncation mutations in the MTMR2 gene.
Laforin	Progressive myoclonus epilepsy	Mutations or microdeletions of Lafora disease phosphatase.
Cdc25	Cell cycle control	Target of *Myc* oncogene and overexpressed in primary breast cancer.
VH1	Infectious disease	Essential for production of infectious virions of *Vaccinia*.

The table summarizes some of the links that have been established between members of the PTP family and disease states. A detailed discussion of information summarized in this Table, including a comprehensive list of citations, is presented in "A genomic perspective on protein tyrosine phosphatases: Gene structure, pseudogenese and genetic disease linkage" by Anderson, J. N. *et al*., submitted 2002). The information will also be added to the web sites http:// ptp.cshl.edu and http:// science.novonordisk.com/ptp.

would offer a strategy to counteract both obesity and diabetes, which is provoked by obesity. Given the increasing prevalence of obesity and its related illnesses in Western society, the potential for a PTP1B-based drug is obvious. The crystal structure of a complex between PTP1B and the activation loop of the insulin receptor as a substrate has revealed unique features of the interaction that could be targeted by an inhibitor [71]. Highly potent inhibitors have been developed [85]; however, the charged nature of the PTP active site presents several challenges to the development of small-molecule inhibitors that maintain the ability both to inhibit PTP function potently and to cross the plasma membrane.

Perspectives

In this post-genomic era, in which composition of the family of PTPs has largely been defined, one can move toward a characterization of function. It is now apparent that the PTPs have the capacity to display selectivity in their recognition of target substrates *in vivo* and that such substrate specificity underlies functional specificity. Broadly speaking, PTPs have the ability to function as either inhibitors or activators of particular signaling pathways, depending upon the signaling context. At present, although data attest to the importance of several members of the PTP family as regulators of cell signaling under normal conditions and in the etiology of human disease, the majority of these enzymes are known primarily by their sequence with little information available regarding their function. It is anticipated that in the near future studies of the PTP family as a whole will generate novel insights into function and perhaps establish new links to disease with the possibility of new avenues for therapy.

Acknowledgments

I apologize for the fact that, due to space limitations, many exciting papers in the PTP field have not been cited. Work in my lab is supported by grants from the NIH, CA53840, and GM55989, as well as the Cold Spring Harbor Laboratory Cancer Center Grant CA45508. I am indebted to my colleagues May Chen and Jannik Andersen for their help in compiling Tables 1 and 2, respectively.

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CHAPTER 109

Protein Tyrosine Phosphatase Structure and Mechanisms

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Introduction

The protein tyrosine phosphatase (PTP) family of enzymes dephosphorylate target signaling proteins and are involved in the diverse regulation of numerous cell functions. PTPs comprise a large gene-family (112 genes) with the minimal catalytic motif CX_5R, where C is the cysteine nucleophile that attacks the phosphate group, R is the arginine residue that binds phosphate and stabilizes the transition state, and X represents any amino acid. A large subgroup of the PTPs are capable of efficient hydrolysis of both phosphotyrosine and phosphothreonine/serine residues and are often referred to as dual-specificity PTPs. Members of the PTP family can be soluble or membrane-associated proteins, as in the receptor-like PTPs. The common feature of these phosphatases appears to be the basic catalytic mechanism involving the formation of a phospho-cysteinyl enzyme intermediate, using the conserved cysteine, arginine, and general acid/base aspartate residue. The catalytic domain of PTPs consists of an α/β fold composed of a highly twisted core of β-strands flanked by α-helices. Domains outside of the catalytic fold serve as regulatory and/or targeting modules. The structure, substrate recognition, catalytic mechanism, and modes of regulation are discussed in this review.

Introduction to the Protein Tyrosine Phosphatase Family

Protein tyrosine phosphatases (PTPs) are signaling enzymes involved in the regulation of numerous cell functions. PTPs dephosphorylate target proteins such as mitogen-activated protein (MAP) kinases and receptor kinases, leading to the appropriate regulation of a variety of signal transducing pathways. PTP families include receptor-like transmembrane and soluble proteins. Dual-specificity phosphatases (DSPs) are a subfamily of intracellular PTPs that catalyze dephosphorylation of the three most prevalent phospho-amino acids (phosphotyrosine, phosphoserine, and phosphothreonine). Analysis of the human genome indicates that tyrosine-specific protein phosphatases and DSPs are one of the more abundant gene families (112 genes, ranked 29th overall) [1].

Structure

Determining the three-dimensional structures of over a dozen PTPs has facilitated the identification of critical residues involved in catalysis, substrate binding, and regulation. Although PTPs share a low percentage of amino acid sequence identity among all family members, their overall structures are similar. The catalytic domain of PTPs consists of an α/β fold, composed of highly twisted core β-strands flanked by α-helices [2–4]. The PTP signature motif HCXXGXXR(T/S) (and minimally CX_5R) defines the active site center, where the catalytic cysteine resides at the base of the active site cleft. Residues in the PTP signature motif form the phosphate-binding loop, where the main-chain N–H groups and the guanidinium side chain of the invariant arginine residue are oriented to coordinate oxygens of the phosphate group during substrate binding and catalysis. The active site is surrounded by intervening loops that are important in providing additional residues for catalysis and substrate specificity. A highly conserved aspartic acid

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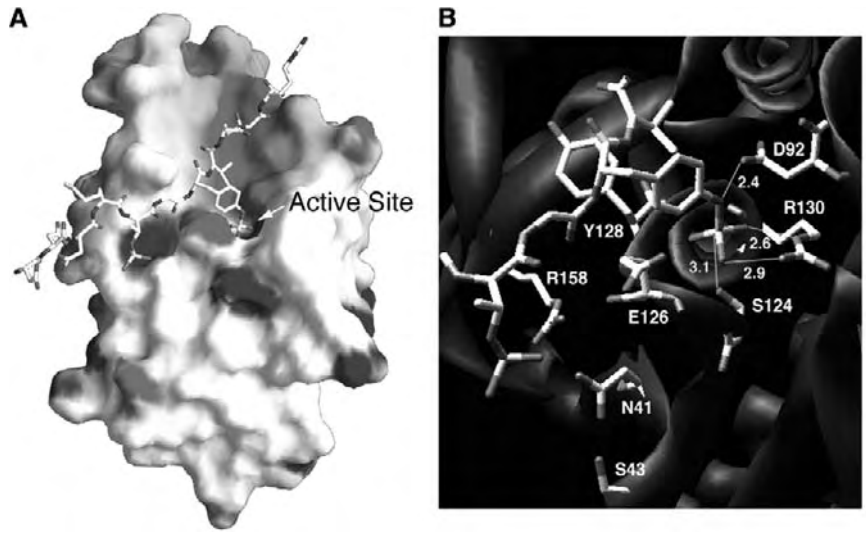


Figure 1 Crystal structure of C124S mutant VHR bound to bisphosphorylated peptide DDE(Nle)pTGpYVATR [24]. (A) Electrostatic surface of the VHR–peptide complex. Surfaces are shaded according to the local electrostatic potential, ranging from –13 V in gray to +13 V in dark gray. The peptide is represented as a stick, with carbon, nitrogen, oxygen, and phosphate atoms. This figure was generated with GRASP. (B) Close-up view of the phosphotyrosine and phosphothreonine binding sites. The peptide is shown as a stick, and the atoms are colored as in panel (A). This figure was generated with Swiss Pdb Viewer v3.5 and POV-Ray v3.1. A color representation of this figure can be viewed on the CD version of *Handbook of Cell Signaling*.

residue is required for general acid/base catalysis and is located on a separate loop (general acid loop) near the top of the active site.

Outside the catalytic domain, amino acid sequences vary dramatically among the PTPs. Additional regions may include modular domains such as SH2 (Src homology 2) domains, fibronectin repeats, and immunoglobulin domains [5]. SH2 domains serve as protein interaction modules recognizing specific phosphorylated tyrosines in proteins or peptides. The SH2 domains of SHPs (Src homology phosphatases) target these phosphatases to specific tyrosyl phosphorylated proteins within cells. Also, the N-terminal SH2 domain of SHPs regulate catalytic activity directly. In the absence of an appropriate phosphotyrosine ligand, the N-SH2 binds to and inactivates the PTP domain by blocking substrate access. This restricts SHP activation to particular locations within the cell where the substrates reside [6,7]. Fibronectin repeats and immunoglobulin domains are found in many receptor protein tyrosine phosphatases (RPTPs). Several RPTPs are believed to play a role in the regulation of cell–cell contact and adhesion through homophilic binding interactions between adjacent cells. RPTPs contain one or two intracellular catalytic domains (membrane-proximal D1 and membrane-distal D2). D1 domains are catalytically active, but most D2 domains lack several of the critical catalytic residues, resulting in a domain that displays little or no phosphatase activity [8–10].

Despite low sequence identity between the tyrosine-specific phosphatases and DSPs, crystal structures of several DSPs (VHR, Pyst1/MKP3, PTEN, and KAP) show a highly conserved active site core similar to PTPs [11–14]. Figure 1 shows the crystal structure of VHR (*Vaccinia* H1 related) bound to a bisphosphorylated peptide substrate [15]. VHR represents the minimal catalytic domain among PTPs, which has made VHR a good model in studies of PTP structure and mechanism. The crystal structure of the DSP Cdc25 catalytic domain reveals that Cdc25 has a unique topology [16,17] that identifies Cdc25 as a more distinct family member of the PTPs. Cdc25 upregulates cyclin-dependent serine/threonine protein kinases (Cdks) by dephosphorylating two adjacent phosphothreonine and phosphotyrosine residues, which are inhibitory to Cdk kinase activity. Although Cdc25 appears to use a similar catalytic mechanism [18], sequence homology within the catalytic domains of other PTPs and DSPs is restricted to the CX_5R motif. Like Cdc25, low-molecular-weight PTPs (LMW-PTPs) constitute a distinctive class. The crystal structure of bovine LMW-PTPs reveals a unique fold [19,20]. The LMW-PTPs also contain the conserved arginine, aspartate, and cysteine residues within their active sites.

Structural features of PTPs have provided evidence for peptide substrate specificity and for selectivity toward the nature of the phosphorylated residue. Peptide specificity appears to be defined largely by residues both N- and C-terminal to the substrate pTyr residue. The structure of PTP1B in a complex with insulin receptor peptides indicates that a second pTyr residue adjacent to the substrate phosphorylation site plays a critical role in specificity [21]. Similarly, the DSP VHR displays a preference for diphosphorylated peptide substrates [15,22]. The peptide-interacting residues in PTP1B and VHR are poorly conserved throughout the entire PTP family, implying that PTPs have distinct protein substrate specificity.

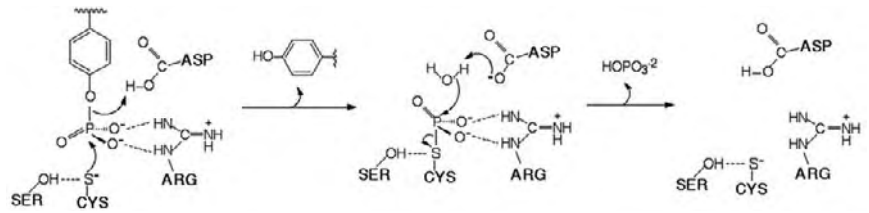


Figure 2 Catalytic mechanism of protein tyrosine phosphatases.

The phosphorylated residue specificity appears to be determined by the depth of the active site pocket, the general acid loop, and the PTP signature motif. Tyrosine-specific PTPs have an ≈9-Å-deep active site cleft; therefore, only phosphotyrosine residues can reach the cysteine nucleophile in the active site. For example, the structure of PTP1B in a complex with a peptide derived from epidermal growth factor receptor (EGFR) revealed that Arg221 at the base and Asp48 at the rim of the active site exactly match the length of pTyr residues [23]. The general acid loop also provides some level of substrate specificity. The PTP1B general acid loop (also called WPD loop, where D is Asp181 general acid) closes over the active site upon phosphorylated peptide binding. This allows the Asp181 to be positioned to act as a general acid in the catalytic reaction and for Trp179 and Pro180 to interact with Arg221 in the active site. These interactions stabilize the catalytically competent conformation of the loop [24]. The crystal structure of the *Yersinia* PTP also shows the ligand-induced conformational change of the general acid loop [25].

In contrast, DSPs have a shallower active site cleft for accommodating both phosphotyrosine and phosphoserine/threonine residues. Also, the PTP signature motif in the DSPs provides substrate discrimination. Though VHR belongs to the DSP family, VHR prefers phosphotyrosine over phosphoserine/threonine [15,22]. While most DSPs contain alanine and isoleucine in the X_2 and X_3 positions of the signature motif $HCX_1X_2GX_3X_4R(S/T)$, VHR harbors a glutamate and a tyrosine, respectively. The crystal structure of VHR bound to a bisphosphorylated peptide (shown in Fig. 1) reveals that the side chains of glutamic acid-126 and tyrosine-128 in the signature motif impart substrate specificity for phosphotyrosine by creating a deep and narrow active site [15]. The smaller residues (isoleucine and alanine, found in many DSPs) allow more efficient phosphothreonine and phosphoserine dephosphorylation activity [15]. Another putative DSP family member, PTEN (phosphatase and tensin homolog deleted on chromosome 10), is unique among known PTPs, as it has two basic lysine residues within the signature motif. These positive charges are believed to interact with the negative charges of inositol phospholipid PIP3 (phosphatidylinositol 3,4,5-triphosphate), the biological substrate for PTEN [13,26].

Mechanism

Protein tyrosine phosphatases share a similar active site structure despite their low amino acid sequence homology. A common mechanism employed by PTPs is represented in Fig. 2. In the first step of the reaction, the cysteine nucleophile in the active site attacks the phosphorus atom of the substrate, forming a phosphoenzyme intermediate. As the ester bond is cleaved, a general acid (conserved aspartic acid residue) donates a proton to the leaving group oxygen, releasing dephosphorylated substrate. In the second step of the reaction, a water molecule is activated by the aspartic acid acting as a general base. The activated water molecule hydrolyzes the phosphoenzyme intermediate, yielding free enzyme and inorganic phosphate.

Numerous kinetic and biochemical data support the reaction mechanism outlined here. The cysteinyl-phosphate intermediates have been observed using a variety of methods [24,27,28]. The conserved cysteine nucleophile has been shown to be essential in all PTP families. The pK_a value of the active site cysteine is quite low, ranging from 4.7 to 5.5. Under physiological pH, the low pK_a of cysteine ensures that it exists as the thiolate anion. The hydroxyl group of a serine or threonine residue in the signature motif is important for facilitating the hydrolysis of the phosphoenzyme intermediate, perhaps by stabilizing the leaving group thiolate [29,30]. The conserved histidine residue in the signature motif has a considerable effect on lowering the pK_a of the cysteine [31,32]. The invariant arginine residue in the signature motif is important for both substrate binding and transition-state stabilization, as it coordinates two of the oxygen atoms on the phosphoryl group via its guanidinium side chain. When the arginine is replaced with lysine or alanine in the *Yersinia* PTP, these interactions are disrupted and the resulting enzyme displays substantially reduced catalysis and substrate binding [33]. As mentioned, the conserved aspartic acid residue in the general acid loop facilitates general acid/base catalysis in PTPs. Substitution of the aspartic acid residue produces an enzyme that is incapable of general acid catalysis [27,30,34,35].

To better understand the transition-state structure, heavy-atom kinetic isotope effects have been measured using *para*-nitrophenyl phosphate (*p*NPP) labeled with ^{15}N and ^{18}O isotopes. These studies have indicated that phosphoenzyme intermediate formation is highly dissociative, where bond formation to the incoming nucleophile cysteine is minimal and bond breaking between phosphorus and the leaving group oxygen is substantial [36,37]. Moreover, the analysis of conserved aspartic acid mutants of PTPs indicates that the leaving group departs as the *p*-nitrophenolate anion and that the aspartic acid is responsible for the protonation of the leaving group in the wild-type enzymes.

Regulation

Although PTPs do not appear to be governed by a universal regulatory mechanism, three basic types of mechanisms are described in the literature: (1) redox regulation of the catalytic cysteine, (2) phosphorylation, and (3) regulation mediated by the inherent flexibility in the general acid loop. In the redox regulatory model, PTP activity is inhibited when the nucleophilic cysteine is reversibly oxidized to either a sulfenic acid (–SOH) or to disulfide (–S–S–) [38,39]. Some PTPs, such as Cdc25, are phosphorylated themselves, resulting in enhancement in catalytic activity [40]; however, the scope of such phosphorylation-dependent regulation appears to be limited. One intriguing mechanism of regulation takes advantage of the inherent flexibility in the general acid loop of PTPs. Crystal structures of ligand-free and ligand-bound PTPs indicate conformational differences in this general acid loop [41]. For example, the general acid loop of MAP kinase phosphatase 3 (MKP3) is flipped away from the active site, where, upon binding, its substrate ERK induces closure of the general acid loop, converting the low-activity form of MKP3 to the activated form [12,42–44]. The recent crystal structure of KAP (kinase-associated phosphatase) with phosphoCDK2 indicates that CDK2 binding to KAP is responsible for the formation of a complex where the catalytic site of KAP is correctly positioned for catalysis [14]. Consistent with this idea, RPTPα is thought to be regulated by dimerization. Based on the crystal structure of the D1 catalytic domains of RPTPα, it was found that the amino-terminal helix–turn–helix region of one monomer is inserted into the active site of the dyad-related D1 monomer in RPTPα, preventing closure of the general acid loop [45]. Similarly, the catalytic activity of SHP2 is regulated by the N-terminal SH2 domain acting as a conformational switch. The N-SH2 domain blocks the PTP active site and closure of the general acid loop, thus inactivating the enzyme in the absence of phosphorylated substrates. The binding of phosphorylated substrates to the N-SH2 domain relieves this inhibition and results in activation of enzyme [46]. Future questions in the PTP research field will need to address both specific and general regulatory mechanisms, as well as identification of the authentic protein substrates for this large and important class of signaling molecules.

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CHAPTER 110

Bioinformatics: Protein Tyrosine Phosphatases

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Introduction to Bioinformatics

Removal of phosphate from phosphotyrosine residue (pTyr) on cellular proteins plays a key role in many different signaling pathways and is catalyzed by three classes of enzymes [1–6]: (1) classic protein tyrosine phosphatases (PTPs), (2) dual-specificity phosphatases (dsPTPs), and (3) low-molecular-weight phosphatases (LMW-PTPs). The classic (tyrosine-specific) PTPs, which are the focus of this bioinformatics analysis, have traditionally been classified into receptor-like and intracellular PTPs based on the presence or absence of a transmembrane-spanning region (Fig. 1).

Bioinformatics is a relatively novel scientific discipline that combines several areas of research [7]. As it is also a rapidly developing field, there is currently not even general agreement on the definition of the word *bioinformatics*, which nonetheless has gained huge popularity as a buzzword intimately connected with the assembly and analysis of the human genome [8,9]. The definition of bioinformatics depends on the context in which the word is used. As a consequence, the English language has recently been enriched with a number of new terms (e.g., *structural genomics*, *toxicogenomics*, *oncogenomics*, *metabolomics*, *proteomics*, *pharmacogenomics*, *chemogenomics*; see Table 1). However, a unifying feature in bioinformatics is the collection and analysis of large assemblies of biological datasets, most often depending heavily on powerful computers and development of software tools. Despite tremendous progression in the collection and annotation of sequence information at a few central sites, it is still a major challenge to review and compile large datasets from various sources that are updated with different speeds.

In this chapter, we present a template approach to database mining and bioinformatics analyses of classic PTPs that include the following elements: (1) compilation of a comprehensive and nonredundant database of PTP cDNA and protein sequences; (2) utilization of this database to create a homology-based classification of PTP proteins based on amino acid sequence alignments and phylogenetic analysis (neighborhood-joining trees); (3) low-resolution homology modeling to identify conserved regions (PTP structure–function) and nonconserved selectivity-determining regions (substrate specificity and inhibitor design); (4) identification of the genomic complement of the PTP protein family by mapping all PTP-like sequences in the human genome; (5) determination of the chromosomal location and genomic structure of each PTP and use of this information to group novel PTPs as either pseudogenes or true novel PTPs; (6) establishing an initial framework for future disease association studies and studies of the genetic elements controlling PTP expression and regulation.

It is our aim to introduce the reader to the most important bioinformatics databases and analytical tools as we delineate the structural and evolutionary relationships among PTP domains, analyze the PTP family in a genomic context, and finally provide some initial tools for functional analyses of PTPs in health and disease. Although in-house-developed software tools have been employed, we believe that the

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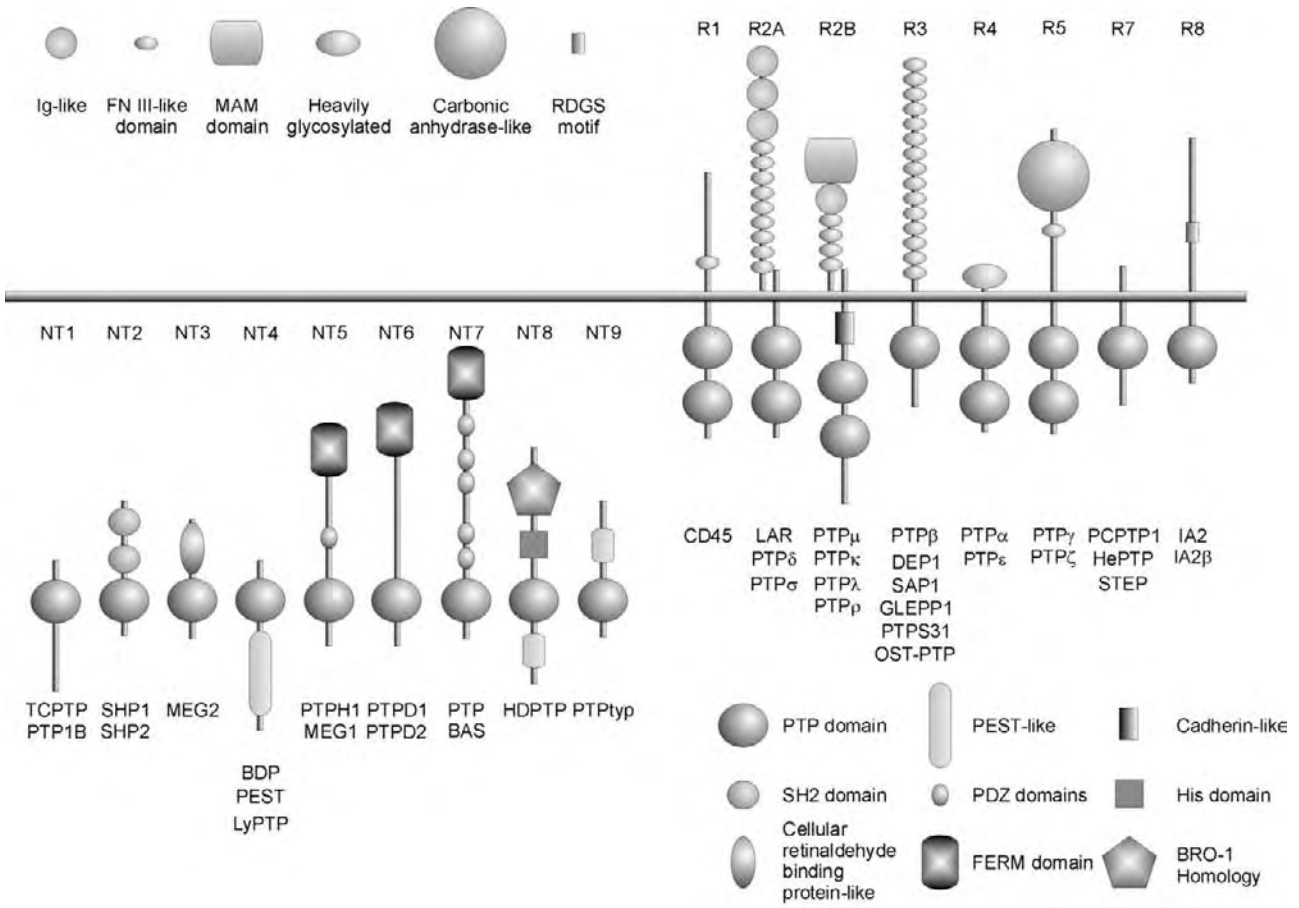


Figure 1 Schematic representation of the PTP family based on sequence similarity among catalytic domains. NT1–NT9: Non-transmembrane or intracellular PTPs; R1–R8: receptor-like PTPs.

Table I Bioinformatics Links

Databases	
EMBL-EBI	http://www.ebi.ac.uk/Databases/ http://www.ebi.ac.uk/embl/index.html
GenBank	http://www.ncbi.nlm.nih.gov/
HGraw	ftp://ftp.ncbi.nih.gov/genbank/genomes/H_sapiens/ (*Genome Project sequences, regardless of chromosome, that have been extracted from GenBank*)
hswall	ftp://ftp.expasy.org/databases/sp_tr_nrdb/ (*human entries from swall and tremble)*
Human genome NCBI	ftp://ncbi.nlm.nih.gov/genomes/H_sapiens
InterPro	http://www.ebi.ac.uk/interpro/
Mouse genome	ftp://genome.cse.ucsc.edu/goldenPath/mmFeb2002
Pfam	http://www.sanger.ac.uk/Software/Pfam/index.shtml
RefSeq	http://www.ncbi.nlm.nih.gov/LocusLink/refseq.html
Swall	ftp://ftp.expasy.org/database/sp_tr_nrdb/ (*a nonredundant concentration of swissprot and tremble*)
SwissProt	http://www.ebi.ac.uk/swissprot/
Genome Browsers and Disease/Phenotype Databases	
Ensembl	http://www.ensembl.org
Fly Base	http://www.flybase.org
Gene Expression Atlas (GNF)	http://expression.gnf.org/cgi-bin/index.cgi
Human Genetic Disease	http://life2.tau.ac.il/GeneDis/
LocusLink	http://www.ncbi.nlm.nih.gov/LocusLink/

(*continues*)

(*continued*)

Mouse knockouts	http://research.bmn.com/mkmd
OMIM	http://www.ncbi.nlm.nih.gov/Omim/
Rat Genome Database	http://www.rgd.mcw.edu/
UCSC (human and mouse)	http://www.genome.ucsc.edu/
Worm Base	http://www.wormbase.org

Tools and Software

Alignments	
Clustalw	http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html
Spidey	http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/
Gene2Est Blast	http://woody.embl-heidelberg.de/gene2est/
Artemis	http://www.sanger.ac.uk/Software/Artemis/ (*A DNA sequence viewer and annotation tool*)
BLAST	http://www.ncbi.nlm.nih.gov/BLAST/ (*BLAST [Basic Local Alignment Search Tool]: a set of similarity search programs designed to explore sequence databases regardless of whether the query is protein or DNA*)
EMBOSS	http://www.emboss.org/ (*a package of high-quality FREE Open Source software for sequence analysis*)
Entrez	http://www.ncbi.nlm.nih.gov/Entrez/ (*a retrieval system for searching several linked databases*)
GFF	http://www.sanger.ac.uk/Software/formats/GFF/ (*an exchange format for feature description*)
Intron/exon predictions	
Metagene	http://www.rgd.mcw.edu/METAGENE/
NetGene2	http://www.cbs.dtu.dk/services/NetGene2/
HmmGene	http://www.cbs.dtu.dk/services/HMMgene/
Genie	http://www.fruitfly.org/seq_tools/genie.html
Alternative splicing	http://www.bit.uq.edu.au/altExtron
MySqI	http://www.mysql.org (*relational database*)
SRS	http://srs.ebi.ac.uk/ (*a powerful data integration platform, providing rapid and user-friendly access to the large volumes of diverse and heterogeneous Life Science data stored in more than 400 internal and public domain databases*)

Drug Discovery and Structural Genomics

Drug discovery	http://www.cgen.com/science/armc-2001.htm
Molecular recognition	
Binding database	http://www.bindingdb.org/bind/index.jsp
Biomolecule interaction	http://www.bind.ca/
Interacting proteins	http://dip.doe-mbi.ucla.edu/
Structural genomics	
CATH	http://www.biochem.ucl.ac.uk/bsm/cath_new/ (*protein structure classification*)
FSSP	http://www2.ebi.ac.uk/dali/fssp/ (*fold classification based on structure–structure alignment of proteins*)
Nature Structural Genomics	http://www.nature.com/nsb/structural_genomics/
SCOP	http://scop.mrc-lmb.cam.ac.uk/scop/index.html (*structural classification of proteins*)
StrucGen	http://www.rcsb.org/pdb/strucgen.html#Resources (*structural genomics overview; worldwide project list*)
Target	http://targetdb.pdb.org/ (*targetDB is a target registration database for structural genomics*)

Other Sites and Links

Celera	http://www.celera.com
Collection of biolinks	http://123genomics.homestead.com/files/home.html http://www.expasy.org/alinks.html#Proteins
EBI	http://www.ebi.ac.uk/
Genomics Institute	http://web.gnf.org/
Incyte	http://www.incyte.com
NCBI site map	http://www.ncbi.nlm.nih.gov/Sitemap/index.html (*important overview with brief description of all NCBI resources*)
Nomenclature	http://www.genomicglossaries.com/content/omes.asp http://www.gene.ucl.ac.uk/nomenclature/
Transgenic/mutation/gene knockouts	http://tbase.jax.org/ http://www.bioscience.org/knockout/knochome.htm http://www.informatics.jax.org/
Tyrosine kinases	http://pkr.sdsc.edu/html/index.shtml
Tyrosine phosphatases	http://science.novonordisk.com/ptp/

approach is generally applicable and that it (with some patience) can be utilized for bioinformatics analyses of other protein families.

Amino Acid Homology Among PTP Domains and Structure–Function Studies

Databases: Genome, mRNA, and Protein Sequences

Monitoring the avalanche of primary sequence data entering archives such as GenBank and EMBL is a daunting task; however, the biological community is increasingly well prepared with secondary databases that extract and annotate subsets from the primary data. The RefSeq resource maintains a nonredundant set of human mRNAs, which are mapped onto genomic sequence via LocusLink or the UCSC genome browser (Table I). A nonredundant catalog of human proteins is maintained in the SwissProt/TrEMBL protein database (Table I, Swall) or in the RefSeq database of mRNA translation products. In addition, protein families and conserved domains (including amino acid sequence alignments and consensus motifs) are found in the InterPro database (Table I), which provides automated annotation of all SwissProt/TrEMBL proteins, including the human proteome (i.e., the gene product inventory of the human genome) [10,11]. Although the human proteome set is some way from completion, the Ensembl project (Table I) provides a source of homology-based predicted gene products based on the current human genome assembly. On the European Bioinformatics Institutes website (Table I, EBI), the current predicted protein index stood at 29,076 sequences as of May 2002. In contrast, the nonredundant mRNA total in RefSeq now stands at 15,846. This means that approximately half of the human genes are already represented as full-length coding sequences if considering only one gene–one-mRNA–one protein.

Collection of Unique Vertebrate PTP Sequences

Establishment of a nonredundant collection of amino acid sequences is a key first step for a comprehensive bioinformatics analysis of any protein family, and a simple text search (Table I, SRS) in the above protein databases retrieves the majority of human PTPs (e.g., the query "tyrosine" and "phosphatase" retrieves 28 human PTPs, including links to their cDNAs). The conserved catalytic domains of these well-characterized cDNAs were next used in a BLAST search to retrieve the entire set of PTP-like sequences deposited in GenBank [12]. Initially, more than 3500 database hits were discovered. Exclusion of expressed sequence tags (ESTs) and high-throughput genome (HTG) sequences reduced this number to 254 sequences, and, after further exclusion of PTP splice variants, partially overlapping clones, and duplicate entries, a total of 113 distinct vertebrate PTP catalytic domains were identified, 37 of which were human. A database of full-length proteins corresponding to this larger set of unique human PTP cDNAs forms the basis for our bioinformatics analyses (Fig. 2). In addition, because most PTPs deposited in GenBank have been identified through low-stringency hybridization and PCR-based cloning techniques, the above review of all PTP accession numbers allowed assignment of synonyms to PTP sequences which were characterized in independent studies and consequently given different names in the literature.

Primary Sequence Alignment: Classification of PTPs and Phylogenetic Trees

Multiple sequence alignments (generated with ClustalW [13]) allow assessment of the degree of residue conservation among homologous proteins and hence provide a powerful basis for their classification. For the classic PTPs, a phylogenetic analysis of 113 aligned vertebrate PTP-domain amino acid sequences (available at http://science.novonordisk.com/ptp/ or http://ptp.cshl.edu) reveals that this group of enzymes can be divided into 17 subtypes: 9 nontransmembrane subtypes (NT-PTPs or intracellular PTPs) and 8 receptor-like PTPs (RPTPs) subtypes (Fig. 1).

The nine intracellular PTP subtypes identified by the phylogenetic analysis correlate well with a classification based on regulatory or targeting domains residing outside the catalytic domains (Fig. 1):*NT1:* PTP1B and TC-PTP; *NT2*: SHP-1 and SHP-2; *NT3*: MEG2; *NT4*: PEST, LyPTP, BDP1; *NT5*: MEG1 and PTPH1; *NT6*: PTPD1 and PTPD2; and *NT7*: PTPBAS. Four of these subtypes each consist of only one enzyme: *NT3*: MEG2; *NT7*: PTPBAS; *NT8*: PTPTyp; and *NT9*: HDPTP.

For tandem-domain RPTPs, the membrane-proximal PTP domains (D1 sequences) cluster into one major trunk of the phylogenetic tree, whereas the single-domain RPTPs define three distinct subtypes (R3, R7, and R8). When including 49 membrane-distal PTP domains (D2 sequences) from vertebrate tandem-domain PTPs in the analysis, these sequences define a separate subfamily that is phylogenetically distinct from the subfamilies defined by the PTP catalytic D1 domains, thus indicating structural and perhaps functional conservation among the D2 sequences [12].

Of note, one RPTP subtype contains both receptor-like and nontransmembrane PTPs (R7), and two RPTP genes also encode cytoplasmic variants as a result of alternative splicing (GLEPP1; mouse RPTPϕ [14]) or alternative promotor usage (PTPε [15]). Except for these discrepancies, the phylogenetic classification based on PTP domain sequence homology is in overall accordance with previous topological classifications based on the presence or absence of an extracellular region.

Conserved Regions and Residues: A Three-Dimensional Comparison

Already during the first years of PTP research, when only a few different PTP cDNAs had been isolated, it became apparent that certain motifs were conserved. At that time, neither the structural nor functional significance of these motifs was known. However, they served as useful priming

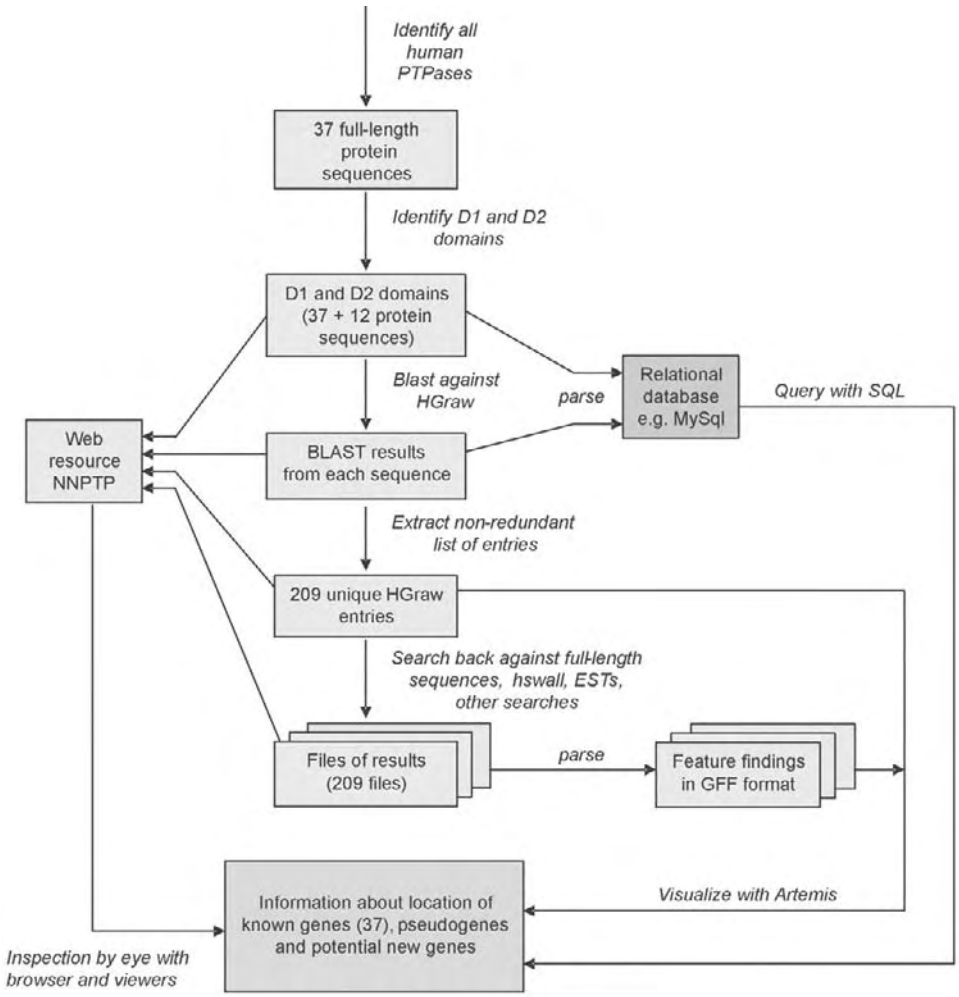


Figure 2 Flow diagram for identification of PTP-like sequences in the human genome. (For abbreviations and links, see Table 1.)

sites for identification of novel PTPs using degenerate oligonucleotide primers and helped significantly in advancing the field in a timely fashion. Equally impressive has been the speed of determining the structures of different PTPs. Thus, following the seminal study on the structure of PTP1B [16], X-ray protein crystallographic structures are currently available for nine different PTP catalytic domains: PTP1B, TC-PTP, SHP-1, SHP-2, PTPμ, PTPα, PTP-LAR, PTP-SL, and *Yersinia* PTP (Table I, Entrez). Moreover, a remarkable number of studies have reported PTPs in complex with various peptide substrates and inhibitors (see below). We and others have superimposed these PTP domains and found a striking conservation of the tertiary structure [12]. This structural conservation allows the combination of primary sequence analysis and low-resolution homology modeling and thus identification of conserved regions and residues at the three-dimensional level. The catalytic domains of PTPs consist of about 280 residues arranged as a three-layer, α–β–α core domain with a central β-sheet sandwiched between α-helices (see Chapter 108).

Primary sequence alignment of the catalytic domains of PTPs reveals 10 discrete, highly conserved motifs (M1–10, detailed at http://science.novonordisk.com/ptp/) and 7 single conserved residues (Glu19, Glu115, Arg156, Arg169, Leu192, Arg254, and Arg257; hPTP1B numbering, which is used throughout this chapter). Several of these motifs play critical roles in maintaining the stability of the PTP domain (e.g., extensive hydrophobic packing is observed for motifs M3–M7), while other motifs and conserved single residues are essential for catalysis. The most highly conserved area within the PTP tertiary structure is defined by the PTP signature motif, HisCysSerXxxGlyXxxArg[Thr/Ser]Gly (M9), and the structural motif [Phe/Tyr]IleAlaXXxGlnGlyPro (M4).

The Catalytic Machinery

While the molecular mechanisms underlying PTP mediated catalysis are treated in detail elsewhere (see Chapter 108), the primary sequence and three-dimensional analyses allow a first glimpse into the intricate catalytic machinery. The PTP signature motif ValHisCysSerXxxGlyXxxGlyArg-[Thr/Ser]Gly (residues 213–223 in PTP1B) defines the PTP family and represents one of Nature's elegant designs of a highly efficient binding pocket for phosphate. The PTP

motif, also called the P-loop or PTP-loop, forms a half-circle with the main chain nitrogens pointing toward the cysteine (Cys215 in PTP1B), which is positioned almost in the center. At physiological pH, this cysteine residue is deprotonated and acts as a nucleophile accepting phosphate transiently during catalysis [17]. Two of the highly conserved single residues help stabilize the PTP-loop (Glu115 and Arg257), which is positioned at the bottom of an approximately 9-Å-deep pocket (i.e., corresponding to the length of the side chain of phosphotyrosine, pTyr, but not the shorter side chains of phosphoserine and phosphothreonine). The phenyl ring of the pTyr substrate interacts with the aromatic Phe182 and Tyr46 residues and the hydrophobic residues Val49, Ala217, and Ile219. Upon binding of pTyr substrates, a major conformational change takes place that moves the WPD loop to close the active site pocket, literally trapping the substrate [18–20]. The WPD loop closure brings Asp181 close to the scissile oxygen of pTyr, where it is in a favorable position to function as a general acid during the first step of catalysis. In the second step, the highly conserved Gln262 positions a catalytic water molecule (for hydrolysis), thereby releasing phosphate from Cys215 [18].

Conserved Surface-Exposed Areas in Tandem PTPs: The D1/D2 Interface

The invariant residues in domain D1, which show considerable substitution in the D2 domains, are positioned close to the active site. In some RPTPs (e.g., PTPα, PTPε, LAR) only two point mutations in D2 domains are required to restore robust catalytic activity against conventional PTP substrates, whereas critical substitutions present in D2 domains of other RPTPs (e.g., CD45, PTPζ, PTPγ) indicate that these domains are truly inactive. While low-resolution homology modeling (so-called Cα-regiovariation score analysis) of the catalytic domains of intracellular PTPs and the membrane-proximal D1 domains of RPTPs shows that the conserved residues converge around the active site, much greater variation is observed in the vicinity of the putative active sites in the D2 domains of receptor-type PTPs [12]. Thus, this analysis supports the notion that most of the catalytic activity is found in proximal D1 domains in tandem RPTPs [21,22], whereas the membrane-distal D2 domains, at least for some RPTPs, seem to play regulatory roles, as has been demonstrated for CD45 [23]. The D2 domains could act as phosphotyrosine recognition units, similar to Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains [24].

So far, the crystal structure of tandem PTP domains has only been reported for PTP-LAR [25]. The relative orientation of the LAR D1 and D2 domains, constrained by a short linker, is stabilized by extensive interdomain interactions. In the present context, it seems significant that the Cα-regiovariation analysis has identified conserved areas on both the D1 and D2 domains that correspond to the interaction area in LAR. In addition, the sequences corresponding to the linker sequence in LAR were found to be conserved in the D2 domains, but not in the D1 domain [12]. Thus, it seems likely that the overall structure of receptor-like PTPs is well represented by the X-ray structure of PTP-LAR.

Nonconserved Residues in the Vicinity of the Active Site: Implications for a Bioinformatics Approach to Structure-Based Drug Design

At this point, the prototype PTP1B has in particular attracted the attention of the pharmaceutical industry. Mice in which the PTP1B gene has been removed (i.e., knocked out) show increased insulin sensitivity and resistance to diet-induced obesity [26,27]. Hence, inhibitors of PTP1B could potentially be useful for the treatment of type 2 diabetes and obesity.

The highly conserved structure of the PTPs and the consequential apparent difficulties related to developing selective active site-directed inhibitors initially discouraged the pharmaceutical industry from considering this group of enzymes as useful drug targets. A similar myth kept the industry away from the kinase field as it was considered impossible to develop kinase inhibitors with the requisite specificity [28]. However, basic research on protein kinases was leading the way for the PTP field, and applied pharmaceutical research has shown that even subtle differences or combinations of differences can be utilized in structure-based design of highly selective inhibitors that bind to the conserved ATP binding pockets in kinases. The PTP inhibitor field is now rapidly catching up, and both academic and industrial laboratories have convincingly demonstrated that selective, active site-directed PTP inhibitors can indeed be made [29–31]. In our laboratory, we used the above Cα-regiovariation score analysis to identify residues or combinations of residues in the vicinity of the active site that would uniquely identify a particular PTP and thus could potentially be used for structure-based design of selective inhibitors. Because the intention is to develop orally active inhibitors, a number of compound characteristics must be taken into account; hence, poor absorption and cell permeation are often observed when the "rule of five" is violated, including exceeding a molecular weight of 500 [32]. Therefore, to allow design of low-molecular-weight, active, site-directed inhibitors, it is a requirement that selectivity be achieved by addressing residues in the vicinity of the active site.

Using the above low-resolution homology modeling approach revealed that four residues (47, 48, 258, and 259) were especially important for the design of selective PTP inhibitors. None of these residues is unique for one specific PTP, but the combination of these four residues constitutes a selectivity-determining region, a signature motif. Even closely related members within one PTP subfamily often differ in this region (e.g., PTPα and PTPε). We and others have used this selectivity-determining region for structure-based design of selective PTP1B inhibitors [33–36] (see Chapter 111 for further details).

From a drug discovery point of view, bioinformatics analyses can contribute significantly to avoiding problems due to lack of specificity which otherwise might show up late in a

development phase as adverse or toxicological effects. Thus, when developing selective PTP inhibitors it is essential to have complete structural knowledge of all members of this enzyme family. As indicated, the conserved three-dimensional fold of PTPs allows relatively accurate structural comparisons of catalytic domains, even of PTPs for which no X-ray structures have yet been obtained. As an example, we have used Asp48 as an important interaction point (for salt-bridge formation) to develop selective PTP1B inhibitors [33]. Using combined structural and genomic analyses, which we have termed *structural bioinformatics*, we have identified all PTPs with an aspartic acid in the equivalent position. By introducing these PTPs as counter screens at an early stage in preclinical development of PTP1B inhibitors, we expect to avoid selectivity problems within the PTP family. In other words, complete mapping and analyses of all PTPs (and all other potential drug target families) are a must in the postgenomic era.

Identification of the Genomic Complement of PTPs

A. Chromosomal Localization of PTP Genes

With access to the human genome assembly (currently "Build" 28 at the National Institutes of Health), it is an easy task to identify the chromosomal localization of the 37 known human PTPs using LocusLink or the BLAST search platform in the UCSC genome browser (Table II). Importantly, the chromosomal localization could be mapped for all published PTPs consistent with the estimated 95% coverage of the human genome. This chromosomal assignment is important to resolve whether homologous proteins are splice variants derived from the same gene or are recently duplicated genes or pseudogenes. In addition, the current refinement of PTP chromosomal localization allows for disease association studies.

Mapping of Intron/Exon Structures: Alignment of mRNA with Genomic Sequences

To visualize PTPs in a genomic context, mRNA or protein sequences were fine-mapped (i.e., unequivocally aligned at the exon level) onto the assembled genome via LocusLink, Ensemble, or the UCSC genome browser (Fig. 2). NCBI provides a convenient tool for aligning mRNA or EST sequences to a genomic sequence (Table I, Spidey) in cases where PTP genome sequences are not present in the genome assembly but are found only in the raw sequences from the sequencing centers.

To distinguish between potentially novel PTPs and PTP pseudogenes in the humane genome, knowledge of the exon/intron structures for the known PTPs is essential. In addition, the genomic organization of a particular PTP is important when analyzing alternative splicing events or promoter elements. Finally, our mapping of PTP exon/intron structure revealed that the above classification of the PTP protein family into 17 PTP subtypes, based exclusively on catalytic domain amino acid sequence homology, is consistent with a classification based on PTP gene structure.

Table II Genomic Annotation of PTPs[a]

PTP name	Chromosome	Gene symbol	OMIM ID.
LyPTP	1p13.2	PTPN22	606986
LAR	1p34.2	PTPRF	179590
PTPlamda	1p35.2	PTPRU	602454
CD45	1q31.3	PTPRC	151460
HePTP	1q32.1	PTPN7	176889
OST-PTP	1q32.1	N.A	N.A
PTPD2	1q32.3	PTPN14	603155
MEG1	2q14.2	PTPN4	176878
BDP1	2p21.1	PTPN18	606587
IA2	2q35	PTPRN	601773
PTPgamma	3p14.2	PTPRG	176886
HDPTP	3p21.31	PTPN23	606584
PTPBAS	4q21.3	PTPN13	600267
PTPkappa	6q22.33	PTPRK	602545
PEST	7q11.23	PTPN12	600079
PTPzeta	7q31.31	PTPRZ1	176891
IA2beta	7q36.3	PTPRN2	601698
PTPdelta	9p24.1	PTPRD	601598
PTPH1	9q31.3	PTPN3	176877
PTPTyp	10q11.22	PTPN20[b]	N.A
PTPepsilon	10q26.2	PTPRE	600926
DEP1	11p11.2	PTPRJ	600925
STEP	11p15.1	PTPN5	176879
GLEPP1	12p12.3	PTPRO	600579
SHP1	12p13.31	PTPN6	176883
PCPTP1	12q15	PTPRR	602853
PTPbeta	12q15	PTPRB	176882
PTPS31	12q21.31	PTPGMC1[b]	603317
SHP2	12q24.13	PTPN11	176876
PTPD1	14q31.3-q32.11	PTPN21	603271
MEG2	15q24.2	PTPN9	600768
TCPTP	18p11.21	PTPN2	176887
PTPmu	18p11.23	PTPRM	176888
PTPsigma	19p13.3	PTPRS	601576
SAP1	19q13.42	PTPRH	602510
PTPalpha	20p13	PTPRA	176884
PTPrho	20q12-q13.11	PTPRT	N.A
PTP1B	20q13.13	PTPN1	176885

[a]This table is based on "Build" 31 and thus contains the most recent information available.
[b]Not a HUGO-approved name.

Searching the Human Genome for PTP-Like Sequences

The collection of unique full-length human PTP protein sequences formed the basis for mining the human genome

for novel PTPs and pseudogenes. A strategy for a BLAST-based homology search [37] with PTP D1 and D2 domain sequences as the origin is shown schematically in Fig. 2. To improve the specificity and reduce noise, the conserved PTP domain sequences were used as origin in this search, rather than the full-length sequences. Using the *tblastn* algorithm (part of the BLAST package) with default settings, D1 and D2 sequences from each of the 37 human protein sequences were searched against the six-frame translation of the human genome (raw sequences from the sequencing centers Human Genome Centers; see Table I). Each of the searches produced a results file with a substantial number of sometimes overlapping hits to various entries in the human genome database (HGraw). A nonredundant list of 295 genome entries was compiled from these files, and each entry was examined by hand for PTP catalytic domains using a number of iterative searches and predictions, including searches against the full-length protein sequences, hswall, and the EST database. In addition, selected genome sequences were analyzed with NetGene2 to predict splice sites, and putative PTP motifs were identified with the *fuzztran* program from the EMBOSS package. To allow quick navigation in the genome entries and the associated search results files, everything was organized in a web environment. To visualize see the Novo Nordisk website, NN-PTP; see Table I. To visualize the genome entries and their associated features (e.g., homology to full-length protein sequences, areas with EST hits, motifs), the results files were parsed to produce feature annotation in GFF format. Artemis provides a user-friendly graphical viewer of genome entries that can be loaded with the associated GFF files.

Using the web environment and the Artemis tool, each genome entry was carefully inspected by hand. This procedure reduced the 295 overlapping hits to: (1) the 37 published PTPs, (2) 9 intron-less PTP pseudogenes, (3) 4 or more potential novel PTPs with exon/intron structure, (4) 11 dsPTPs, and (5) 14 false-positive entries (low complexity hits). Although definitive classification of these potentially novel PTPs awaits a combined analysis of the finished genome sequence and experimental verification of cDNAs, it seems clear that the total number is far below earlier expectations in this protein family [38].

To complement the tedious manual data analyses with brute-force SQL queries, all hits between the origin protein sequences and the genome entries were uploaded to a relational database (MySql), which also assists in (1) keeping track of hits, (2) generating statistics, and (3) more in-depth database mining in the future.

PTP Pseudogenes

As indicated previously, the human genome contains at least nine intronless PTP-like sequences that are closely related (>94% nucleotide identity) to the mRNA of TCPTP (two sequences), SHP2 (five), MEG1 (one), or RPTPα (one). Closer inspection of these sequences revealed multiple inframe stop codons due to insertions and deletions. Only two of the nine sequences had perfect matches with ESTs.

As an example, in addition to the TCPTP gene on chromosome 18 (Table 2), two TCPTP-like sequences were identified on chromosomes 1 and 13 (TCPTP-P1 and TCPTP-P13) which share 94 to 95% nucleotide identity over 1440 bp with the TCPTP cDNA. The lack of introns and the presence of polyadenylated tails indicate that these sequences are pseudogenes that arose by retrotransposition. Both TCPTP sequences harbor frameshift mutations and premature stop codons. If transcribed, this would generate short PTP-unfunctional polypeptides of 41 or 149 amino acids, respectively. Of note, TCPTP-P13 is associated with an EST sequence (aw401979), thus it may be expressed, although the function of such an mRNA/truncated protein is unknown.

When reviewing the *in situ* hybridization data published in the pregenomic era, we discovered that both the TCPTP and SHP2 pseudogenes identified in this study *in silico* also had been detected experimentally [39–41]. In the case of TCPTP, Johnson and coworkers [39] compared the specificity of genomic and cDNA probes and demonstrated that, under identical conditions, the genomic TCPTP probes (containing both exon and intron sequence) readily identified a single specific site of hybridization (18p11.3-p11.2), whereas the TCPTP cDNA probe identified sites of both the gene and its pseudogenes (1q22-q24 and 13q12-q13). Likewise, Jirik and coworkers [40], when using a SHP2 cDNA probe, found hybridization signals over 4q21 and 5p14 as well as to a lesser degree over chromosomes 3q1-3q13.2, 6q23-q24, and 8q12, in addition to 12q24.1 (the SHP2 gene; see Table 2) [40]. In light of today's genome assembly, we conclude that these signals correspond to the exact localization of five intronless SHP2 pseudogenes.

Novel PTPs

In addition to the intronless PTP pseudogenes, our analysis revealed a few novel PTP-like sequences and fragments that have an exon/intron structure consistent with the genomic organization of the PTP gene family. Some of these sequences are only fragments, and the final verification of these putative novel PTP genes awaits completion of the human genome sequence or experimental demonstration of their expression. However, one novel human PTP could be assigned to chromosome 1q32 (Fig. 3). The PTP gene located here has approximately 80% homology to both rat osteotesticular PTP (OST-PTP) and mouse embryonic stem cell phosphatase (PTP-ESP). Together with fluorescence *in situ* hybridization studies, which map OST-PTP to mouse chromosome 1 (region F–G, a region syntenic to human chromosome 1q32-q33), the similarity in gene structure suggests that this novel PTP is the human ortholog of OST-PTP. Because the human OST-PTP has not yet been cloned, the identification of its genomic sequence will facilitate future characterization of this PTP. As a first step, the human genome browser (Table 1, UCSC) allowed us to retrieve a hypothetical amino acid sequence for this PTP, as predicted by the Ensembl project. Only two EST sequences map to this region of chromosome 1 (Fig. 3), suggesting a highly

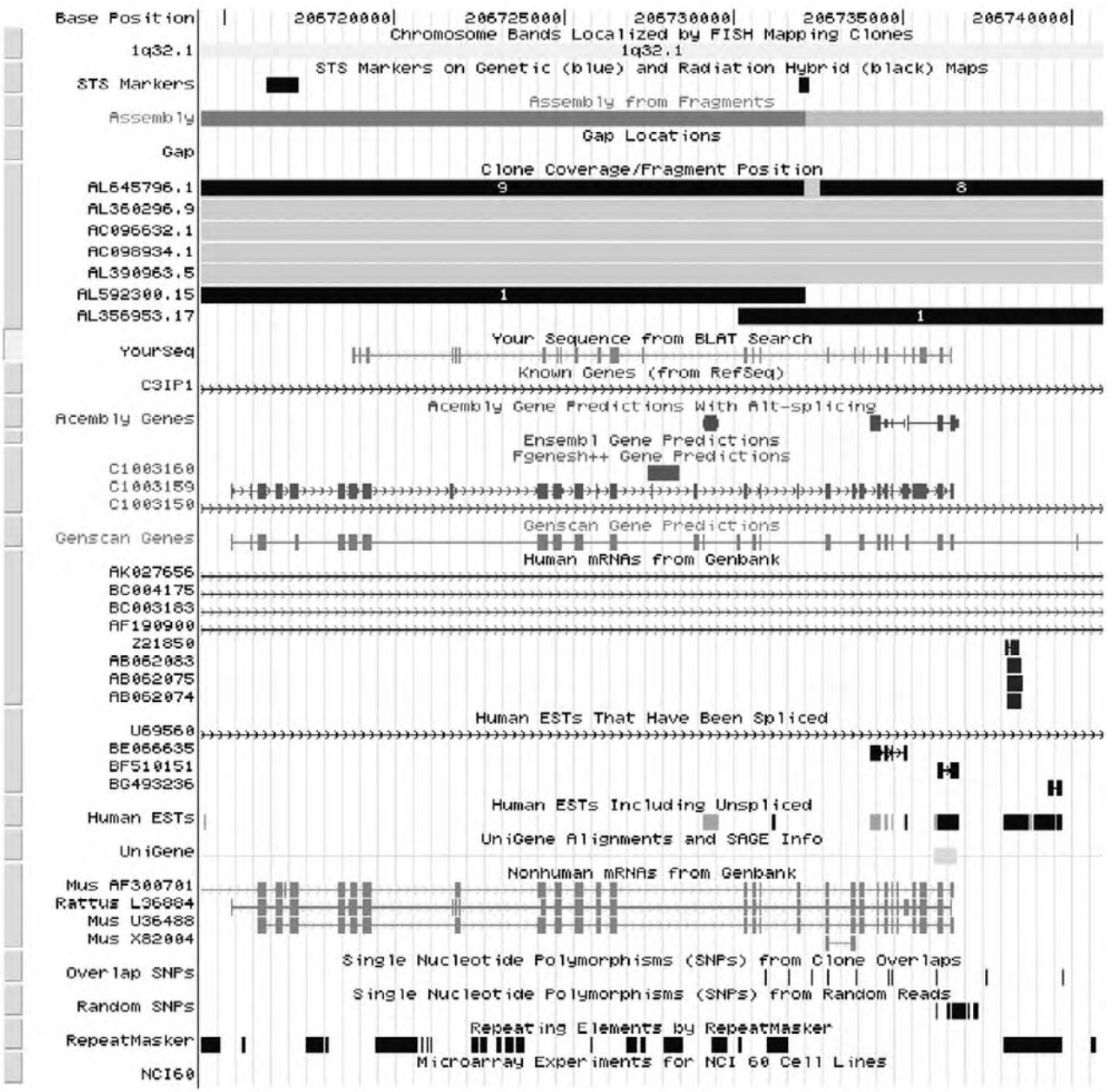


Figure 3 Identification of a novel human PTP (OST-PTP) and its genomic context in a selected browser (*UCSC*, Table I). From top to bottom the features include the nucleotide base position that refers to the coordinates in the NIH genome assembly, with the cytogenetic band immediately underneath, and a graphic view of clone coverage (gaps and overlap) in the assembly and accession numbers of the raw sequence. The identifier 'YourSeq' is the exon-intron structure we have predicted for human OST-PTP and underneath are homology-supported gene models (Acembly, Ensembl, Fgenesh and GenScan). Below the automated gene predictions are mRNA and ESTs sequences from human (black) and rodents (grey) aligned to the genome. Finally, additional display options can be selected, e.g. location of repeats, SNP, sequence-tagged-sites (STS), genetic markers and microarray expression data.

regulated and specific expression pattern similar to that observed for its mouse and rat counterparts [42].

Functional Aspects of PTPs in Health and Disease: Bioinformatics

Phosphorylation of cellular proteins regulates most signal transduction processes, and many diseases have been associated with abnormal phosphorylation patterns. An intricate balance and regulatory machinery are required to maintain the exact levels of phosphorylation. How can less than 50 different classic PTPs be sufficient to regulate and fine-tune phosphotyrosine levels in a human from conception, during embryonic life, and through childhood, puberty, and adulthood? In addition to inherent substrate specificity that resides within the catalytic domains of protein tyrosine kinases and PTPs, other regulatory measures must be at play. As an example, compartmentalization may influence the accessibility to substrates and associated proteins [43,44], and the

subcellular localization may be further affected by proteolytic processing [45]. In addition, the activity of PTPs may be influenced by covalent modification [46]. Bioinformatics analyses of gene structure may provide further insight into PTP regulation by predicting alternative splicing, as recently demonstrated for PTPrho [47].

The present refinement of PTP chromosomal localization allows for disease-association studies using information from genetic disease databases such as the Online Mendelian Inheritance in Man (OMIM) database, which is an authoritative and comprehensive genetic knowledge database integrated in NCBI's Entrez suite (Table 1, OMIM) which provides full-text overviews of genetic disorders and gene loci as well as links to other genetic databases [48,49]. A recent candidate disease gene study illustrates this point. The relatively common Noonan syndrome maps to a 5-cM region on chromosome 12q24.1. Because PTPN11 (encoding SHP2) maps to the same region (Table 2) and SHP2 is known to play an important role in animal development, Tartaglia *et al.* hypothesized that mutations in this gene could be the cause of the syndrome [50]. Indeed, missense mutations in *PTPN11* were found in more than 50% of the cases. Of note, the identified amino acid substitutions affect highly conserved residues of SHP2 that are predicted to be important for the regulatory autoinhibition caused by binding of the N-terminal SH2 domain to the active site of SHP2 [51] (see Chapter 117).

The ultimate definition of the exact functions of a protein is often elusive and requires inter- and multidisciplinary approaches. Rarely is the function of a protein defined unequivocally in one study; rather, a general understanding evolves through a series of stepwise and iterative investigations where corrections are required as information is gathered over the years. Although there still remains much work to be done to complete the sequencing and analysis of the human genome, the next gold rush is on to accelerate the process of defining the function of proteins: proteomics [52].

While the human proteome may be considered endless or at least one order of magnitude more complex than the genome, from a practical point of view the functions of a gene product may be inferred from combined experimental studies, such as (1) tissue-distribution analyses (mRNA/protein), (2) over-expression of dominant-negative mutants, (3) *in vitro* and *in vivo* gene knockouts and transgenes, (4) antisense or RNA-mediated interference (RNAi) studies, (5) disease-association studies, or (6) the use of selective small molecule inhibitors or drugs. Provided that an evolutionary relationship has been established, deletion of an ortholog of a human gene in *Caenorhabditis elegans* or *Drosophila melanogaster* may further assist in assigning a function to such a gene [53]. Novel public and proprietary databases (e.g., Incyte, Celera) and bioinformatics tools will undoubtedly in the postgenomic area contribute significantly to initial insights or formulation of hypotheses for the functions of a given protein, from both a structural and functional genomics/proteomics point of view (important resources and links may be found via NCBI or 123Genomics; see Table I).

In the field of protein tyrosine phosphatase, PTP1B has received particular attention, and an understanding of its function is emerging from the above combined approaches. Thus, it seems likely that PTP1B is a key regulator of leptin signaling [54–56] and potentially an important drug target in obesity and type 2 diabetes (see Chapter 118). In the current bioinformatics context, it is of particular interest that PTP1B maps to 20q13.1-q13.2 (Table 2), a region linked to obesity and diabetes [57–60], and recently a rare P387L variant of the PTP-1B gene was found to be associated with relative risk of type 2 diabetes in a Danish study [61].

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CHAPTER 111

PTP Substrate Trapping

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Introduction

In the early 1990s, many protein tyrosine phosphatases (PTPs) had been identified yet very few had been linked to a physiological function. For some, very little was known about them except for their primary amino acid sequence, which was sufficient only to identify them as members of a rapidly growing family of PTPs. During this time, many efforts were underway to attempt to ascribe functions to some of these PTPs. In the lab of Tonks *et al.* [1,2] at Cold Spring Harbor, one approach that was developed with this purpose in mind was termed *substrate trapping*. The fundamental concept behind this method was to identify a mutant form of a PTP that met three criteria: (1) it retained normal substrate binding specificity, (2) it was incapable of dephosphorylating substrates, and (3) it would form a sufficiently stable dead-end complex such that the substrates could be isolated in association with the mutant PTP. Any substrates present in the complex could be visualized with antiphosphotyrosine antibodies and upon identification could suggest potential physiological functions of that PTP. Since its initial development, this method has been applied widely to this class of enzymes, members of which utilize a similar catalytic mechanism but vary in their ability to dephosphorylate phosphotyrosine, phosphoserine, phosphothreonine, or phospholipids.

By far, the most common use of the method has been to carry out immunoprecipitation-type experiments similar to those originally described [1,2], in attempts to further one's understanding of the physiological function of a particular PTP. Other experiments have utilized trapping mutants to perturb cellular function. Interpretation of these experiments is not always simple, however, as sometimes the mutant PTP serves only to block the action of the normal PTP and in other cases the trapping mutant not only subverts the action of the active PTP but also interferes with the normal function of the substrate itself. Studies of the tumor suppressor gene PTEN illustrate the former case as well as demonstrate that trapping mutants can be effective when the substrate is a lipid, in this case phosphatidylinositol 3,4,5-triphosphate (PIP3) [3,4]. Expression of the C124S mutant of PTEN in 293 cells overwhelmed endogenous PTEN to cause an increase in levels of the substrate PIP3 and did not decrease survival rates of LnCaP cells, while active PTEN did [4]. Conversely, experiments with D181A-PTP1B and $p210^{bcr\text{-}abl}$ showed that not only did this mutant antagonize endogenous PTP1B, resulting in increased tyrosine phosphorylation of $p210^{bcr\text{-}abl}$ [5], but it also blocked the downstream actions of $p210^{bcr\text{-}abl}$ by binding to the tyrosine phosphorylation site required for signaling via grb2 [6].

Practical considerations related to use of the substrate-trapping methodology, including purifying or expressing appropriate mutants, choosing a source of potential substrates, deciding whether to trap proteins from a cell lysate or to isolate a complex following expression of the substrate-trap ping phosphatase in cells, and isolating the trapping mutant/substrate complex, have been thoroughly described [7]. This chapter first discusses the current understanding of how trapping mutants work, some new and improved trapping mutants and how noncatalytic site regions of PTPs can contribute to substrate recognition. Next, some new approaches to identifying substrates with these mutants are discussed as well as some uses of these mutants for purposes other than the identification of substrates.

Original C → S and D → A Substrate-Trapping Mutants

The substrate-trapping methodology developed in the Tonks lab focused originally on a conserved catalytic aspartate residue (181 in PTP1B) [8], the mutation of which results in a severely reduced catalytic capacity (0.001% of wt activity for D181A-PTP1B assayed with RCML) [1].

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Whereas mutation of the essential catalytic cysteine to either serine or alanine was known previously to produce a protein without measurable catalytic activity [9], this mutant form of several tyrosine-specific phosphatases (e.g., PTP1B, TCPTP, PTP-PEST, PTP-H1) did not effectively trap any tyrosine-phosphorylated substrate proteins. However, replacement of the catalytic aspartate residue with alanine (D → A) created a mutant with the ability to form a sufficiently stable complex with substrates that allowed the complex to be isolated and studied.

The reason why complexes between PTP1B-D181A and its substrates survive isolation (e.g., immunoprecipitation and washing conditions) better than those formed with PTP1B-C215A (or S) is still not completely understood. Presumably significant differences exist in the kinetics (dissociation and/or association rate constants) of substrate binding to each mutant. Nonetheless, a significant increase in understanding of the thermodynamic parameters of substrate binding by these mutants has been gained. Isothermal titration calorimetry measurements of binding of a nonhydrolyzable difluoromethylene-phosphono-phenylalanine peptide by wild-type and mutant forms of PTP1B have shown that the D181A mutant of PTP1B exhibits a fivefold higher affinity for this ligand than does the wt or C215S protein [10]. For both the D181A and C215S mutants, a reduction in electrostatic repulsion between the bound dianionic phosphate-containing ligand and the negatively charged D181 or thiolate C215 might have been predicted to result in higher affinities for both mutants; however, for the C215S protein the gain in enthalpy upon ligand binding is offset by an increased entropic barrier. The explanation that the C215S protein exhibits greater mobility or flexibility in the catalytic site region [10] has been confirmed visually in rather dramatic fashion in a crystal structure of the C215S protein [11]. In this structure, the PTP loop that contains the C215S mutation and contributes to binding the phosphate oxygens in substrates has adopted a conformation in which it is almost perpendicular to its normal orientation [11]. This conformation is clearly incompatible with substrate binding, and the increased entropic term measured by Zhang *et al.* [10] likely reflects the energy required to flip the loop back to the conformation observed in the crystal structures of PTP1B-C215S (or C215A) bound to phosphotyrosine-containing peptide substrates [12–14].

Second-Generation Trapping Mutants

While the use of D → A mutants initially appeared to be a useful, somewhat general method for identifying PTP substrates it was not long before modifications and improvements in the technique began to appear. For the phosphatase PTPH1, the D → A single mutant was used successfully to isolate tyrosine-phosphorylated VCP out of a lysate from pervanadatetreated cells and to identify it by peptide sequencing [15]. However, when the D811A mutant was expressed in 293 cells, to confirm that the interaction between PTPH1 and VCP could occur in cells where each protein would be present in its correct subcellular location, the PTP itself was the only tyrosine-phosphorylated protein present in the complex. Further study showed that mutation of a second residue within the catalytic site, a conserved tyrosine that forms one side of the substrate binding site, was critical for successful intracellular trapping of the substrate VCP [15]. With the Y676F-D811A double mutant, the tyrosine phosphorylation of PTPH1 itself was almost completely eliminated, and the VCP substrate was now trapped in a complex with the isolated PTPH1 [15].

Recently, the combination of the D181A mutant of PTP1B with Q262A has been shown to result in a substrate binding protein with an affinity for substrate 6-fold greater than for the D181A mutant alone and 30-fold greater than for wt enzyme [16]. Several other PTP1B mutations were tested in combination with D181A. The rationale for each was either to decrease further the residual amount of phosphatase activity present in the D181A mutant or to increase the affinity for substrate. Completely eliminating PTP activity by combing D181A with mutations of C215 resulted in unusually complex binding curves (C215S) or reduced instead of improved affinity (C215E) [16]. In agreement with these binding data, combining the D181A and C215A mutations of PTP1B did not result in any better trapping of substrates (Flint and Tonks, unpublished data) compared to D181A alone [1]. However, introduction of the Q262A mutation into D181A-PTP1B further reduced the k_{cat} toward pNPP by 10-fold and enhanced the affinity for a nonhydrolyzable peptide substrate by 6-fold as compared to D181A alone [16]. Q262 normally functions in PTP1B to promote hydrolysis of the phosphoenzyme intermediate via activation of a water molecule. Mutation of this residue enabled Pannifer *et al.* to trap this unstable catalytic intermediate and examine it crystallographically [17]. The Q262A mutant enzyme has a reduced K_m for substrate as well as a lower k_{cat} [1,18]. When the D181A-Q262A double mutant was transfected into COS cells, it trapped slightly more tyrosine phosphorylated epidermal growth factor (EGF) receptor substrate than did the single mutant D181A-PTP1B [16].

Accessory or Noncatalytic Site Contributions to Substrate Recognition

For some PTPs, mutation of the catalytic aspartate residue renders them much better trapping mutants than does a mutation of the catalytic cysteine residue—for example, PTP1B, TC-PTP, and PTP-PEST. However, for others, a C → S mutation at the catalytic site has been an effective tool for identifying potential substrates. These differences may be explained by variations in the way the enzymes recognize their substrates. For PTPs in which recognition of substrate is restricted to the portion of the substrate protein immediately surrounding the phosphorylation site, the intrinsic affinity of the D → A mutant vs. the C → S may be

a critical factor for isolating the complex. However, for PTPs in which the recognition of substrate utilizes contacts beyond the immediate vicinity of the phosphorylation site, then a C→S mutant can form a catalytically inert complex with the substrate that is stable enough to be isolated and detected. PTP-PEST is a useful illustrative example of this point. The D199A mutant of PTP-PEST uniquely identified $p130^{cas}$ out of a complex array of tyrosine-phosphorylated proteins from pervanadate-treated HeLa cells. This interaction was mediated via the catalytic site as it was disrupted by vanadate and was detected using a truncated form of PTP-PEST containing little more than the phosphatase domain [2]. However, when the full-length form of PTP-PEST was used in trapping experiments, the tyrosine phosphorylated substrate $p130^{cas}$ could be isolated with either the D199A or the C231S mutant [2]. Further characterization showed that a proline rich region around P337 was responsible for enhancing the interaction with $p130^{cas}$ through binding to its SH3 domain [19].

Other examples of phosphatases for which accessory substrate binding sequences or domains have been discovered include CD45, YopH, KAP, and the MAP kinase phosphatases (MKPs). For CD45, the zeta-chain of the T-cell receptor complex was identified as a substrate and its second (noncatalytic) PTP domain (D2) was required for the interaction [20,21]. Replacement of its D2 with the second PTP domain of the closely related receptor PTP LAR prevented the association with the phosphorylated zeta-chain. YopH is a tyrosine-specific PTP that is a virulence factor from the pathogenic bacterium *Yersinia*. It dephosphorylates paxillin and $p130^{cas}$ upon infection of HeLa cells, and expression of a C→S mutant traps these substrates [22,23]. Additionally, a noncatalytic N-terminal domain of YopH has been identified as an important contributor to recognition of these substrates [23,24].

The crystal structure of the C→S mutant of KAP in a complex with its substrate phospho-Thr-160 cdk2 provides an example of the majority of the interaction between the substrate and PTP being remote from the catalytic site, with interactions at the catalytic site restricted to little more than binding of the phosphate group [25]. A final, well-studied class of noncatalytic, site-mediated interactions has been found for the MKPs and their substrates, the mitogen-acti vated protein kinases (MAPKs), also known as extracellular signal-regulating kinases (ERKs). Before the advent of D→A trapping mutants, the C→S mutant of MKP-1 was shown to effectively trap phospho-ERK2 from serum-stimulated cells [26]. An extensive body of more recent literature documents the specificity of MKP3 for its substrate ERK2. The N-terminal, noncatalytic domain of MKP3 is responsible for mediating a high-affinity interaction with ERK2 that not only brings the two proteins together but also activates the catalytic function of MKP3 [27–30]. One facet of the activation appears to be the induction of a conformational change that swings the catalytic aspartic acid into a position where it is now capable of greatly enhancing the rate of dephosphorylation [31].

New Twists on Trapping

One natural extension of more commonly practiced substrate-trapping procedures is to carry out the final analysis of the trapped complexes on two-dimensional polyacrylamide gels instead of by western blotting after the usual one-dimensional SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Potential substrate proteins in the complex can be visualized by blotting to specifically detect phosphotyrosine or could be silver stained to detect all components. With the current sensitivity of mass spectrometry (MS) methods for identifying minute amounts of proteins, the contents of the complex could be determined directly. A different two-dimensional approach was described by Pasquali *et al.* [32] in which substrate-trapping mutants are used in a "far-western" procedure. A complex mixture of tyrosine-phosphorylated proteins (e.g., lysate from cells treated with pervanadate) is separated on two-dimensional gels and transferred to a membrane. The trapping mutant is used as a probe to seek out its substrates on the membrane, and the putative substrate is subsequently identified by MS methods. While these authors were technically successful in identifying α-tubulin as the spot on the gel to which FAP-1 bound, they also pointed to limitations of the method, such as a requirement that the PTP recognize its substrate as it is stuck to a membrane in a partially folded state, that the conditions of the probing might require optimization, and that under these conditions a PTP would have the opportunity to associate with a protein that it might never normally encounter in the cellular milieu. Nonetheless, for substrates of dual-specificity phosphatases that may not necessarily be detectable with antiphosphotyrosine antibodies, methods such as these may be required to detect and identify these substrates.

An alternate approach to substrate trapping that bypasses the need to detect the phosphorylated substrate directly was developed by Kawachi *et al.* [33]. Using a variation of the yeast two-hybrid method developed for identifying SH2-domain-binding proteins [34], the authors engineered a strain of yeast with inducible expression of v-Src, a substrate-traping PTP–lexA fusion, and a library of potential substrates fused to the GAL4 activation domain. Transcriptional activation of the reporter gene occurs when v-Src phosphorylates one of the GAL4 fusions which is then bound by the substrate-trapping PTP–lexA fusion. Sequencing of the plasmid encoding that particular GAL4 fusion identifies the potential substrate. Transcriptional activation should not occur in the absence of v-Src expression when the GAL4 fusion should not be phosphorylated. With this system, the GIT1/Cat-1 protein was identified as a substrate for the neuronal receptor PTPζ [33]. Two obvious advantages of this method are that it might enable identification of low- abundance substrates and that interactions having rapid on/off rates that would likely be disrupted by standard immunoprecipitation wash procedures might now be detected. A limitation of this method is that for the substrate trapping to occur, the PTP substrate also must be a substrate for the kinase

used (v-Src in this case). Presumably, one could tailor the choice of kinase to be relevant to any particular signal transduction pathway of interest.

Along the lines of tuning the substrate-trapping system to a specific signaling paradigm, Walchli *et al.* [35] published a "brute-force" approach to substrate trapping that might be more accurately referred to as phosphatase trapping. Instead of attempting to identify substrates for a PTP of interest, this method uses a substrate of interest, the autophosphorylated kinase domain of the insulin receptor, to screen through a collection of 37 different PTP trapping mutants to identify which phosphatases exhibit a preference for that substrate. Wild-type/active forms of the candidate PTPs were then tested for their ability to dephosphorylate a phosphotyrosine peptide substrate derived from the insulin receptor kinase. Interestingly, these activity assays matched the binding data reasonably well except for some unresolved exceptions. With such a collection of mutant PTPs in hand, the potential exists to screen all of them against other substrates of interest to trap the corresponding phosphatases.

Other Applications of Substrate Trapping Mutants

One other area in which substrate trapping mutants of PTPs have shown interesting results is in the identification of the subcellular sites at which substrate dephosphorylation is thought to occur. For the C → S mutant of YopH, described above, it was shown to localize to focal adhesions [22] in cells infected with *Yersinia* harboring this form of YopH. This localization pattern matched the subcellular location of the focal-adhesion-related substrates $p130^{cas}$ and paxillin that YopH dephosphorylates and its C → S mutant traps. In a remarkable, high-tech application of substrate trapping, the intracellular site of dephosphorylation of EGF and platelet-derived growth factor (PDGF) receptors by PTP1B was visualized with fluorescence resonance energy transfer (FRET) measurements and fluorescence lifetime imaging microscopy (FLIM) [36]. The D181A mutant of PTP1B was expressed in PTP1B-deficient mouse fibroblasts and then examined for its interaction with transiently expressed GFP fusions of the EGF and PDGF receptors. The images revealed that 30 minutes after growth factor stimulation distinct, punctate, vesicular structures contained both D181A-PTP1B and endocytosed growth factor receptors. Experiments such as these suggest that in the future we can look forward to seeing other innovative uses of the substrate trapping mutants of PTPs.

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CHAPTER 112

Inhibitors of Protein Tyrosine Phosphatases

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Introduction

Protein tyrosine phosphatases (PTPs) are a large family of enzymes whose structural diversity and complexity rival those of protein tyrosine kinases (PTKs). Unlike PTKs, however, which share sequence identity with protein serine/threonine kinases, the PTPs show no structural similarity with the protein Ser/Thr phosphatases. Not surprisingly, inhibitors of protein Ser/Thr phosphatases such as okadaic acid and microcystin are not effective against PTPs. In addition, these two classes of protein phosphatases have evolved to employ completely different strategies to accomplish the dephosphorylation reaction. Thus, while the protein Ser/Thr phosphatases are metalloenzymes with bimetallic centers containing iron and effect catalysis by direct attack of an activated water molecule at the phosphorus atom of the substrate [1], the PTPs do not require metals and proceed through a covalent phosphocysteine intermediate during catalytic turnover [2]. The hallmark that defines the PTP superfamily is the active-site amino acid sequence (H/V)**C**$(X)_5$**R**(S/T), also called the PTP signature motif, in the catalytic domain. An analysis of the nearly completed human genome sequence suggested that humans may have more than 100 PTPs, including both the tyrosine-specific and dual-specific phosphatases, which can utilize protein substrates that contain pTyr, as well as pSer and pThr.

Although all PTPs share a common catalytic mechanism and catalyze the same biochemical reaction, the hydrolysis of phosphoamino acids, they have distinct (and often unique) biological functions *in vivo* [3]. Genetics and biochemical studies indicate that PTPs are involved in a number of disease processes [4,5]. However, because PTPs can both enhance and antagonize cellular signaling, it is essential to elucidate the physiological context in which PTPs function. One of the major challenges of the PTP field is to establish the exact functional roles for individual PTPs, both in normal cellular physiology and in pathogenic conditions. Potent and specific PTP inhibitors could serve as very powerful tools to delineate the physiological roles of these enzymes. They can also be good starting points for therapeutic developments. This chapter provides a summary and update of currently known PTP inhibitors. Both specific and nonspecific small-molecule competitive and reversible PTP inhibitors are discussed. In addition, some examples of covalent and time-dependent PTP inactivators are also presented.

Covalent PTP Modifiers

The PTPs employ covalent catalysis, utilizing the thiol group of the active-site Cys residue as the attacking nucleophile to form a thiophosphoryl enzyme intermediate (E-P) [6]. Substitutions of the Cys residue completely abrogate PTP activity. The nucleophilic cysteine is housed within the active-site architecture specifically designed to bind a negatively charged phosphoryl group. Consequently, the pKa for the sulfhydryl group of the active-site Cys is extremely low (≈5) [7]. Thus, the PTPs are very sensitive to thiol-specific alkylating agents. For example, the PTPs can be irreversibly inactivated by iodoacetate, *N*-ethylmaleimide, and 5,5′-dithio-2-nitrobenzoic acid [7-9]. In addition, PTPs can also be inactivated by heavy metals including Zn^{2+}, Cu^{2+}, and *p*-(hydroxymercuri)benzoate, possibly through covalent bond formation with the active-site thiol group. There were

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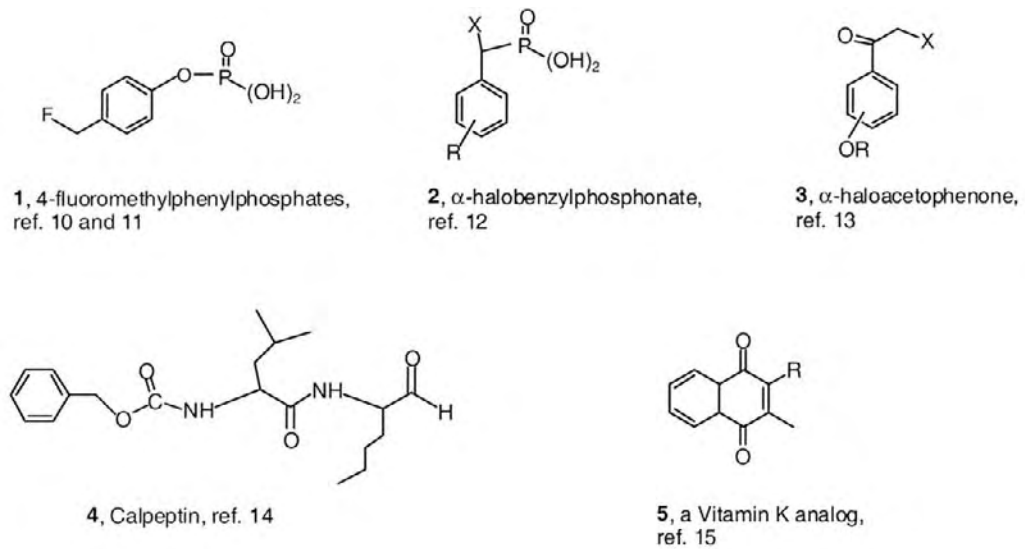


Figure 1 Structures of covalent inactivators of PTP.

several attempts to design specific, PTP-active-site-directed, alkylating agents, taking into consideration the architecture of the PTP catalytic site and the nature of the thiol-mediated phosphate hydrolysis. The 4-fluoromethylphenylphosphate (Fig. 1, compound **1**) was designed as a mechanism-based phosphatase inactivator [10,11] that, upon cleavage of the phosphate ester bond by the phosphatase, rapidly liberates the fluoride ion and forms a reactive quinone methide intermediate. Subsequent attack by PTP nucleophilic residues would result in formation of covalent adducts. Unfortunately, the lack of selectivity among various phosphatases and the unfavorable kinetics prevent the wide use of this compound in PTP research. The α-halobenzylphosphonate (Fig. 1, compound **2**) is an irreversible inactivator of the *Yersinia* PTP and PTP1B [12]. Mechanistically, this compound would be expected to undergo nucleophilic displacement of the halide without cleavage of the carbon–phosphorus bond. More recently, α-haloacetophenone derivatives (Fig. 1, compound **3**) have also been shown to be capable of covalently modifying SHP1 and PTP1B, possibly via nucleophilic displacement of the halide by the active-site thiol group [13]. A unique feature of these compounds is that the inhibition can be reversed upon photoactivation. Interestingly, the dipeptide aldehyde calpain inhibitor Calpeptin (Fig. 1, compound **4**) was recently shown to preferentially inhibit membrane-associated PTPs [14]. Although the exact mechanism of inhibition has not been investigated, it is possible that the aldehyde functionality may react with residues in the PTP active site to form covalent adducts. Finally, several vitamin K analogs (Fig. 1, compound **5**) have been shown to be effective PTP inactivators, possibly involving Michael-type nucleophilic addition of the active site Cys to the menadione moiety [15].

Due to the extremely low pKa of the active-site thiol group, the PTPs are also prone to metal ion-catalyzed oxidation by O_2 in the air. Thus, it is a common practice to include EDTA and DTT in PTP assay buffers in order to keep the active-site Cys in the reduced form. In addition to molecular oxygen, exposure of the PTPs to reactive oxygen species (ROS) can also result in PTP inactivation. For example, it has been shown that treatment of various PTPs with hydrogen peroxide [16,17], superoxide radical anion [18], and nitric oxide [19] all lead to the oxidation of the active-site Cys. Because ROS can be generated endogenously in the cell and because the oxidation of the active-site Cys by ROS in many cases is reversible, it has been suggested that PTP inactivation by ROS may provide a means for temporal negative regulation of PTP activity.

Oxyanions as PTP Inhibitors

Inorganic phosphate is a hydrolytic product of the PTP reaction and serves as a reversible competitive PTP inhibitor ($K_i \approx 5$ m*M*) [20]. Because of the similar physical and chemical properties, many oxyanions, including sulfate, arsenate, molybdate, and tungstate, can competitively inhibit PTPs to various degrees (10 μ*M* to millimolar levels). These oxyanions could inhibit PTPs by simply mimicking the tetrahedral geometry of the phosphate ion. The crystal structure of the PTP complexed with tungstate reveals the interactions between the enzyme and the phosphoryl moiety of the substrate [21]. The oxyanions adopt a tetrahedral configuration, and the oxygen atoms of the oxyanion make hydrogen bonds with the guanidinium group of the invariant Arg residue in the active site as well as the NH amides of the peptide backbone making up the PTP signature motif (H/V)**C**$(X)_5$**R**(S/T).

Vanadate is by far the most potent oxyanion inhibitor of PTPs, with K_i values of less than 1 μ*M* [22]. Thus, the binding affinity of vanadate for PTPs is several orders of magnitude higher than that of phosphate. Because vanadate is known to be able to adopt five-coordinate structures readily,

such observations have led to the hypothesis that vanadate may inhibit the PTPs by forming complexes that resemble the trigonal-bipyramidal geometry of the transition state. The crystal structures of the PTP–vanadate complexes show that indeed the vanadate moiety in the PTP complex is trigonal bipyramidal, with three short, equatorial, nonbridging V–O bonds, one apical bridging V–O bond, and one long V–S bond to the active site Cys [23–25]. Interestingly, a detailed kinetic and Raman spectroscopic study indicates that the PTP–vanadate complex may not be a true transition state analog for the PTP reaction [26], which occurs via a highly dissociative metaphosphate-like transition state [27]. However, even if only a fraction of the transition-state energy is captured by vanadate, it may be sufficient to account for its higher potency against PTPs.

It is important to point out that vanadate does not display any selectivity against individual members of the PTP family. Because there are no readily available PTP inhibitors with potencies comparable to that of vanadate, vanadate has been widely used as a pharmacological reagent for global inhibition of PTPs. Of note is the ability of vanadate to mimic the effects of insulin and to stimulate cellular proliferation. In many of the *in vivo* studies, the effective vanadate concentration used was well into the millimolar range. This may be caused by the presence of chelating and reducing agents, which reduce the free vanadate concentration. Because vanadate inhibits many classes of phosphoryl transfer enzymes, including phosphatases, ATPases, and nucleases, interpretation of cellular effects by vanadate should always be exercised with care.

Pervanadate, which is the complex of vanadate with hydrogen peroxide, is a more potent general PTP inhibitor than vanadate [28]. Unlike vanadate, which inhibits the PTPs reversibly and competitively, pervanadate inhibits the PTPs by irreversibly oxidizing the catalytic Cys [22]. Because pervanadate retains the vanadyl moiety, which is directed to the phosphate-binding pocket, and at the same time gains a peroxide group, which targets the active site Cys, it serves as a more efficient and specific reagent for global suppression of PTP activity at concentrations much lower than those employed for vanadate.

pTyr Surrogates as PTP Inhibitors

X-ray crystallographic studies have revealed a very similar active site (the pTyr binding site) for PTPs. In the substrate-bound form of PTP1B, the terminal non-bridge phosphate oxygens of pTyr form an extensive array of hydrogen bonds with the main-chain nitrogens of the PTP signature motif (residues 215–221) and the guanidinium side chain of Arg221. The phenyl ring of the pTyr is engaged in hydrophobic interactions with the active-site cavity, formed by the nonpolar side chains of Val49, Ala217, Ile219, and Gln262 and the aryl side chains of Tyr46 and Phe182, which sandwich the pTyr ring and delineate the boundaries of the pTyr-binding pocket [29,30]. Consistent with the fact that pTyr is essential for peptide/protein substrate binding, the interactions between pTyr and the PTP active site represents the dominant driving force for pTyr-containing peptide recognition. With this in mind, major efforts have been made to develop nonhydrolyzable pTyr surrogates that contain both a phosphate mimic that substitutes the phosphoryl group and an aromatic scaffold that can occupy the active-site pocket in a manner reminiscent of the benzene ring in pTyr. A variety of nonhydrolyzable pTyr surrogates have been reported (Fig. 2).

The most commonly used nonhydrolyzable pTyr surrogate is phosphonodifluoromethyl phenylalanine (F_2Pmp; Fig. 2, compound **6**) [31], which is over 1000 times more potent than phosphonomethyl phenylalanine (Pmp; **7**) when incorporated into a peptide. This may be attributed to a direct interaction between the fluorine atoms and PTP active-site residues [32]. Other nonhydrolyzable pTyr surrogates include sulfotyrosine (**8**) [33], *O*-malonyltyrosine (**9**) [34], fluoro-*O*-malonyl tyrosine (**10**) [35], aryloxymethyl phosphonate (**11**) [36], cinnamic acid (**12**) [37], 3-carboxy-4-(*O*-carboxymethyl) tyrosine (**13**) [38], salicylic acid (**14**) [39], aryl difluoromethylene phosphonate (**15**) [40], aryl difluoromethylene sulfonate (**16**) and tetrazole (**17**) [41], 2-(oxalylamino)-benzoic acid (**18**) [42], 5-carboxy-2-naphthoic acid (**19**) [43], and α-ketocarboxylic acid (**20**) [44]. Like pTyr, none of these nonhydrolyzable pTyr surrogates alone exhibits high affinity toward PTPs. However, when attached to an appropriate structural scaffold, the pTyr surrogate-containing compounds can be very effective PTP inhibitors.

Bidentate PTP Inhibitors

Although pTyr is essential for peptide/protein substrate recognition, pTyr alone does not possess high affinity for PTPs [45]. This and the fact that the PTP active site (pTyr binding site) is highly conserved among various PTPs present a serious challenge for the development of potent and selective PTP inhibitors targeted primarily to the active site. The discovery of a second aryl phosphate-binding site in PTP1B (defined by residues Arg24, Arg254, Met258, Gly259, and Gln262), which is not conserved among the PTPs and is adjacent to the active site, provides a novel paradigm for the design of tight-binding and specific PTP1B inhibitors that can span both sites [30]. Moreover, kinetic and structural studies have shown that amino acid residues flanking the pTyr are also required for efficient PTP substrate recognition [29,45–48]. These results suggest that subpockets adjacent to the PTP active site may also be targeted for inhibitor development. Consequently, an effective strategy for PTP inhibitor design is to attach a nonhydrolyzable pTyr surrogate to a properly functionalized structural element, which interacts with the immediate surroundings beyond the catalytic site. This strategy produces bidentate PTP inhibitors that simultaneously bind both the active site and a unique adjacent peripheral site, thereby exhibiting both enhanced affinity and specificity.

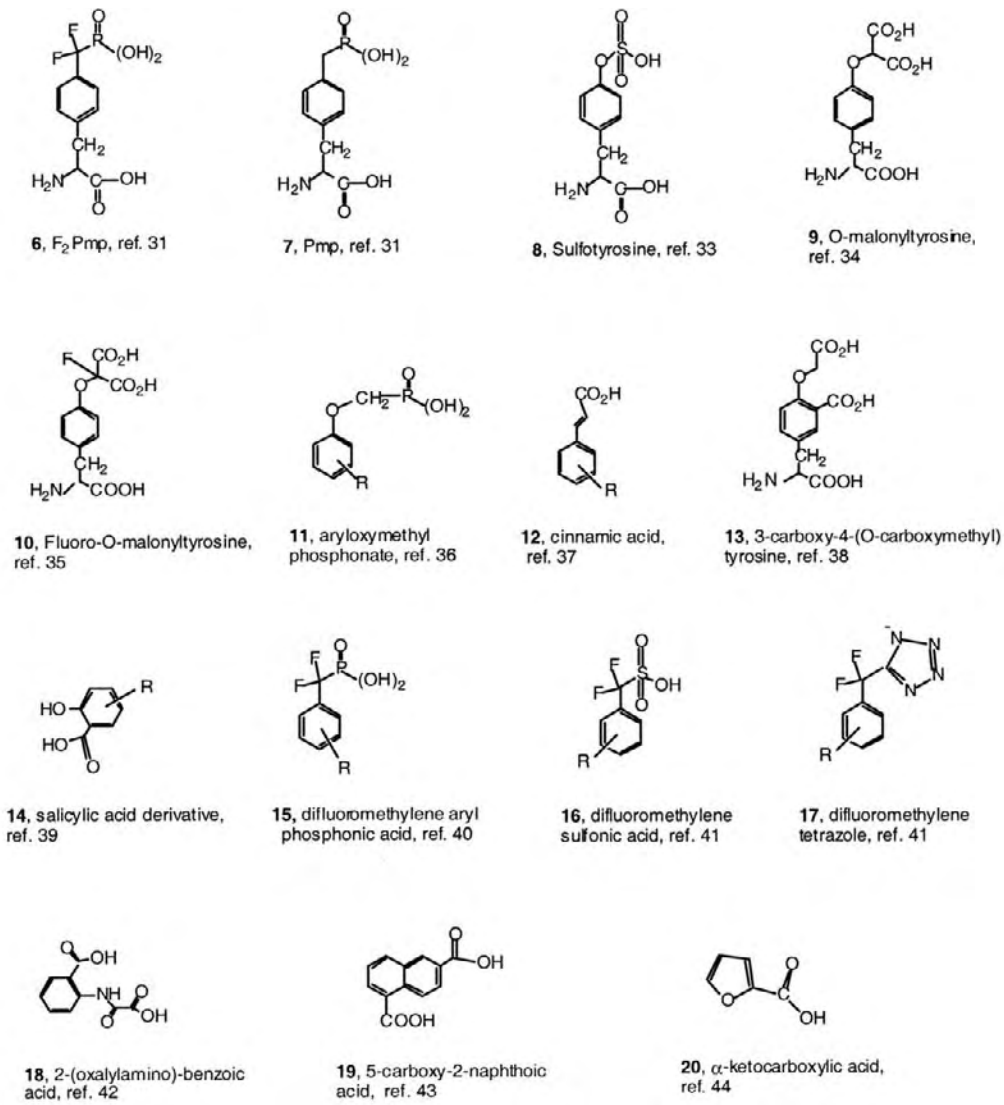
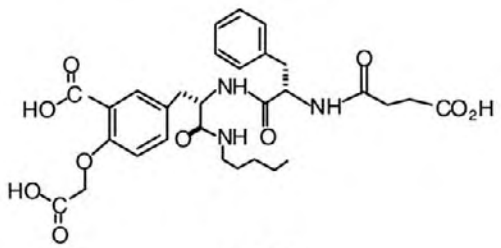


Figure 2 Structures of nonhydrolyzable pTyr surrogates.

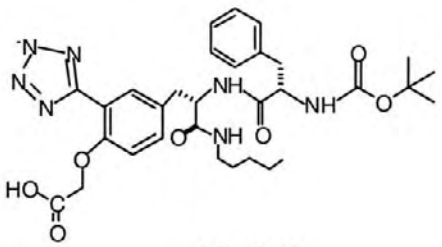
Initial attempts to exploit this strategy generated several *bis*-aryldifluorophosphonate inhibitors that display modest selectivity for PTP1B [40,49,50]. Recent medicinal chemistry efforts directed to optimization of the 3-carboxy-4-(*O*-carboxymethyl) tyrosine core (Fig. 2, compound **13**) and its attached peptide template led to several small molecule peptidomimetics (e.g., compounds **21** and **22** in Fig. 3) that displayed sub- to micromolar potency against PTP1B and augmented insulin action in the cell [51,52]. Using a structure-based approach, the Novo Nordisk group was able to introduce a substituent into the core structure of 2-(oxalylamino)-benzoic acid (Fig. 2, compound **18**) to address the second aryl phosphate-binding pocket in PTP1B [53]. This transformed a general, low-affinity, and nonselective PTP inhibitor into a reasonably potent ($K_i = 0.6$ μ*M*) and selective inhibitor for PTP1B (Fig. 3, compound **23**). A completely different approach (namely, combinatorial chemistry) was employed to identify bidentate PTP1B inhibitors capable of simultaneously occupying both the active site and a unique peripheral site in PTP1B [54]. This effort resulted in the identification of compound **24** in Fig. 3, which displays a K_i value of 2.4 n*M* for PTP1B and exhibits several orders of magnitude selectivity in favor of PTP1B against a panel of PTPs. Compound **24** is the most potent and selective PTP1B inhibitor identified to date. Subsequent structural and mutagenesis studies reveal that the distal element in compound **24** does not interact with the second aryl phosphate-binding pocket, but rather occupies a distinct area involving residues Lys41, Asn44, Tyr46, Arg47, Asp48, Lys116, and Phe182 [62]. The interactions between compound **24** and PTP1B are unique and provide the molecular basis for its potency and selectivity for PTP1B. Collectively, these results demonstrate that it is feasible to acquire potent, yet highly selective, PTP inhibitory agents.

Other PTP Inhibitors

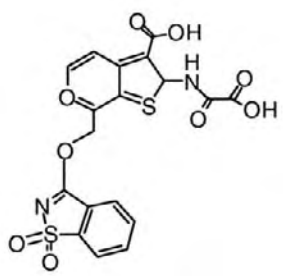
PTP1B was the founding member of the PTP family, and a large amount of structural and mechanistic information is



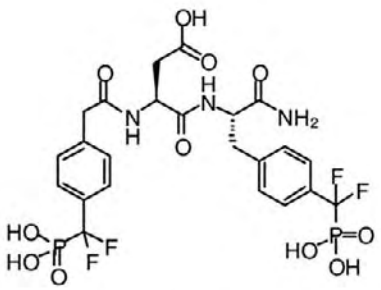
21, ref. 51



22, ref. 52



23, ref. 53



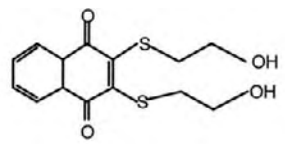
24, ref. 54

Figure 3 Structures of potent and selective bidentate PTP1B inhibitors.

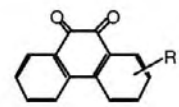
available for PTP1B. Furthermore, biochemical and genetic data suggest that PTP1B is a negative regulator for both insulin and leptin signaling. Consequently, most of the PTP inhibitors that are reported in the literature are directed to PTP1B. However, other PTPs have also received considerable attention, notably CD45 and Cdc25. Most of the inhibitors described earlier for CD45 and Cdc25 display only modest potency (≈10 μ*M*) with very limited selectivity [55–57]. Many of them were identified from natural product screens. In most cases, the manner in which these compounds interact with the target PTPs is unclear, rendering structure-based optimization of new analogs difficult. A recent high-throughput evaluation of 10,070 compounds in a publicly available chemical repository of the National Cancer Institute led to the discovery of NSC 95397 (2,3-*bis*-[2-hydroxyethylsulfanyl]-[1,4]naphthoquinone) (Fig. 4, compound **25**), which displayed mixed inhibition kinetics, with *in vitro* K_i values for Cdc25A, -B, and -C of 32, 96, and 40 n*M*, respectively [58]. Compound **25** showed significant growth inhibition against human and murine carcinoma cells and blocked G_2/M phase transition. Medicinal chemistry efforts around the 9,10-phenanthrenedione core resulted in potent CD45 inhibitors (Fig. 4, compound **26**), some of which inhibit T-cell-receptor-mediated proliferation with activities in the low micromolar range [59]. Interestingly, suramin (Fig. 4, compound **27**), one of the oldest synthetic therapeutics, which has long been used for the treatment of sleeping sickness and onchocerciasis, has been shown to be a potent reversible and competitive inhibitor of PTPs [60]. This is consistent with the observation that suramin leads to enhanced levels of tyrosine phosphorylation in several cell lines. More recently, sodium stibogluconate (Fig. 4, compound **28**), a pentavalent antimonial used for the treatment of leishmaniasis, has been suggested as a potent inhibitor of PTPs [61]. Although sodium stibogluconate augments cytokine responses in hemopoietic cell lines, its exact mode of action against PTPs is not clear and requires further investigation.

Concluding Remarks

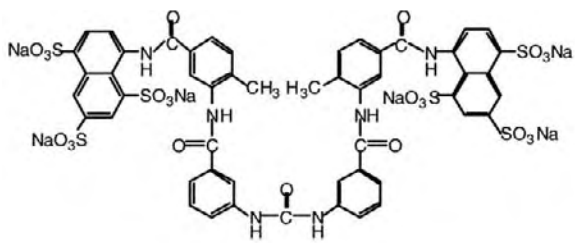
The importance of PTPs in the regulation of cellular signaling is well established. In spite of the large number of PTPs identified to date and the emerging roles played by PTPs in human diseases, a detailed understanding of the role played by PTPs in normal physiology and in pathogenic conditions has been hampered by the absence of PTP-specific agents. Such PTP-specific inhibitors could potentially serve as useful tools in determining the physiological significance of protein tyrosine phosphorylation in complex cellular signal transduction pathways and may constitute valuable therapeutics in the treatment of several human diseases. Despite the difficulties in obtaining such compounds, there are now several relatively specific inhibitors for PTP1B. It appears that significant differences exist within the active site and the immediate surroundings of various PTPs such that selective, tight-binding PTP inhibitors can be developed. In principle, an identical approach (i.e., to create bidentate inhibitors that could span both the active site and a unique adjacent peripheral site) used for PTP1B could also be employed to produce specific small-molecule inhibitors for all members of the PTP family that would enable the pharmacological modulation of selected signaling pathways for treatment of various diseases. Combinatorial solid-phase library synthesis is



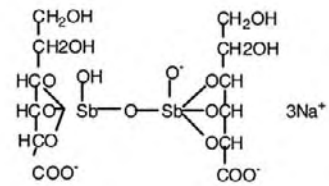
25, ref. 58



26, ref. 59



27, ref. 60



28, ref. 61

Figure 4 Other PTP inhibitors.

finding wide applicability throughout the pharmaceutical industry, and, not surprisingly, this technique has begun to yield fruitful results in the area of PTP inhibitors.

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CHAPTER 113

Regulating Receptor PTP Activity

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Introduction

The receptor protein tyrosine phosphatases (RPTPs) are a family of transmembrane phosphatases that catalyze the removal of the phosphate moiety from a phosphotyrosine residue (pTyr), resulting in a variety of intracellular responses including long-term potentiation, axonal path finding and neural transmission, and transformation. RPTPs consist of an extracellular domain, a transmembrane region, and two tandem intracellular tyrosine phosphatase domains, with the exception of four of the RPTPs that contain only one intracellular phosphatase domain. The greatest dissimilarity between RPTPs occurs in the extracellular region, in which diverse protein modules including immunoglobin (Ig)-like domains, fibronectin type III (FnIII)-like domains, and extensively glycosylated domains are found. In contrast, the intracellular tyrosine phosphatase domains share high sequence and structural homology not only within the RPTP family but with the nonreceptor tyrosine phosphatases as well. The majority of the phosphatase activity resides in the first, membrane-proximal domain (D1). The second, membrane distal domain (D2), although very homologous to D1, possesses little or no catalytic activity. Notably, the primary sequence of this domain is well conserved among RPTPs, suggesting that D2 plays a functionally significant role in overall RPTP activity in cells.

In order to maintain control over pTyr-mediated cellular signaling, the phosphatase activity of a given RPTP must be carefully regulated. To date, various modes of regulation have been associated with directing RPTP enzymatic activity, including receptor dimerization, phosphorylation, substrate recruitment via protein–protein interactions, and extracellular domain ligand binding. Here, we review some of the mechanisms employed by the RPTP family to ensure its proper regulation in the context of pTyr signaling cascades.

Regulation by Dimerization

Dimerization plays a critical role in the regulation of another family of transmembrane proteins, the receptor tyrosine kinases. Specifically, ligand binding to the extracellular domain allows the intracellular kinase domain to dimerize and cross-phosphorylate at regulatory sites, leading to activation of the intracellular kinase domain. Two independent crystal structures of the membrane-proximal phosphatase domain, D1, of RPTPα reveal a symmetrical dimer in which the active site of one domain is occluded by a helix-turn-helix wedge of its dimer forming partner [1]. Disulfide-bonding experiments demonstrate that this dimeric configuration renders RPTPα catalytically inactive *in vivo* [2]. Since the initial RPTPα crystal structure was revealed, a plethora of evidence has surfaced supporting the idea that dimerization is an important regulatory tool in RPTPs. Most notably, Tertoolen *et al*. [3] used fluorescence resonance energy transfer to demonstrate that RPTPα dimerizes constitutively in living cells and that the transmembrane region is sufficient for dimer formation. RPTPα dimerization is a negative regulatory event, in contrast to activation of receptor tyrosine kinases by dimerization (see Fig. 1A). Dimerization also plays a crucial role in the regulation of another RPTP family member, CD45. Recombinant D1 from CD45 exists primarily as a dimer as assessed by gel filtration chromatography [4]. EGF-enhanced dimerization of the CD45 intracellular domain linked to the extracellular ligand binding and transmembrane domain of EGFR results in CD45 inactivation consistent with a regulatory model in which dimerization serves as a negative regulatory signal [5]. Like RPTPα, CD45 dimerization is dependent on the wedge region located in the membrane-proximal D1 phosphatase domain. A knock-in mutant mouse containing a point mutation in the wedge region of CD45 (Glu^{613} to Arg) that inhibits dimer formation

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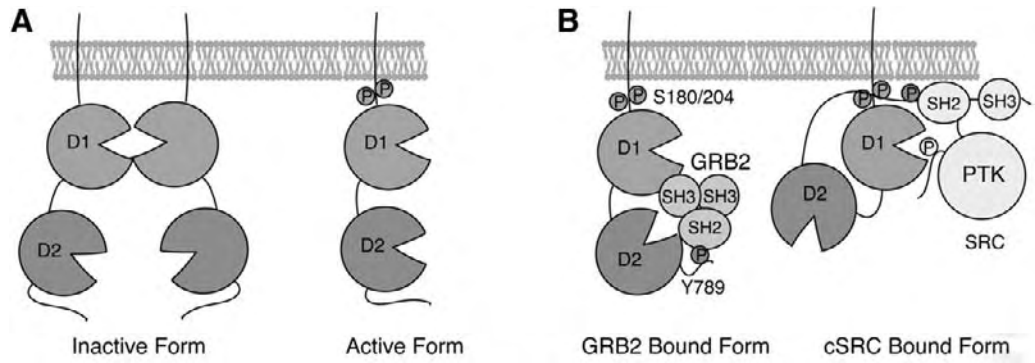


Figure 1 (A) The membrane-proximal phosphatase domain, D1, of RPTPα forms an inhibited dimer in which the active site of one monomer is sterically occluded by a wedge region of its dimer partner; perhaps serine phosphorylation in the juxtamembrane region serves to regulate dimer formation. (B) The C-terminus of RPTPα is tyrosine phosphorylated, allowing binding of the SH2-domain-containing protein Grb-2. This binding does not result in the recruitment of associated factors but may mask the site to inhibit binding of the SH2 domain of cSRC. Under the proper conditions, cSRC can bind the pTyr C-terminus, allowing RPTPα to dephosphorylate and activate cSRC.

exhibits a variety of phenotypes, including polyclonal lymphocyte activation consistent with an increase in cellular CD45 activity [6].

The dimerization model for RPTP regulation may not be a universal mechanism employed by all RPTPs. The crystal structures of the membrane-proximal phosphatase domain of RPTPμ and the tandem phosphatase domains of LAR failed to show dimer formation through the inhibitory wedge region [7,8]. Nevertheless, both structures contained an intact wedge that is not shared with cytosolic PTPs. It is important to note that the constructs used to crystallize both RPTPμ and LAR did not contain the transmembrane region that Tertoolen *et al.* demonstrated is sufficient for dimer formation in RPTPα [3]. It is unlikely that the wedge region provides sufficient binding energy to drive dimerization of RPTPα *in vivo*; therefore, additional regions such as the transmembrane segment are likely important for dimer formation [8A].

Regulation by Phosphorylation

Phosphorylation is a ubiquitous modification used to regulate the catalytic activity of a myriad of signal transducing proteins, and the RPTPs are no exception. RPTPs use both serine and tyrosine phosphorylation to regulate phosphatase activity and the formation and dissociation of protein–protein interfaces. RPTPα and CD45 have been the most extensively studied with respect to phosphorylation and its regulatory implications for *in vivo* RPTP activity.

RPTPα is phosphorylated on Ser^{180} and Ser^{204} located in the membrane-proximal region in response to treatment of cells with phorbol ester, a potent protein kinase C activator, resulting in an increase in RPTPα phosphatase activity [9,10]. Further evidence that serine phosphorylation regulates catalytic activity surfaced upon investigation of RPTPα activity at different stages in the cell cycle. Zheng and Shalloway [11] identified an increase in RPTPα activity during mitosis, coincident with serine phosphorylation. Dephosphorylation of RPTPα with the serine/threonine phosphatase PP2A reduces RPTPα phosphatase activity to premitotic levels, although the specific residues undergoing phosphorylation and dephosphorylation were not identified. The importance of serine phosphorylation in the regulation of RPTPα was further demonstrated by over-expression of a $Ser^{180/204}$ Ala double mutant that results in the elimination of ERK/MAPK stimulation [12]. These observations clearly show that serine phosphorylation plays a role in regulating the catalytic activity of RPTPα; however, further work is necessary to elucidate the exact mechanism of action. One possibility is that phosphorylation affects the dimeric state of RPTPα, perhaps favoring the activated monomeric form through electrostatic repulsion of the phosphorylated juxtamembrance region (see Fig. 1A).

In addition to RPTPα, CD45 is also regulated by serine phosphorylation but through a distinct site. CD45 differs from other RPTPs in that it contains an acidic 19-amino-acid insert in the second phosphatase domain, D2, that is phosphorylated *in vivo* on multiple serine residues by CK2. Mutation of these residues to glutamates, which serve as effective phosphate mimics, results in a threefold increase in CD45 phosphatase activity. The mechanism of this activation is unclear [13].

Most RPTPs contain a conserved tyrosine residue at the extreme C terminus that for some RPTPs is constitutively phosphorylated. Much of the work examining the role of this pTyr residue has been carried out on RPTPα (Tyr789). The C-terminal segment encompassing the Tyr789 residue is also a consensus Src homology domain 2 (SH2) binding domain that serves as a docking platform for the SH2-domain-containing protein GRB2. Curiously, the GRB2-associated protein SOS is not detected in immunoprecipitates with RPTPα, suggesting that GRB2 binding is functionally distinct from SOS-mediated signaling events [14]. The C-terminal phosphorylation site of RPTPα is also capable of binding the SH2 domain of the tyrosine kinase cSRC. This binding event is necessary to open up the inhibited cSRC conformation and allow subsequent RPTPα-mediated hydrolysis of the inhibitory pTyr site of cSRC, resulting in cSRC activation.

One attractive hypothesis to explain the role of GRB2 in RPTPα regulation involves masking of the RPTPα C-terminal pTyr from cSRC by GRB2 binding when cSRC activation is not desirable. Recent work by Zheng *et al.* [15] suggests that binding of GRB2 or cSRC to the C-terminal pTyr of RPTPα is regulated by phosphorylation at $Ser^{180/204}$. Specifically, a $Ser^{180/204}$ to Ala double mutant abolishes the ability of RPTPα to dephosphorylate and coimmunoprecipitate cSRC, while enhancing GRB2 coimmunoprecipitation with RPTPα. The authors propose that, upon $Ser^{180/204}$ phosphorylation, the C terminus of RPTPα forms an intramolecular interaction with the juxtamembrane region, causing the C terminus to adopt an extended conformation preferred by the SH2 domain of cSRC. In contrast, the SH2 domain of GRB2 binds pTyr residues in a β-turn conformation, presumably the conformation of the C terminus of RPTPα in the absence of $Ser^{180/204}$ phosphorylation (Fig. 1B) [15].

These brief examples demonstrate the potential of RPTPs to utilize serine and tyrosine phosphorylation to regulate their catalytic activity and proximity to pTyr-containing substrates such as cSRC. It remains to be seen how widely phosphorylation is used to regulate RPTP activity and whether there are shared mechanisms of regulation within the family of tyrosine phosphatases. Interestingly, RPTPε contains an SH2 binding motif identical to RPTPα at its C terminus, making it an excellent candidate for similar modes of SH2 domain binding and regulation.

Regulation by D2 Domain

The membrane-distal phosphatase domain of RPTPα (D2) is highly conserved among all RPTPs and exhibits little or no phosphatase activity, despite the fact that most D2 domains possess the catalytic cysteine residue required for pTyr turnover. The X-ray crystal structures of both D1 and D2 domains of LAR [8] and RPTPα [1] (Sonnenburg *et al.,* in preparation) reveal that both phosphatase domains share a very similar overall three-dimensional architecture. The lack of D2 enzymatic activity appears to be the result of two residues, Val^{555} and Glu^{690} in RPTPα. Mutation of these residues to the corresponding residues found in the D1 domain (Tyr and Asp, respectively) restores catalytic activity to levels comparable to D1 [16]. Therefore, D2 possesses the architecture necessary for efficient pTyr turnover, yet has maintained low or nonexistent activity through replacement of two key catalytic residues in the RPTP active site. One possible noncatalytic role for D2 in RPTP function is the regulation of target protein turnover through participation in protein–protein interactions critical for either substrate recognition or RPTP sequestration. X-ray crystallographic studies of the D2 domain of RPTPα reveal that the linker between the D1 and D2 domains is flexible, an observation confirmed by limited proteolysis studies (Sonnenburg *et al.*, in preparation). Perhaps the D2 domain has been maintained to recruit substrates by way of its catalytically inert active site, similar enough to an active phosphatase to bind substrate but not capable of rapid catalysis. Through such multipoint binding, the substrate remains in close proximity to the active D1 domain to be acted upon when the proper signal is transmitted. D1/D2 interdomain flexibility may allow substrates bound to the D2 domain to be presented to the active D1 domain. The RPTP LAR binds to phosphorylated insulin receptor, a substrate, via its D2 domain, an association weakened by mutation of the active site cysteine to serine [17].

In addition to interacting with substrate molecules, D2 domains can interact with RPTPs in either an intramolecular or intermolecular fashion. An intramolecular interaction between D2 and D1 was observed for CD45, as well as between D2 and the region N-terminal to D1 (juxtamembrane region) in RPTPμ [4,18]. Although an exact role for these interactions has not been established, it has been proposed that they may regulate dimerization by inhibiting intermolecular D1 homodimer formation. Yeast two-hybrid screens have identified a variety of intermolecular interactions between D2 domains and the wedge region of D1 domains from various RPTP family members [19,20]. This raises the possibility that RPTPs are capable of forming heterodimers *in vivo*. Heterodimer formation may serve multiple roles in RPTP signaling, including enhancement of the diversity of signaling roles possible for RPTPs or activating RPTPs by disrupting wedge-mediated D1 inhibitory dimer formation. Further work is required to determine how both intra- and intermolecular interactions in RPTPs affect their catalytic activity, location, and downstream signaling events.

RPTPs employ multiple mechanisms to ensure the proper regulation of their catalytic activity. These mechanisms include, but are not limited to, dimerization, phosphorylation, and potentiation and dissolution of regulatory protein–protein interactions. An additional area of growing exploration is the identification of ligands for the extracellular domains of RPTPs. Recently, RPTPσ has been shown to bind to the heparan sulfate side chains of heparan sulfate proteoglycans via its first extracellular Ig domain [21]. Another cell-surface ligand, the neuronal glycosylphosphatidylinositol (GPI)-anchored receptor contactin, binds the extracellular domain of both RPTPα and β in a *cis* conformation. Interestingly, contactin is able to recruit SRC family member kinases, a known substrate of RPTPα, perhaps creating an efficient signal transducing complex [22,23]. All RPTP ligands, however, are not membrane associated. A soluble cytokine, pleiotrophin is a ligand for RPTPβ and RPTPγ which, upon binding, leads to inactivation of RPTP phosphatase activity through receptor dimerization [24]. Subcellular localization also appears to play an important role in regulating RPTP function. RPTPμ localizes to regions of cell–cell contact in complex with cadherins, the function of which is regulated by reversible tyrosine phosphorylation, perhaps through RPTP phosphatase activity [25]. The RPTP LAR binds to LAR-interacting protein 1 (LIP1) through its association with the D2 domain, resulting in localization of LAR to disassembling focal adhesions, potentially regulating this cellular phenomenon [26]. However, RPTPs are not confined to locations near the cell membrane. Gil-Henn *et al.* [27]

have shown that a cytoplasmic form of RPTP consisting of the intracellular D1 and D2 domains from RPTPα and RPTPε exists in cells as a result of calpain cleavage. Coincident with the appearance of this soluble form of RPTP is a decrease in cSRC activation. Presumably, cytosolic RPTPs would access different cellular substrates than when attached to the membrane, opening up the possibility of an entirely novel set of RPTP substrates and downstream signaling cascades. Recent observations indicate that oxidative stress may be another regulator of RPTP phosphatase activity, in this case by inducing the inhibitory dimeric state of RPTPα [28]. It is clear that a complete picture of all the molecular and cellular mechanisms used by RPTPs for biological function will require a multidisciplinary approach carried out in numerous cooperating laboratories.

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CHAPTER 114

CD45

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Introduction

CD45 (also known as LCA, EC3.1.3.48, T200, Ly5, PTPRC, and B220) constitutes the first and prototypical receptor-like protein tyrosine phosphatase. CD45 was originally identified as leukocyte common antigen (LCA) and is expressed on all nucleated hematopoietic cells as one of the most abundant cell-surface glycoproteins. Its homologs have been identified in various mammals, chicken, shark, and the pufferfish *Fugu rubripes* [1,2].

Structure

CD45 is a type I transmembrane molecule consisting of a heavily glycosylated extracellular domain, a single transmembrane domain, and a large cytoplasmic tail (Fig. 1). The extracellular domain contains an N-terminal region with three alternatively spliced exons (4, 5, and 6), which encode multiple sites of O-linked glycosylation that are variably modified by sialic acid. Alternative splicing generates various isoforms with molecular weights ranging from 180 kDa for RO (lacking all three) to 235 kDa for RABC (including all three) and differing substantially in size, shape, and negative charge (Fig. 1). Isoform expression is highly regulated in a cell- and activation-state-specific manner. For example, naïve T cells predominantly express the larger isoforms (including one or two of the exons) while activated, and memory T cells primarily express the smallest RO isoform [1,2]. The remaining extracellular domain is heavily N-glycosylated and contains a cysteine-rich region followed by three fibronectin type III (FnIII) repeats. An analogous cysteine-rich region exists in the receptor tyrosine kinase EGFR (epidermal growth factor receptor), where it is important for ligand binding [3]. The three FnIII repeats are unusual because of their high cysteine content [4].

The cytoplasmic region is highly conserved between all mammalian species analyzed. It contains two tandemly duplicated protein tyrosine phosphatase (PTPase) domains [1,2]. Only the first one has enzymatic activity and is necessary to rescue T-cell receptor (TCR) signaling in a CD45-deficient cell line [5]. The function of the second domain is currently unclear. It has a unique 19-amino-acid acidic insert that can be phosphorylated by casein kinase II [6,7]. In addition, the crystal structure of the membrane-proximal phosphatase domain of RPTPα and sequence similarity between RPTPα and CD45 suggest that the juxtamembrane region may form a structural wedge [8].

Function

Studies using CD45-deficient T and B cell lines demonstrate that CD45 is an obligate positive regulator of antigen receptor signaling. Ablation of the murine CD45 gene by three independent groups reveals its critical positive role in lymphocyte development and activation [2,9]. For example, thymocyte development is largely blocked and the few mature T cells produced are refractory to TCR stimulation. Loss of CD45 in humans results in a form of severe combined immunodeficiency (SCID) [2,9].

Src family kinases (SFKs) are a primary substrate for CD45. SFKs are responsible for initiating antigen receptor signaling. They also modulate signal transduction cascades

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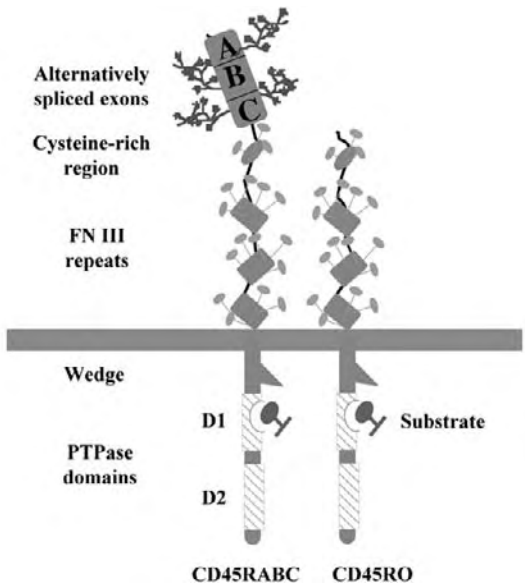


Figure 1 Structure of CD45. CD45 exists as multiple isoforms due to alternative splicing of three exons (4, 5, and 6, designated A, B, and C) in the extracellular domain. The largest isoform, RABC (including all three exons), and the smallest isoform, RO (lacking all three exons), are shown. These three exons encode multiple sites of O-linked glycosylation. As a result, various isoforms differ substantially in size, shape, and negative charge. The remaining extracellular domain is heavily N-glycosylated and contains a cysteine-rich region followed by three fibronectin type III repeats. CD45 has a single transmembrane domain and a large cytoplasmic tail containing two tandemly duplicated PTPase domains, D1 and D2. Only D1 has enzymatic activity and is necessary to rescue T-cell receptor (TCR) signaling in a CD45-deficient cell line. The function of D2 is currently unclear. In addition, molecular modeling indicates that the juxtamembrane region may form a structural wedge.

emanating from growth factors, cytokines, and integrin receptors [1,2,10]. In most CD45-deficient cells, SFKs are hyperphosphorylated at the negative regulatory tyrosine [1,2]. Moreover, expression of a constitutively active Lck Y505F mutant in CD45-deficient mice largely rescues the block in T-cell development [11]. By preferentially dephosphorylating the negative regulatory tyrosine, CD45 can maintain SFKs in a primed, or signal-competent, state capable of full activation upon receptor stimulation.

Although CD45 clearly plays a positive role in antigen receptor signaling, it can also function as a negative regulator in other settings. For example, CD45-deficient macrophages and T cells are abnormally adherent [12,13]. Despite hyperphosphorylation of the negative regulatory tyrosine of the SFKs, kinase activity is enhanced due to hyperphosphorylation at low stoichiometry of the autophosphorylation site, explaining the increased adhesiveness of these cells. This finding suggests that both the autophosphorylation site and the negative regulatory tyrosines can serve as CD45 substrates in some contexts. Interestingly, similar findings have been described for antigen receptor signaling in some CD45-deficient T and B cell lines [9,10]. The discrepancy of positive and negative effects of CD45 can be explained by its inclusion in or exclusion from clustered signaling complexes. Physical separation from the TCR during antigen recognition at the immunological synapse results in a net positive effect, while access to its substrate during integrin-mediated adhesion results in a negative effect [10].

In addition to SFKs, CD45 may also negatively regulate cytokine- and interferon-receptor-mediated activation by dephosphorylating Janus tyrosine kinases (JAKs) [14]. Other possible, but controversial, substrates include ZAP-70 and CD3ζ [2].

Regulation

The alternative splicing of CD45 is highly conserved and tightly regulated [1]. Naïve T cells predominantly express the larger isoforms and, following activation over the course of 3 to 5 days, switch to expression of the smallest RO isoform [15]. This regulated event is likely under the control of splicing factors that are induced in a PKC- and Ras-dependent manner after T-cell activation [16]. A point mutation in exon 4, which disrupts the function of an exonic splicing silencer [17] and causes abnormally high levels and persistent expression of the larger isoforms [18], has been linked to the development of multiple sclerosis in German patient cohorts [19]. These observations provide support for a contribution by the extracellular domain in regulating CD45 activity and suggest differences in regulation of the various isoforms. Surprisingly, despite the structural similarity between CD45 and receptor tyrosine kinases, a definitive ligand for CD45 has not been identified. Alternative means of regulation include spontaneous homodimerization, membrane localization, and interactions with other molecules.

Dimeric forms of CD45 can be detected through chemical cross-linking of cellular lysates [20] or by using a cysteine dimer-trapping method [21]. Dimerization of a CD45 chimera inactivates its catalytic function via the putative juxtamembrane wedge that blocks the catalytic site of the partner monomer during dimerization [3]. Introduction of a point mutation at the tip of this wedge abolishes the inhibitory effect of dimerization on TCR signaling in a transformed T cell line [8]. Mice bearing this wedge mutation develop a lymphoproliferative syndrome and severe autoimmune nephritis with autoantibody production, resulting in early death [21]. In addition, fluorescence resonance energy transfer (FRET) analysis suggests preferential homodimerization of the smallest RO isoform [22]. Together, these data indicate a role for differential homodimerization in negative regulation of CD45 function.

Cellular localization and access to substrate may contribute to the effect of CD45 on signaling. Redistribution of an intracellular pool of CD45 upon T-cell activation has been observed [23]. Most studies on the localization of CD45 show that it is absent from membrane lipid rafts and excluded from the central region of the interface between the T cell and the antigen-presenting cell. The latter is presumably due to the large size of CD45 and the relatively small size of molecules involved in antigen-specific recognition [2].

The function of CD45 may also be modulated through its interactions with other proteins. CD45 has been reported to associate at the cell surface with CD2, LFA-1, IFN receptor α chain, Thy-1, CD100, and CD26 [1,2,24]. Moreover, compared to larger isoforms, RO is found to preferentially associate with CD4 and TCR via its extracellular domain [22,25]. CD22, galectin 1 and glucosidase II can bind CD45 and other glycoproteins through specific sugar residues [1,2,26], although the functional consequences of these interactions are unclear. The transmembrane domain of CD45 mediates its interaction with lymphocyte phosphatase-associated phosphoprotein (LPAP) [27]. The cytoplasmic tail of CD45 is associated with the cytoskeletal protein fodrin [1]. Other possible means to modify CD45 function include serine phosphorylation of its second PTPase domain by casein kinase II [6,7] and inhibition of CD45 activity during neutrophil activation by reactive oxygen intermediates [28].

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CHAPTER 115

Properties of the Cdc25 Family of Cell-Cycle Regulatory Phosphatases

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Introduction

In all eukaryotic cells, the progression of the cell cycle is regulated by a family of cyclin-dependent kinases (Cdks). The first identified member of this family is maturation-promoting factor (MPF), which consists of three subunits. In vertebrates, MPF contains the protein kinase Cdc2, a regulatory partner called cyclin B, and a small ancillary subunit known as the Suc1 or Cks protein [1]. The control of MPF involves a number of distinct regulatory mechanisms, including phosphorylation, proteolysis, and changes in intracellular localization. For example, the activity of the Cdc2 subunit is dramatically dependent on its state of phosphorylation. In the case of human Cdc2, protein kinase activity absolutely requires phosphorylation on threonine-161 (Thr-161). However, Thr-161 appears to be phosphorylated throughout the G_2 and M phases of the cell cycle. The abrupt activation of MPF at the G_2/M transition can be explained by the existence of two inhibitory phosphorylation sites on Cdc2, namely tyrosine-15 (Tyr-15) and threonine-14 (Thr-14).

For MPF to become active at M phase, Cdc2 must undergo dephosphorylation at Tyr-15 and Thr-14 by a phosphatase in the Cdc25 family (Fig. 1). Prior to mitosis, Tyr-15 and Thr-14 are phosphorylated by the inhibitory kinases Wee1 and Myt1. Wee1 is a predominantly nuclear kinase that phosphorylates Tyr-15. The kinase Myt1, which is an integral membrane protein that resides in the endoplasmic reticulum and Golgi apparatus, modifies Thr-14 and to a lesser extent Tyr-15 [2,3]. Due to its central role in the biochemistry of Cdc2, Cdc25 plays a pivotal role in mitotic control and is the target of extensive regulatory networks. The focus of this chapter is on the biochemistry and regulation of the Cdc25 family of phosphatases.

Physiological Functions of Cdc25

Cdc25 was identified initially in the fission yeast *Schizosaccharomyces pombe* [4]. In this organism, cells with conditional mutations in the Cdc25 protein are unable to enter mitosis at the restrictive temperature and thus continue to grow into highly elongated cells. In further studies, it was shown that the timing of mitosis is highly dependent on the intracellular concentration of Cdc25. Cells with a reduced amount of active Cdc25 undergo mitosis at abnormally late times. Conversely, cells with elevated levels of Cdc25 enter mitosis with accelerated kinetics.

Biochemical studies in the early 1990s established that Cdc25 contains an intrinsic phosphatase activity [5–7]. Cdc25 is capable of dephosphorylating both phosphotyrosine and phosphothreonine and is thus a member of the dual-specificity phosphatase family [8]. Like other dual-specificity phosphatases, Cdc25 absolutely requires a key cysteine residue for catalysis. Accordingly, Cdc25 requires a

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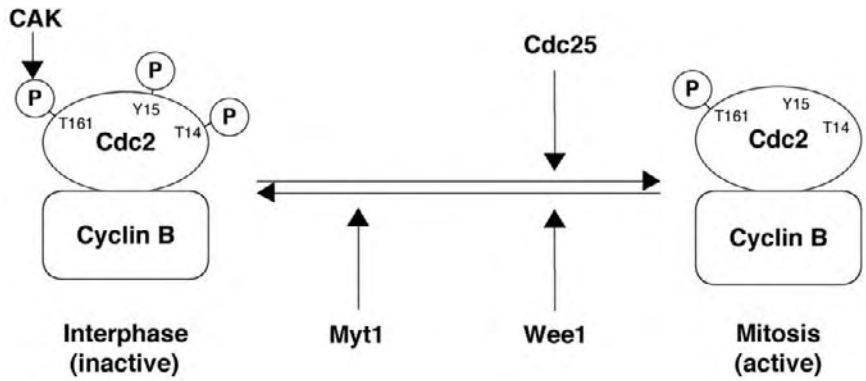


Figure 1 Posttranslational regulation of MPF. Cdc2 is positively regulated by phosphorylation on Thr-161 by the Cdk-activating kinase (CAK). Cdc2 is negatively regulated by phosphorylation on Thr-14 and Tyr-15.

reducing agent (e.g., dithiothreitol) for activity and is highly sensitive to alkylating agents such as N-ethylmaleimide. Cdc25 is also potently inhibited by phosphomimetic compounds such as sodium orthovanadate.

In vertebrates, the Cdc25 family contains three distinct members: Cdc25A, Cdc25B, and Cdc25C [9–11]. The existence of these distinct enzymes can be rationalized by the fact that the Cdk family is more elaborate in vertebrates than in yeast. For example, a complex consisting of Cdk2 and cyclin E has a role in the G_1/S transition. Cdc25A can remove inhibitory phosphate groups from Cdk2 [11]. Cdc25B and Cdc25C have both been implicated in mitotic regulation. Cdc25C can dephosphorylate the Cdc2–cyclin B complex effectively. Evidence has been presented that Cdc25B acts at some point upstream of Cdc25C in the mitotic control circuit [11]. Intriguingly, knockout mice that do not express Cdc25C are viable, suggesting that there is a functional redundancy among Cdc25 family members [12].

Regulation of Cdc25

Among the Cdc25 family, our understanding of Cdc25C regulation is perhaps the most comprehensive at this time. Broadly speaking, two general mechanisms regulate the action of Cdc25C. A kinase network activates Cdc25C at mitosis by catalyzing the stimulatory phosphorylation of its regulatory domain, and, prior to mitosis, suppressive controls downregulate Cdc25C.

Activation of Cdc25C at M Phase

At the G_2/M transition, Cdc25C undergoes extensive hyperphosphorylation, which results in a substantial decrease in its electrophoretic mobility [13,14]. This phosphorylation elicits a marked increase in the phosphatase activity of Cdc25C toward Cdc2. The mitotic phosphorylation of Cdc25C is carried out by the Cdc2–cyclin B complex itself and the Polo-like kinase (Plx1 in *Xenopus*, Plk1 in humans) [15–17]. The Cdc2–cyclin B complex appears to act in an "autocatalytic" activation loop. According to this scheme, a small amount of active Cdc2–cyclin B would contribute to the activation of Cdc25C, which in turn would produce more active Cdc2–cyclin B. This scenario would nicely explain the abrupt and precipitous activation of MPF at M phase; however, this model would apparently require some distinct triggering mechanism to initiate the process. The Polo-like kinase is an excellent candidate for a factor that would kick-start the activation of Cdc25C. The phosphorylation of Cdc25C by the Polo-like kinase also increases its activity but occurs at sites that are largely distinct from those that are phosphorylated by Cdc2. Thus, the phosphorylation of Cdc25C by the Polo-like kinase represents a discrete pathway that could regulate the timing of mitosis.

Suppression of Cdc25 during Interphase

Cdc25 is maintained in a low-activity state during interphase by inhibitory mechanisms that suppress its action or activation or both. These inhibitory mechanisms remain in place if checkpoint controls detect the presence of incompletely replicated or damaged DNA. This facet of regulation involves phosphorylation of Cdc25 on one or more sites by the so-called effector checkpoint kinases [18,19]. These kinases include Chk1 (in fission and budding yeast, *Xenopus*, and humans). Another effector kinase with this substrate specificity has different names according to the species (Rad53 in budding yeast, Cds1 in fission yeast and *Xenopus*, and Chk2 in humans).

In vertebrates, Chk1 phosphorylates Cdc25C on a major serine residue (Ser-216 in humans and Ser-287 in *Xenopus*) [20–22]. This serine group resides in consensus site for the binding of 14-3-3 proteins. 14-3-3 proteins are widespread polypeptides that recognize phosphopeptide motifs in target proteins. In *Xenopus* and humans, the 14-3-3 binding site is immediately adjacent to a bipartite nuclear localization sequence (NLS). For this reason, binding of 14-3-3 has a dramatic effect on the localization of Cdc25C [23–27]. The phosphorylation of Cdc25 by Chk1 most likely has other functional consequences. For example, the binding of 14-3-3

to Cdc25C appears to reduce its catalytic activity modestly and inhibit the interaction of Cdc25C with cyclin B [22,28]. In fission yeast, the phosphorylation of Ser-99 on Cdc25 by Chk1 leads to an inhibition of its phosphatase activity [29]. This inhibition apparently does not require binding of 14-3-3 proteins. Overall, it appears that phosphorylation by Chk1 can suppress Cdc25 by 14-3-3-dependent and 14-3-3-independent mechanisms. Moreover, the suppression of Cdc25 by 14-3-3 may involve multiple effects.

Localization of Cdc25

In vertebrates, the localization of Cdc25 is very dynamic. In *Xenopus* and humans, Cdc25C contains both an NLS and one or more nuclear export sequences (NESs) [23,24,26, 27,30,31]. Cdc25C can be predominantly nuclear or cytoplasmic, depending on how the NLS and NES regions are modulated. For example, binding of 14-3-3 can occlude recognition of the NLS by nuclear import factors. Furthermore, the NES region appears to be regulated by phosphorylation. For example, Ser-198 in the NES of human Cdc25C undergoes phosphorylation at mitosis [31]. This phosphorylation reduces the effectiveness of the NES and thus promotes nuclear accumulation. Human Plk1 has been implicated as the enzyme that phosphorylates Ser-198. In this event, the Polo-like kinase may regulate both the activity and localization of Cdc25C. The nuclear accumulation of Cdc25C correlates strongly with mitotic entry in vertebrates, which implies a causal relationship between nuclear entry of Cdc25C and mitotic initiation. In fission yeast, however, nuclear accumulation of Cdc25 is not required for mitosis [32].

Stability of Cdc25

Like other key cell-cycle proteins, one or more members of the Cdc25 family are subjected to regulated proteolysis. For example, Cdc25A undergoes prompt destruction following DNA damage in human cells [33,34]. Chk1 and Chk2 have been implicated in these processes, depending on the type of DNA damage. In *Drosophila*, the Tribbles protein regulates the stability of String, a fly homolog of Cdc25, at a key point in morphogenesis [35]. In fission yeast, the ubiquitin ligase Pub1 has a role in controlling the abundance of Cdc25 [36].

Concluding Remarks

The importance of Cdc25 phosphatases in cell-cycle regulation is underscored by the diversity of molecular mechanisms that are employed in their regulation. In vertebrates, there is still much to learn about how different Cdc25 family members collaborate in progression through the phases of the cell cycle and how distinct regulatory mechanisms contribute to the coordinated regulation of these enzymes.

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CHAPTER 116

Cell-Cycle Functions and Regulation of Cdc14 Phosphatases

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Introduction

The *CDC14* gene of the budding yeast *Saccharomyces cerevisiae* encodes a protein phosphatase that is essential for cell-cycle progression [1] and serves as a prototype for a group of closely related enzymes within the protein tyrosine phosphatase (PTP) family. Orthologs of yeast Cdc14 have been identified in protists, fungi, flowering plants, and animals, suggesting that this phosphatase, like many other cell-cycle regulators, is conserved among all eukaryotes. Cdc14 from budding yeast is the founding member of this subgroup of protein phosphatases and has been most thoroughly studied.

The Cdc14 Phosphatase Subgroup of PTPs

The Cdc14 phosphatases [1,2] utilize the Cys-dependent catalytic mechanism shared by all PTPs, but outside of a short segment surrounding their active sites they exhibit no sequence similarity to the classical tyrosine-specific enzymes of this family. Cdc14 phosphatases dephosphorylate Ser/Thr as well as Tyr residues in artificial substrates *in vitro* [1,2], placing them among the dual-specificity phosphatases (DSPs), a distinct subgroup of the PTP family. The Cdc14 orthologs have little in common with other DSPs, many of which regulate MAP kinases. Cdc14 orthologs and these MAP kinase phosphatases differ in their domain organization, and the only sequence similarity is restricted to a 60-residue region flanking their active sites.

The basic structural organization of the prototypical budding yeast Cdc14 is shared by all orthologs identified to date (Fig. 1). The 62-kDa yeast enzyme contains a conserved N-terminal catalytic domain (residues 1–374) and an Asn/Ser-rich, noncatalytic C-terminal segment that is not essential for its cell-cycle function [1]. The oligomerization of budding yeast Cdc14, observed both *in vitro* and *in vivo*, is mediated through an interaction requiring the catalytic domain [1,3]. A noncatalytic domain is present at the C termini of all Cdc14 orthologs, but it varies in length and has diverged during speciation (Fig. 1). Apart from a nuclear export sequence identified in human Cdc14A [4], no other functions have been assigned to the noncatalytic domain.

Budding Yeast Cdc14 is Essential for Exit from Mitosis

Exit from Mitosis

Following their association with B-type cyclins, the activation of cyclin-dependent kinases (Cdk) triggers the onset of mitosis. At anaphase after sister chromatids have separated, mitotic Cdks must be inactivated in order for cells to exit from mitosis. During exit from mitosis, cells restore the nucleus to its premitotic state (e.g., disassemble the mitotic spindle) and prepare for cytokinesis (for review, see Morgan [5]). A prevailing mechanism for mitotic Cdk inactivation is the regulated destruction of mitotic cyclins.

The anaphase-promoting complex (APC) ubiquitinates cyclins and other mitotic regulators, triggering their recognition and proteolysis by the 26S proteosome [5]. In budding yeast, specificity factors known as Cdc20 and Cdh1/Hct1 interact with the APC to govern substrate selectivity and the order in which crucial regulators are ubiquitinated and

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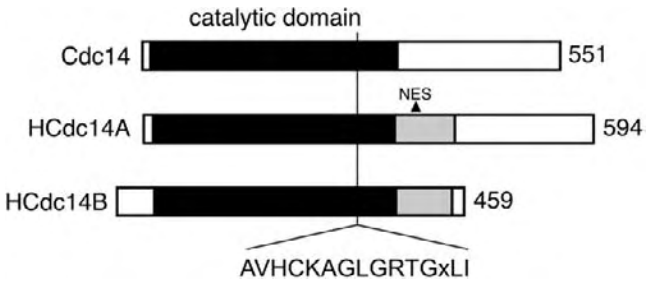


Figure 1 Schematic diagram illustrating the structural organization of budding yeast and human Cdc14 phosphatases. The yeast and human Cdc14A and B phosphatase sequences are depicted (accession numbers NP_116684, NP_003663, and NP_003662, respectively) with the total number of amino acid residues shown on the right. The solid black boxes delineate the position of the catalytic domain (≈330 residues) conserved among all Cdc14 orthologs, whereas the open boxes show divergent non-catalytic regions. The gray boxes depict additional sequences conserved only among the human enzymes and several other vertebrate orthologs. The vertical line denotes the position of the catalytic site; the active site sequence that is identical among all Cdc14 phosphatases is shown underneath (x indicates a variable position). The position of the nuclear export signal (NES) identified in human Cdc14A [4] is indicated by the triangle.

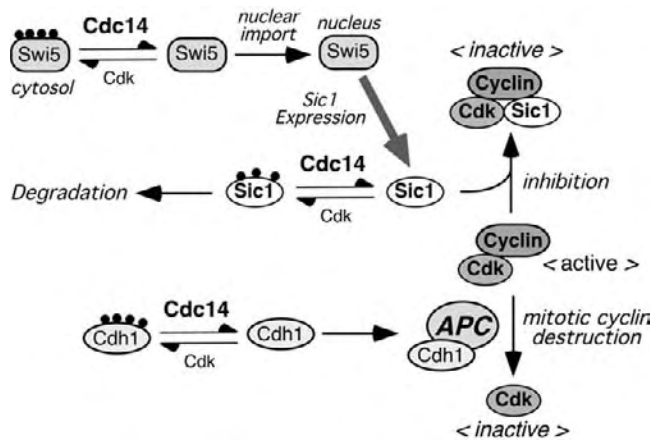


Figure 2 The role of budding yeast Cdc14 in promoting exit from mitosis. The schematic diagram illustrates how Cdc14 dephosphorylates Swi5, Sic1, and Cdh1 to drive Cdk1 inactivation by two mechanisms: APC-mediated cyclin destruction and protein inhibition [6].

destroyed during mitosis [5]. APC^{Cdc20} acts first to initiate anaphase by ubiquitinating the yeast securin Pds1. Upon its destruction, Pds1 liberates a protease necessary for sister chromatid separation. Subsequently, Cdh1 promotes the APC-mediated ubiquitination of mitotic cyclins and other targets that are destroyed during exit from mitosis. Cdh1 is expressed throughout the cell cycle but Cdk-mediated phosphorylation prevents its interaction with the APC during early mitosis [5].

Cdc14 Substrates

In budding yeast, Cdc14 dephosphorylates at least three substrates (Cdh1, Swi5, and Sic1) [6,7] that ensure the inactivation of mitotic Cdk activity through two pathways: degradation of mitotic cyclins and expression of Sic1, a Cdk inhibitor (see Fig. 2). Upon its dephosphorylation by Cdc14, Cdh1 activates the APC and directs the ubiquitination of mitotic cyclins and other protein targets [7]. Expression of the Cdk inhibitor Sic1 is dependent on the zinc finger transcription factor, Swi5. Prior to anaphase, Swi5 accumulates in the cytoplasm but is prevented from entering the nucleus because of Cdk-dependent phosphorylation at Ser residues adjacent to its nuclear localization signal (Fig. 2). Cdc14 dephosphorylates Swi5, thus permitting it to enter the nucleus and activate Sic1 transcription [6]. Cdc14 also targets the Sic1 protein itself, preventing its destruction as a result of inopportune phosphorylation [6]. Cdh1, Swi5, and Sic1 undergo Cdk-dependent phosphorylation, and it is generally assumed that Cdc14 phosphatases prefer substrates phosphorylated by this group of kinases. Considerable evidence supports this notion, but it is premature to assume that Cdc14 opposes only Cdks as no sites dephosphorylated by this phosphatase, either *in vitro* or *in vivo*, have been directly mapped, and the substrate preference of Cdc14 has not yet been investigated.

The Nucleolus and Cdc14 Regulation

Genetic and biochemical studies have begun to reveal how Cdc14, which is present at constant levels throughout the cell cycle, is held in check until its activity is required between anaphase and early G_1. Net1 (also known as Cfi1) [8,9], a major player in the cell-cycle-dependent regulation of Cdc14, is a core subunit of the nucleolar RENT complex [8]. The RENT complex is also involved in maintenance of nucleolar integrity, repression of recombination among tandem rDNA repeats, recruitment of Pol I, and stimulation of rDNA transcription [10]. In interphase and early mitosis, most if not all Cdc14 is sequestered in the nucleolus by Net1 [8,9], where its activity is fully inhibited [11] and its access to substrate is limited. Net1 is a highly specific and potent competitive inhibitor ($K_i = 3$ n*M*) that contains a Cdc14-binding region (residues 1–341) at its N terminus [11].

Two distinct signaling pathways, known as the FEAR [12] and MEN [13] networks, control Cdc14 release from Net1 (Fig. 3). For both pathways, it is not known how the protein kinases and other signaling components act on the RENT complex to induce the release of Cdc14, but it may involve phosphorylation of Net1 [14]. The FEAR pathway (Fig. 3) is activated first at early anaphase when the securin Pds1 is degraded and active separase is released [12]. FEAR signaling triggers a transient release of Cdc14 into the nucleus that is not sufficient for exit from mitosis but ensures it occurs with proper timing [12]. The MEN pathway (Fig. 3) is activated at late anaphase when the dividing nucleus spans the bud neck bringing the Tem1 G-protein into contact with its guanine nucleotide exchange factor Lte1 [15,16]. Activation of MEN signaling is essential for exit from mitosis and produces a sustained release of Cdc14 into the nucleus and cytoplasm that promotes Cdk inactivation. Cdc14 released by FEAR signaling may act to potentiate subsequent signaling through the MEN pathway [12] by dephosphorylating the Cdc15 kinase that is known to be a Cdc14 substrate [17,18]. It is likely that the FEAR network has additional

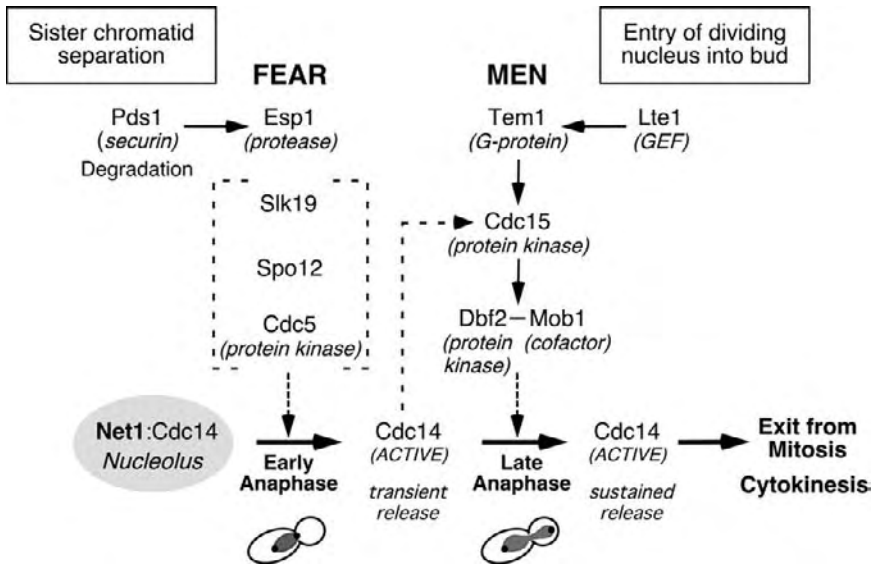


Figure 3 A model for the cell-cycle-dependent regulation of Cdc14 by the FEAR and MEN networks. The signaling proteins involved in the FEAR [12] and MEN [26] pathways are depicted. Arrows are shown where the order of signaling within the pathways is known. The components (Slk19, Spo12, and Cdc5) enclosed in large brackets are necessary for FEAR signaling, but it is not clear how they interact or in what order they operate in the network. The dashed arrow depicts the potential role of Cdc14 in potentiating MEN signaling by targeting Cdc15 [12]. Although Cdc5 is thought to act in both pathways, it is shown here only in the FEAR network. As indicated by the dashed arrow, the exact mechanism triggering Cdc14 release from Net1 is not known for either pathway.

roles during early anaphase. The dependency of Cdc14 release on the proteolysis of Pds1 [19–21] is not explained by the FEAR pathway alone, indicating there must be at least one other mechanism linking the two events. The FEAR and MEN pathways in conjunction with the requirement for Pds1 degradation ensure that mitotic exit does not occur unless sister chromatids are separated and the segregated chromosomes are correctly partitioned to mother and daughter cells (Fig. 3). Like several other proteins of the MEN pathway, the role of Cdc14 may not be limited to mitotic exit but could also include functions required for cytokinesis [22].

Fission Yeast Cdc14 Coordinates Cytokinesis with Mitosis

An ortholog of *S. cerevisiae* Cdc14, named clp1 [23] or flp1 [24], has been identified in the fission yeast *Schizosaccharomyces pombe*. The role of fission yeast Cdc14 in cell-cycle progression differs considerably from that of the budding yeast enzyme. *S. pombe* Cdc14 is not an essential phosphatase and is not necessary for mitotic cyclin degradation or exit from mitosis [23,24]. This is not completely surprising, as the fission yeast Cdc20 ortholog instead of Cdh1 appears to control the APC-dependent destruction of mitotic cyclins. Instead of exit from mitosis, *S. pombe* Cdc14 is involved in controlling the onset of mitosis [23,24]. Through an undefined mechanism, Cdc14 suppresses Cdk activation at the G_2/M transition by opposing Tyr 15 dephosphorylation, a requirement for full mitotic kinase activity.

Recent analyses suggest that *S. pombe* Cdc14 is also involved in coordinating cytokinesis with the events of late mitosis [23]. In contrast to budding yeast, *S. pombe* divides by medial fission instead of budding [25]. During mitosis, *S. pombe* first assembles a medial ring containing actomyosin and then forms a septum at the middle of the cell. At the end of anaphase, a signaling pathway initiates septation, contraction of the medial actomyosin ring, and completion of cytokinesis [25]. Interestingly, most of the components of this signaling pathway, known as the septation initiation network (SIN), are orthologs of the MEN pathway of budding yeast, and the two pathways are thought to have the same organization and to propagate signals via similar mechanisms [25,26]. Surprisingly, Cdc14 is not a major effector or target of the SIN pathway [24]. Instead, Cdc14 appears to potentiate the SIN pathway by suppressing Cdk activity that is known to antagonize SIN signaling and cytokinesis.

Like its budding yeast counterpart, *S. pombe* Cdc14 is localized to the nucleolus during interphase [23,24]. Upon its release at early mitosis, Cdc14 diffuses throughout the nucleus and cytoplasm and accumulates at the spindle pole bodies, mitotic spindle, and medial ring [23,24]. Fission yeasts have no homolog of budding yeast Net1 and it is not known whether Cdc14 is active within the nucleolus, but its sequestration could restrict access to substrates. The SIN network does not trigger Cdc14 release; instead, it is required to exclude the phosphatase from the nucleolus until cytokinesis is complete [23]. How Cdc14 is initially released

is unknown. *S. pombe* Cdc14 is phosphorylated during mitosis, but how this modification might regulate the enzyme is not known [24]. Identification of substrates will be required to define how *S. pombe* Cdc14 modulates the G_2/M transition and coordinates cytokinesis with mitosis.

Potential Cell-Cycle Functions of Human Cdc14A and B

Two distinct Cdc14 phosphatases are expressed in humans [2] and several other vertebrates. Human Cdc14A and B exhibit 62% sequence identity over a 400-residue segment. Evidence suggests that Cdc14A is involved in regulating cell division, but so far there are few clues about the function of the B form.

Although many details differ, regulation of the APC during vertebrate mitosis is fundamentally the same as that observed in yeast. A Cdh1 ortholog must be dephosphorylated to direct the APC-dependent ubiquitination of mitotic cyclins that results in Cdk inactivation and exit from mitosis. A recent study [27] showed that human Cdc14A dephosphorylates Cdh1 *in vitro*, allowing it to activate APC-mediated cyclin ubiquitination. Moreover, human Cdc14A is found in a major fraction of Cdh1 phosphatase activity isolated from HeLa cell lysates [27]. Although this study [27] using *in vitro* reactions is not definitive, it provides evidence that human Cdc14A has the capacity to regulate the APC and to promote exit from mitosis. Thus, the function of budding yeast Cdc14 in promoting mitotic exit may have been conserved in humans.

Besides Cdh1, the only other potential substrate identified for human Cdc14 phosphatases is the tumor suppressor p53 [28]. Cdc14A and B associate with p53 *in vivo* and both dephosphorylate Ser 315 *in vitro* [28]. Ser 315 is targeted by Cdks, consistent with the notion that Cdc14 phosphatases oppose these kinases. Its binding to sequences in the N termini of the Cdc14 phosphatases [28] suggests that the interaction with p53 may be independent of its recognition as a phosphosubstrate and could permit the constitutive association of the two proteins. Thus far, evidence that Cdc14 controls the phosphorylation state of Ser 315 in cells is lacking, and there are conflicting reports regarding the role of this site in p53 regulation.

Several observations suggest that the regulation of human Cdc14A and B may differ from that observed in budding yeast. Both human phosphatases are insensitive to the yeast Net1 inhibitor, and no gene encoding a Net1 homolog can be identified in the human genome [11]. Targeting to specific organelles or subcellular compartments is at least partly responsible for human Cdc14 regulation. The majority of Cdc14A is localized to the centrosome, but some enzyme is also found in the cytosol [4]. During mitosis, most but not all of the Cdc14A leaves the centrosome and appears in the cytosol. A nuclear export signal (residues 352–367) (Fig. 1) is necessary for the translocation of Cdc14A out of the nucleus and to prevent its sequestration in nucleoli, where Cdc14B is localized [4]. The nuclear export signal as well as N- and C-terminal sequences appear to be required for localization to the centrosome [4].

Recent findings have implicated human Cdc14A in centrosome duplication [4]. Like chromosomes, centrosomes must be duplicated exactly once in every round of cell division, and defects in this process lead to aberrant chromosome segregation and aneuploidy [29]. Over-expression or depletion of Cdc14A in human cells resulted in defective chromosome segregation that could be attributed to aberrations in the centrosome duplication cycle [4]. These data are fully consistent with the well-documented role of phosphorylation in regulating centrosome duplication. It will be important to identify substrates in order to define the role of Cdc14A in centrosome duplication. In this regard, it is intriguing that the potential Cdc14 substrate p53 has been linked to centrosome function [30,31]. Cells lacking p53 accumulate multiple centrosomes, suggesting that they have defects in the duplication cycle [30]. The phosphorylation of Ser 315 is required for the binding of p53 to unduplicated centrosomes [31]. The possibility that Cdc14A could modulate centrosome duplication by controlling the phosphorylation state of Ser 315 in p53 certainly merits further study. Research on the human Cdc14 phosphatases is in its infancy; nevertheless, the clues we have obtained highlight the importance of investigating potential links between this group of enzymes and tumorigenesis.

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CHAPTER 117

MAP Kinase Phosphatases

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Introduction

Mitogen activated protein kinases (MAPKs) are functionally dependent on specific upstream MAPK kinases (MAPKKs) that in turn are activated by MAPKK kinases (MAPKKKs). Together, these enzymes constitute a functional cassette that is highly conserved in a wide-range of animal species. Genetic analysis and molecular and biochemical studies have revealed the existence of several distinct MAPK cascades that play an essential role controlling functions as diverse as morphological development, learning and memory, stress responses, proliferation, differentiation, and apoptosis. One property that all MAPK pathways share is their transient activation. Hence, while cell stresses and growth factors induce MAPK enzymatic activation by phosphorylation on critical threonine and tyrosine residues, this activity generally peaks within a few minutes and thereafter falls back to basal levels. Such observations suggest that, as with pathways leading to MAPK activation, processes controlling dephosphorylation are also likely to play a critical role controlling cell function. Consistent with this, over recent years a pivotal role for protein phosphatases acting at the level of MAPKs in modulating the extent and duration of MAPK enzymatic activation has been demonstrated in organisms as diverse as yeast, worms, flies, and mammals (Fig. 1).

Eukaryotic protein phosphatases comprise three classes of enzymes: the serine/threonine phosphatases PPP and PPM and the protein tyrosine phosphatases (PTPs). The PPP family includes the phosphatases PP1, PP2A, and PP2B, whereas PP2C is the prototypic member of the PPM family [1]. The PTP superfamily is characterized by the structural CX5R motif in the active site and can be further subclassified into four classes based on protein structure: (1) the tyrosine specific phosphatases, (2) low-molecular-weight phosphatases, (3) Cdc25-like phosphatases, and (4) VH1-like dual-specificity phosphatases [2]. In the following sections, we will describe how organisms as diverse as yeast and mammals have used similar classes of phosphatases to achieve tight regulation of MAPK.

MAPK Phosphatases in Yeast

In the budding yeast *Saccharomyces cerevisiae*, five distinct MAPK pathways have been identified. These pathways regulate mating, sporulation, filamentous growth, cell wall integrity, and responses to osmotic shock. One of the first genetic screens for genes controlling MAPK revealed a functional redundancy, as several distinct phosphatases appeared to be important in the inactivation of a single target MAPK, in this case Hog1. Hence, double deletion of the *PP2C* serine/threonine phosphatase *PTC1* together with the tyrosine-specific phosphatase *PTP2* is lethal, and this phenotype is reversed by inactivating components in the Hog1 MAPK pathway. This implies that in the double-phosphatase-mutant yeast the Hog1 pathway is constitutively activated. Subsequent studies revealed that the osmotic response MAPK Hog1 is regulated by Ptc1, Ptp2, and its homolog Ptp3, although Ptp2 appears to be the major regulator in this pathway [3].

While Hog1 controls responses to osmotic stress, Fus3 is a distinct MAPK underlying pheromone responses in *S. cerevisiae*. When screening for gene suppressors of the yeast mating response, the VH1-like dual-specificity phosphatase *MSG5* was identified as an inactivator of Fus3 activity [4]. Importantly, *MSG5* is also induced by mating pheromone, indicating that this phosphatase functions as a negative-feedback regulator of Fus3 actions. Subsequent studies revealed that Msg5 is not the only phosphatase regulating this MAPK, and that Ptp2 and Ptp3 also play a role, although, in contrast to the case of Hog1, Ptp3 appears to be more important.

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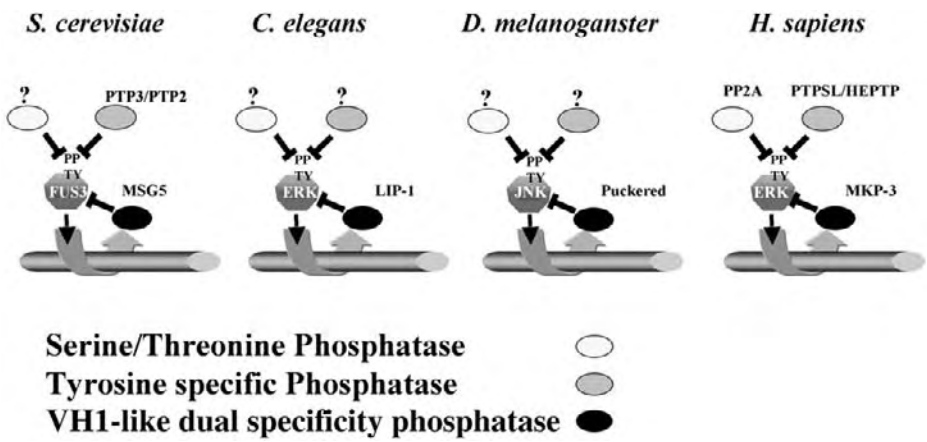


Figure 1 Schematic representation of protein phosphatases important for inactivation of MAPKs in yeast, worm, fruit fly, and human. Genetic and biochemical studies reveal that conserved members of serine/threonine, tyrosine-specific, and VH1-like dual-specificity phosphatases all play a role in inactivating various target MAPKs. Shown are specific phosphatase gene family members known to inactivate selected MAPKs. Powerful transcriptional induction of phosphatase genes, tight MAPK binding, and phosphatase catalytic activation, as well as regionalized subcellular localization, are emerging as important mechanisms for allowing tight and specific control of MAPKs by these various phosphatases (see text for details).

As with Hog1, Fus3 therefore represents a further example of a MAPK inactivated by both a tyrosine-specific as well as a VH1-like dual-specificity phosphatase. In fact, it appears that Ptp3 is required for maintaining a low basal activity of Fus3, while Msg5 plays a major role following pheromone stimulation [3]. It is of note that Ptp3, similar to mammalian MKPs, interacts with Fus3 via a cryptic CH2 (Cdc25 homology) domain, and this targeted binding is responsible for its *in vivo* substrate selectivity. Mutations in either Ptp3 or Fus3 that abolish this interaction cause a dysregulation of the Fus3 MAPK [3]. When considering specificity of interaction, it is worth noting that although Msg5 does not affect Hog1 activity it has been shown to act on a third yeast MAPK, Mpk1 [3].

A MAPK Phosphatase in *C. elegans*

Despite a recognized role for the ERK MAPK pathway in vulval development and the identification of both stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) and p38/Hog MAPK components in the nematode *Caenorhabditis elegans*, only one MAPK phosphatase (LIP-1) has so far been described. A genomic sequence search identified LIP-1 as a homolog of the human MKP3/PYST1 (see below). LIP-1 was shown to be genetically upregulated by the notch signaling pathway in the vulval neighboring cells P5.p and P7.p. This upregulation seems likely to result in inactivation of the MAPK MPK1, an event critical for generating an appropriate pattern of cell differentiation in developing worms [5].

MAPK Phosphatases in *Drosophila melanogaster*

A screen for genes regulating *Drosophila* embryonic dorsal closure identified Puckered, a VH1-like dual-specificity phosphatase, mutations of which lead to cytoskeletal defects and a failure in dorsal closure. Such phenotypic effects are similar to those associated with mutations in the MAPK basket, a *Drosophila* JNK homolog. Loss-of-function mutations in Puckered result in hyperactivation of DJNK, and mRNA expression is regulated by the JNK pathway. This indicates that Puckered regulates SAPK/JNKs and that, as with Msg5, it is a feedback regulator of this MAPK pathway [6]. Remarkably, the Puckered catalytic domain is related to the mammalian dual-specificity phosphatase MKP-5, which selectively interacts and inactivates p38 and SAPK/JNK MAPK family members and is regulated by stress stimuli.

Fruit fly eye differentiation is driven by the *ras* ortholog RAS1 and is dependent on a downstream MAPK belonging to the ERK family. In searching for regulators of this pathway a tyrosine-specific family phosphatase, PTP-ER, was isolated as a negative regulator of eye development acting downstream of RAS1. PTP-ER complexes with and inactivates wild-type ERK but not the gain-of-function ERK mutant Sevenmaker. PTP-ER is a homolog to mammalian PTP-SL and STEP and, like its mammalian counterparts, also contains stretches of basic residues that act as docking sites for binding and specific inactivation of the target ERK MAPK [7]

Despite its functional importance, PTP-ER might not act alone to inactivate ERK, as a recent search of the *Drosophila* EST Expressed Database identified a homolog of the mammalian ERK-specific dual-specificity phosphatase MKP-3/PYST1. Like its mammalian counterpart, DMKP-3 inhibits ERK but is ineffective on SAPK/JNK and p38 MAPKs. Furthermore, DMKP-3 interacts with *Drosophila* ERK via its N-terminal domain and is catalytically activated following interaction with this target MAPK [8].

MAPK Phosphatases in Mammals

The human protein phosphatase CL100, its mouse ortholog 3CH134 (also named Erp), and PAC1 were the first phosphatases recognized to play a role in the inactivation of mammalian MAPKs. Similar to yeast MSG5, CL100 and PAC1 undergo rapid transcriptional activation following exposure to growth factors. Furthermore, inactivation of ERK in fibroblast and Jurkat cell lines correlates with accumulation of CL100 and Pac1 protein, respectively, suggesting a direct role of these phosphatases as feedback regulators of MAPK [9,10]. CL100 belongs to the VH1-like dual-specificity family of phosphatase and consistently was found to dephosphorylate both threonine and tyrosine residues of ERK. These studies also showed that CL100 was specific for MAPK when compared with a number of other phosphoproteins [11]. These observations combined with the ability of catalytic inactive CL100 to coprecipitate with ERK suggested that CL100 is specific for MAPK, leading to its being renamed MAPK phosphatase-1 (MKP-1) [12].

CL100/MKP-1 was originally characterized as an ERK-specific phosphatase but was later also shown to inactivate SAPK/JNK and p38 MAPKs. In fact, another dual-specificity phosphatase gene family member, MKP-3/PYST1 [13,14], has emerged as a more specific inactivator of ERK MAPKs. Interestingly, the MKP-3/PYST1 N-terminal domain binds selectivity to ERK1 and ERK2, but not JNK2, JNK3, or p38 which mirrors its selectivity for inactivating these MAPKs [15]. It turns out that MKP-3/PYST1 binding results in a powerful increase in phosphatase activity with consequent inactivation of the ERK MAPK to which it is complexed [16]. MKP-3/PYST1 enzymatic activation is independent of ERK kinase activity but requires specific charged residues within the noncatalytic N terminus for binding to this MAPK [17–19]. Such a mechanism of MKP-3/PYST1 enzymatic activation is supported by its crystal structure, which shows that in the absence of its target substrate the catalytic domain displays a distorted, probably less active, conformation [20]. Interestingly, a Sevenmaker ERK mutant (ERK2 D319N) is disabled in its ability to either bind or catalytically activate MKP-3/PYST1, suggesting that this deficiency contributes to the gain-of-function activity displayed by this MAPK mutant when expressed in cells.

CL100/MKP-1, PAC1, and MKP-3/PYST1 turned out be founding members of a large family of MKP dual-specificity phosphatases which now include 10 distinct gene products [3,17,21]. MKP family members share a common VH1-like phosphatase catalytic domain as well as noncatalytic regions homologous to the cell-cycle phosphatase regulator CDC25, designated as CH2 domains. These MKP N-terminal regions contain stretches of basic residues that appear essential for mediating specific and tight binding to target MAPKs [3,17–19]. Interestingly, matching these basic charges is a stretch of acid groups present on MAPKs within a conserved motif that has been shown to mediate interaction with dual-specificity phosphatases and upstream MAPKKs, as well as substrates [18,19].

Despite the importance of MKP-3/PYST1 for inactivating ERK, other phosphatases also appear likely to play a role in control of mammalian MAPKs. Hence, biochemical studies, using protein phosphatase inhibitors, have revealed that serine/threonine phosphatases, such as PP2A, are also important for rapid dephosphorylation and inactivation of ERK following EGF stimulation in PC12 cells [22]. Similarly, a serine/threonine phosphatase of the PP2C class, PP2Cα, was recently shown to inactivate stress-responsive SAPK/JNK as well as p38 MAPK pathways in mammalian cells [23]. In addition to serine/threonine and VH1-like phosphatases, several tyrosine-specific phosphatases have also been implicated in the inactivation of MAPK in mammals. Hence, PTP-SL and its homolog STEP were shown to associate with ERK1/2 *in vitro* and to inactivate this MAPK in transfected cells [24]. Another tyrosine-specific phosphatase selectively expressed in hematopoetic tissue, HEPTP/LCPTP, also inactivates ERK1/2 and p38 in transfected cell [25,26]. As seen with the dual-specificity MKPs, binding of HEPTP/LCPTP and STEP to target MAPKs is dependent on conserved basic residues within their noncatalytic N-terminus. This motif is also present in the *Drosophila* homolog PTP-ER [7,23].

Summary

Overall, a wide range of genetic and biochemical studies now indicate an emerging theme in which a combination of serine/threonine, tyrosine-specific, and VH1 dual-specificity phosphatases all play an important role in inactivation of different MAPKs. In many cases, the tight binding of the phosphatase, which appears to be critical for specific MAPK inactivation, together with highly targeted subcellular localization for some family members, indicates a regionalized inactivation of MAPK by different classes of phosphatases. Powerful transcriptional induction also suggests that some phosphatases play selective roles in inactivating MAPK function under different states of stress, endocrine, or growth factor stimulation. Future studies of the complexities of protein phosphatase functions will no doubt reveal further details on the importance this diverse enzyme family in controlling MAPK function.

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CHAPTER 118

SH2-Domain-Containing Protein–Tyrosine Phosphatases

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History and Nomenclature

Shp1 was cloned by four groups, using polymerase chain reaction (PCR) methodology [1–4]. Later, mammalian Shp2s were cloned using similar approaches [5–9], and Shp homologs in *Xenopus* [10] and chicken [11] were reported. This caused a profusion of synonyms for the same genes. A subsequent agreement resulted in the adoption of single names for each mammalian Shp [12], with "Shp1" replacing PTP1C [1], SH-PTP1 [2], HCP [3], and SHP [4]; SH-PTP2 [5], Syp [6], PTP1D [7], PTP2C [8], and SH-PTP3 [9] are now termed "Shp2". Recently, genome sequencing efforts have reintroduced some confusion. The Human Gene Mapping Nomenclature Committee employs a standardized naming system for protein–tyrosine phosphatases (PTPs) in which human Shp1 is termed PTPN6 and Shp2 is PTPN11 (the "N" indicates "non-transmembrane"; the number specifies the order in which the PTP was reported to the database). The *Drosophila corkscrew* (*csw*) gene was identified in a screen for modifiers of the Torso receptor tyrosine kinase (RTK) pathway [13]. Although initially believed to be an Shp1 homolog, sequence analysis [5] and functional studies [14] indicate that Csw is the homolog of Shp2. *Caenorhabditis elegans* has a single Shp, *ptp-2*, the function of which also appears most analogous to Shp2 [15]. It remains unclear whether Csw and Ptp-2 also have some functions similar to Shp1, or if Shp1 evolved to carry out functions unique to vertebrates.

Structure, Expression, and Regulation

Primary Structure

Shps (Fig. 1A) all have two SH2 domains at their N termini (hereafter, N-SH2 and C-SH2), a classical protein–tyrosine phosphatase (PTP) catalytic domain, and a C-terminal tail (C-tail). The Csw PTP domain is split by a cysteine/serine-rich insert (≈150 amino acids) that is unrelated to known protein motifs [13]. The function of the Csw insert is unknown, but its conservation in other *Drosophila* species implies an important role, perhaps in protein–protein interaction. Because vertebrate Shp2 orthologs lack an insert, its function either is specific to insect signaling pathways or is encoded on another vertebrate protein. Some *csw* splice variants fail to encode the insert, suggesting that it is important only in some signaling pathways (L. Perkins, personal communication).

Shps also differ in their C-tails (Fig. 1A, B). Shp1 and Shp2 have two tyrosyl phosphorylation sites in this region, which are phosphorylated differentially by RTKs and nonreceptor protein–tyrosine kinases (PTKs) [6,7,16–23]. The Csw C-tail retains only the more proximal tyrosine (Y542 in Shp2), whereas Ptp-2 lacks both sites. Shp2 and Csw (but again, not Ptp-2) have proline-rich domains that may bind SH3-domain-containing proteins, although specific interacting proteins have not been reported. Shp1 lacks a proline-rich domain but has a basic sequence that functions as a nuclear localization sequence (NLS) [24,25].

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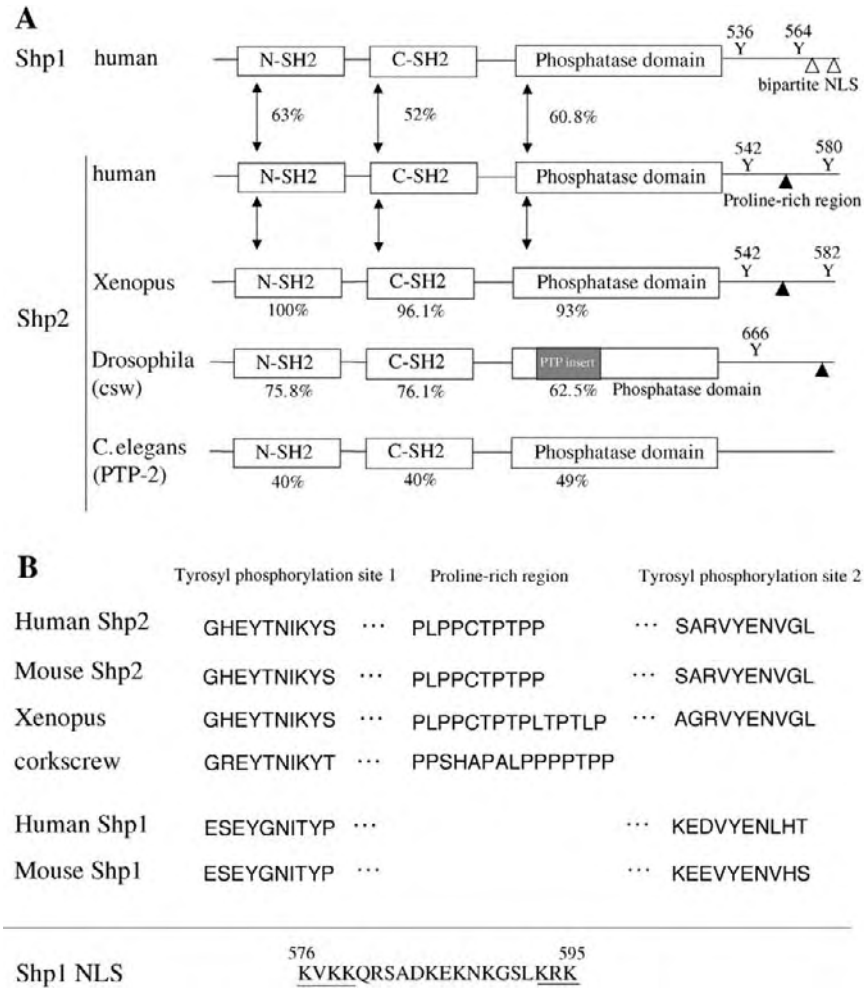


Figure 1 The Shp family. (A). Schematic structures of Shp family members. The SH2 and PTP domains and C-tail are indicated, and the relative positions of tyrosyl phosphorylation sites, proline-rich domains, and nuclear localization sequences are shown. (B) Features of Shp C-tails. Shown are sequences surrounding potential tyrosyl phosphorylation sites and proline-rich domains. Also shown is the potential bipartite NLS found in Shp1, but not other family members.

Expression

Shp2 and its orthologs are expressed ubiquitously, although at variable levels in different tissues [5,6,8,13]. Shp1 is more restricted, with high levels in hematopoietic cells, lower amounts in most epithelia and some neuronal cells, and few or none in fibroblasts [2,4,26]. The Shp1 gene has two promoters that function in a tissue-specific fashion, generating Shp1 isoforms with slightly different N termini [27,28]. In humans, the more 3′ (downstream) of these promoters is expressed only in hematopoietic cells, whereas the upstream promoter is expressed only in epithelia. The murine 3′ promoter may be active in epithelial cells as well, but the upstream promoter retains epithelial-specific expression [28]. A third Shp1 isoform, generated by alternative splicing, has a C-terminal extension [29]. Splice variants within the PTP domain of Shp2 and Csw also have been defined [5,13,30]. The Shp2 isoforms reportedly have different PTP activities [30], but their physiological significance has not been determined. Although differential expression may explain some differences in the roles of the Shps, they clearly are not the whole story. Many cells and tissues, particularly hematopoietic cells, express high levels of both Shps, yet the consequences of loss of either molecule are strikingly different.

SH2 Domain Function

Not surprisingly, the SH2 domains of Shps target them to phosphotyrosyl-containing (pTyr) proteins. Little is known about the binding properties of invertebrate Shps, but multiple proteins are known to bind the SH2 domains of the mammalian orthologs. Most fall into three distinct categories: receptors (RTKs or cytokine receptors), scaffolding adapters (e.g., IRS, DOS/Gab, and FRS proteins), and so-called immune inhibitory receptors (commonly termed *inhibitory receptors*). The latter comprise a large number of glycoproteins, first described in immune cells, hence the name [31]. However, several inhibitory receptors are

expressed more widely. Some Shp-binding proteins (e.g., Shps1/Sirpα, Pir-B) bind both Shps [32–35], whereas others (e.g., Gab/Dos family proteins) exhibit specificity in Shp binding [36].

Unlike the case for most SH2 domains, residues at both the N and C terminals of pTyr contribute to binding. The consensus for binding an Shp SH2 domain conforms to the immunoreceptor tyrosine-based inhibitory motif (ITIM), I/V/L-X-pY-X-X-I/V/L [37–45]. However, why some pTyr-peptides that fit the ITIM consensus bind the N- vs. C-SH2 of one or both Shps had remained unclear. Recently, mass spectrometric screening of a combinatorial peptide library was used to assess the specificity of the N- and C-SH2 domains of Shp1 [46]. This novel approach, together with direct affinity measurements, suggests a single consensus for binding to the C-SH2 (V/I/L-X-pY-A-X-L/V) and two distinct motifs for the N-SH2 (L-X-pY-A-X-L and L-X-pY-M/F-X-F/M). Notably, in this analysis, the N-SH2 strongly prefers Leu at Y−2 (although Ile, Val, and Met substitute equally well in direct binding assays), whereas peptides with other hydrophobic residues at −2 can bind the C-SH2. Importantly, these consensus sequences show strong agreement with earlier binding studies of known Shp1-interacting proteins. It will be interesting to apply this approach to the SH2 domains of Shp2. A recent study did assess Shp2 SH2 domain binding, using a different combinatorial method. This analysis locked in a valine at Y−2, preventing assessment of the relative contribution of this position to binding [47]. However, it did reveal similar binding preferences for the two SH2 domains, with positions +1 (threonine/alanine/valine), +3 (valine, isoleucine, leucine), and +5 (tryptophan, phenylalanine) being most important for specificity. Although the same general consensus appears to apply to N- and C-SH2s of Shp2, the two domains differ quantitatively in their preferences for individual amino acids at each position. Consistent with previous binding [48] and structural [49] studies, and in marked contrast to many other SH2 domains, selection for residues at the +5 position (which was not tested in the earlier work on Shp1 binding preferences) was quite strong. Surprisingly, however, there was no apparent preference for acidic residues at the +2 and +4 positions, in contrast to earlier work on Shp2 binding sites in IRS-1, which identified Y1172 (YIDLD) as the optimal site [48].

Some reported Shp SH2-domain-binding interactions fail to follow the above rules. CTLA-4 reportedly binds Shp2 via a G-X-pY-X-X-M motif [50]. Mast cell function-associated antigen (MAFA) contains an ITIM-like motif with Ser at Y−2, and a pTyr peptide bearing this sequence can bind to both Shps [51]. Also, Shp1 reportedly binds several tumor necrosis factor (TNF) family (death) receptors via a conserved A-X-pY-X-X-L motif. Even more surprisingly, binding could be competed by a short peptide lacking any residue at Y−2 [52]. These reports stand in marked contrast to earlier studies, which revealed an essential role for positions upstream of pTyr [39,53,54] and a specific requirement for hydrophobic residues at the −2 position. Conceivably, some of these nonconforming interactions are indirect, but further work is required to resolve these inconsistencies.

Additional complexity arises from the ability of some ITIMs to bind to the SH2 domain of the inositol phosphatase SHIP; however, the SHIP SH2 does not require a hydrophobic residue at the Y−2 position [51,53–55]. Also, leucine at the Y+2 position favors SHIP binding [56], whereas bidendate ligands enjoy an obvious avidity advantage for Shps. The binding sites for Shp2 and the E3 ubiquitin ligase SOCS3 on several cytokine receptors also overlap [57–61]. Peptide library screening confirms the similar specificity of the Shp2 and SOCS3 SH2 domains, although pTyr peptides with considerable (>30-fold) specificity for the latter can be identified [47]. SOCS SH2 domains bind with markedly higher affinity than Shp2 to these shared binding sites [47]. Nevertheless, the overlapping specificity of their SH2 domain has complicated analyses of the respective roles of the Shps, SHIP, and SOCS proteins in several signaling pathways.

Regulation of PTP Activity

Shps have very low basal activity, but addition of a pTyr ligand for the N-SH2 substantially enhances catalysis [48,62–65]. Appropriate bis-phosphorylated (bidentate) pTyr ligands have an even greater effect, resulting in a ≈50-fold increase in activity [66]. Comparable stimulation results from N-SH2 truncation [63,64,67,68]. A molecular explanation for these findings was provided by the crystal structure of Shp2 lacking its C-tail (i.e., containing the N- and C-SH2 and PTP domains) [69]. In the structure, the backside of the N-SH2 (the surface opposite the pTyr-peptide-binding pocket) is wedged into the PTP domain. This physically and chemically inhibits the catalytic cleft, and contorts the N-SH2 pTyr-peptide-binding pocket. Thus, in the basal state, the PTP domain is inhibited by the N-SH2, *and* pTyr-peptide binding is incompatible with binding of the N-SH2 backside to the PTP domain. The C-SH2 has minimal interactions with the PTP domain, and its pTyr-binding pocket is unperturbed in the basal state. Thus, the C-SH2 probably serves a search function, surveying the cell for appropriate pTyr targets. If it binds to a bidentate ligand (one that also has an N-SH2 binding site), the effective increase in local concentration of the N-SH2 ligand can reverse inhibition by the PTP domain, allowing release of the N-SH2 and enzyme activation. Recent studies support such a model for Shp1 interactions with gp49B [70]. Alternatively, high-affinity ligands for the N-SH2 might be able to cause activation in the absence of C-SH2 binding (Fig. 2A).

The biological relevance of the Shp2 structure was verified by analyzing mutants of the N-SH2/PTP domain interface. Such mutants have increased basal activity *in vitro*, retain the ability to bind N-SH2 ligands, and behave as gain-of-function (activated) mutants *in vivo* [71]. More dramatic confirmation came with the recent finding that analogous mutants are the cause of Noonan syndrome in humans. The structure of full-length Shp1 (or a form lacking its C-tail)

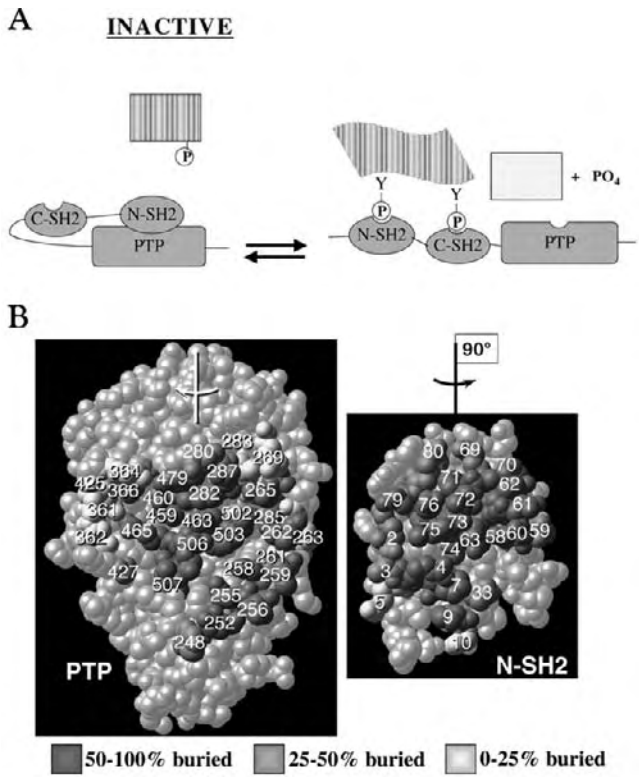


Figure 2 (A) Regulation of Shps. Drawing illustrates regulation of a prototypical Shp by a pTyr peptide. In the basal state, the Shp is largely inactive, because the backside of the N-SH2 is inserted into the catalytic cleft. This results in mutual allosteric inhibition, with the N-SH2 inhibiting the PTP domain and the PTP domain contorting the pTyr binding pocket of the N-SH2, which is on the opposite surface. The C-SH2 is left essentially unperturbed in the basal state, with its pTyr-peptide binding pocket in a conformation suitable for binding an appropriate ligand. Most likely, the C-SH2 has the primary targeting function to most binding proteins. If an appropriate binding protein has an adjacent pTyr peptide sequence capable of binding the N-SH2, the increase in local concentration overcomes the mutual allosteric inhibition, resulting in opening of the enzyme and activation. For further details, see text. (B) Location of mutations in Noonan syndrome. Shown is an "open book" representation of the Shp2 crystal structure in which one is looking directly onto the surfaces of the N-SH2 backside loop and the PTP domain involved in basal inhibition. The positions of known Noonan syndrome mutations (e.g., D61G and E76D) can be found in these domains. Note the high correspondence between NS mutations and residues involved in basal inhibition. (Figure courtesy of S. Shoelson, Joslin Diabetes Center).

has yet to be solved, but all of the N-SH2 and PTP domain residues involved in basal inhibition are either identical or highly conserved between the two Shps [69]. An Shp1 PTP domain structure has been reported [72]. Its similarity to the Shp2 PTP domain and the stimulatory effects of pTyr peptide binding and N-SH2 truncation on Shp1 activity strongly suggest that the molecular details of Shp1 regulation are analogous.

Alternative Mechanisms of Regulation: The Role of the C-Tail

The effects of C-tail tyrosyl phosphorylation remain controversial. Insulin receptor-induced tyrosyl phosphorylation of Shp1 (at Y536) stimulates activity [21], although others [19] found no effect of tyrosyl phosphorylation, perhaps due to autodephosphorylation. One group [7] reported that tyrosyl phosphorylation of Shp2 increases activity, but this study could not distinguish the effects of phosphorylation from SH2 domain association. Recently, protein ligation techniques were used to replace Y542 or Y580 of Shp2 with a nonhydrolyzable pTyr mimetic [73]. Phosphorylation at either position was found to stimulate catalysis by two- to threefold (the same fold stimulation observed with a stimulatory pTyr peptide in these experiments). Mutagenesis, combined with protease resistance studies, suggested that stimulation by pY-542 involves intramolecular engagement of the N-SH2 domain, whereas pY-580 stimulates activity by binding to the C-SH2.

Although these studies are novel and provocative, they are difficult to reconcile completely with previous studies. It is unclear how pY-580, upon engaging the C-SH2, would cause enzyme activation while the N-SH2 remains wedged in the PTP domain; notably, pY580-phosphorylated Shp2 is further activated by a pTyr peptide for the N-SH2, suggesting that the N-SH2/PTP domain interaction mechanism remains intact. The Shp2 crystal structure provides no obvious explanation for how C-SH2 engagement could affect catalytic activity, and previous enzymological studies showed no effect of C-SH2 engagement alone [65,69]. Also, given the closed conformation of Shp2 in the absence of pTyr-peptide binding, it is difficult to see how binding of pY542 to the N-SH2 leads to increased proteolytic susceptibility. Further work will be required to resolve these issues.

Serine–threonine phosphorylation of the Shps also has been reported. Shp1 undergoes serine phosphorylation in response to PKC agonists [74,75], and phosphorylation may inhibit catalytic activity [75], although this was not noted by others [74]. Specific PKC isoforms phosphorylate Shp2 *in vitro* [76,77] and in transfected cells [77]. Mutagenesis suggests that S576 and S591 are the sites of phosphorylation, but mutation of these sites has no apparent effect on catalytic activity or biological function [77]. Extracellular signal-regulating kinase (ERK)-dependent phosphorylation of Shp2 was reported by one group [78] to be inhibitory, but not to be by another [76]. The sites of serine–threonine phosphorylation were not identified, but sequence analysis predicts these are likely within the C-tail. Shp2 is required for ERK activation in many signaling pathways, raising the possibility that phosphorylation of Shp2 by ERK is part of a negative feedback loop.

Certain phospholipids, particularly phosphatidic acid, stimulate Shp1 (but not Shp2) [79] by binding to a specific high-affinity site within the C-tail [80]. The physiological significance of phospholipid binding remains to be determined, although one intriguing possibility is that it relates to the reported ability of Shps to be excluded from lipid rafts [81–83].

The C-tail of Shp1, but not Shp2, has a functional NLS. Substantial amounts of Shp1 are found in the nuclei of epithelial cells [24] and tissues [84; H. Keilhack and B.G.N.,

unpublished observations], whereas under basal (randomly growing) conditions Shp1 is cytoplasmic in hematopoietic cells [24,25,74]. However, cytokine stimulation can result in nuclear translocation of Shp1 in hematopoietic cells [25]. Translocation occurs with delayed kinetics (>1 hr post-stimulation), which might reflect a requirement for synthesis of a new protein to promote nuclear inport or repress nuclear export. Most studies of Shp signaling have focused on immediate events following receptor stimulation; this new work argues that attention should be paid to later events as well.

Besides these conventional mechanisms of regulation, Shp2 (and other PTP family members) may be regulated by reversible oxidation. Increasing evidence suggests that hydrogen peroxide and other reactive oxygen intermediates (ROIs) are generated upon growth factor and cytokine stimulation and act as second messengers [85]. A recent study showed that Shp2 undergoes transient inactivation by ROI in response to platelet-derived growth factor (PDGF) stimulation of Rat 1 fibroblasts, and argued that Shp2 inactivation is required for normal PDGFR function in these cells [86]. However, other work indicates that Shp2 plays a signal-enhancing role in PDGF signaling [87–90]. Further studies are required to clarify these discrepancies and to test whether Shps are targets for ROIs in other pathways.

Biological Functions of Shps

Genetic models for murine Shp1 and for Shp2 orthologs in mouse, *Drosophila*, and *C. elegans* have been invaluable for defining the biological functions of the Shps. The phenotypes of Shp-deficient organisms will be described briefly here; more complete descriptions are available in several other reviews.

The *motheaten* Phenotype

Two naturally occurring point mutations exist in the murine Shp1 gene, each of which causes abnormal splicing of Shp1 transcripts [91,92]. The *motheaten* (*me*) allele generates an early frameshift; consequently, *me/me* mice are protein null. The *motheaten viable* (me^v) allele encodes two aberrant Shp1 proteins; one with a small deletion, the other with a small insertion in the PTP domain. Together, these retain only about 20% of wild-type (WT) Shp1 activity, demonstrating the essential role for PTP activity in Shp1 function.

The phenotypes of *me/me* and me^v/me^v mice differ only in severity, with *me/me* mice succumbing to abnormalities earlier (2–3 weeks) than me^v/me^v (9–12 weeks) [93–98]. For this reason, we use **me** to refer generically to Shp1-deficient mice. The **me** phenotype derives its name from patchy hair loss, which gives the mice a motheaten appearance. The hair loss, in turn, results from sterile dermal abscesses consisting of neutrophils. Inflammation also is prominent elsewhere, including the joints, liver, and lungs. The latter leads to the early demise of **me** mice, due to severe interstitial pneumonitis caused by accumulations of alveolar macrophages and neutrophils. The macrophage population in **me** mice is expanded and exhibits abnormal differentiation, with a dramatic increase in $CD5^+$ monocytoid cells and a decrease in cells expressing tissue/marginal zone macrophage markers [99,100]. Some dendritic cell populations are increased, whereas others are diminished [100]; osteoclast numbers and function are enhanced, leading to osteopenia in **me** mice [101]. Shp1-deficient mice on either a *nude* or *rag* knockout background still develop inflammatory disease [102], and the disease can be reproduced by transplantation of bone marrow cells and prevented by treatment with Mac-1 antibodies [103]. Thus, lymphoid cells are dispensable, and defects in the myeloid lineage are critical, if not sufficient, for development of the **me** inflammatory syndrome.

Although from the host standpoint, the myeloid defects present the gravest problems, every other hematopoietic lineage is affected by Shp1 deficiency [93–98]. The thymus undergoes premature involution, possibly due to defective homing of a thymic accessory cell [104,105]. Indeed, thymocytes and peripheral T cells lacking Shp1 actually exhibit increased mitogenesis in response to T-cell antigen receptor (TCR) stimulation [106,107]. Consistent with enhanced responsiveness, crosses to TCR transgenic mice show that Shp1 deficiency lowers the threshold for thymic selection [108–111]. Normal B (B2 cell) lymphopoiesis is reduced, but there is a marked increase in B1a ($CD5^+$) cells. The remaining B cells appear hyperactivated and produce autoantibodies [93,98]. Proliferation [112,113] and calcium flux [114] in response to B-cell antigen receptor (BCR) stimulation are reportedly enhanced in **me** lymphocytes, and the response threshold of a transgenic BCR is lowered in **me** mice [114]. Natural killer (NK) cell activity is decreased [115], but the remaining NK cells show enhanced lytic activity [116]. Motheaten mice are anemic, probably due to chronic hemolysis, although their erythroid progenitors are hyper-responsive to erythropoietin (EPO) [117–119]. Increased numbers of certain mast cell populations also have been reported [120,121]. Because the lymphohematopoietic system is highly interactive, identifying which **me** abnormalities are primary (i.e., cell autonomous) defects, as opposed to secondary consequences of the myeloid defects, has posed major (and ongoing) challenges. Nevertheless, many of the abnormalities have been ascribed to loss of negative regulation of specific signaling pathways in the absence of Shp1.

Invertebrate Models of Shp2 Deficiency

Csw is a maternal effect mutation affecting the so-called terminal class pathway [13], which is initiated by the RTK Torso and controls embryonic head and tail development [122]. Loss-of-function mutations in *csw* were found to have a phenotype similar to, although less severe than, *torso* mutations, which provided the first evidence of a positive (i.e., signal enhancing) function for an Shp2 ortholog [13]. Csw also is a required positive component of the *sevenless*,

breathless (fibroblast growth factor receptor [FGFR]), and *Drosophila* epidermal growth factor receptor (EGFR; DER) pathways [14,123,124].

Ptp-2 functions in at least two RTK signaling pathways. In vulval development, which is controlled by the EGFR ortholog Let-23, *ptp-2* mutation alone has no obvious effect. However, *ptp-2* deficiency suppresses the multivulva phenotype induced by mutation of the negative regulator *lin-15*. Interestingly, *lin-15* mutations cause Let-23 activation even in the absence of the EGFR ligand, Lin-3, implying that Ptp-2 may play an important role in a ligand-independent Let-23 pathway [15]. Ptp-2 also is important for signaling by the FGFR ortholog EGL-15 [125] and has an essential role in an as yet unidentified pathway required for oogenesis [15].

Functions of Vertebrate Shp2

Studies of *Xenopus* embryogenesis provided initial evidence of a role for Shp2 in vertebrate development [10]. Expression of dominant-negative Shp2 disrupts gastrulation, causing severe tail truncations reminiscent of, but less severe than, the effects of dominant-negative FGFR. Dominant-negative Shp2 also blocks FGF-induced mesoderm induction and elongation of ectodermal explants. Recently, two activated mutants of Shp2 (similar to those found in Noonan syndrome) were found to induce elongation of ectodermal explants in the absence of exogenous FGF. Activated mutants do not, by themselves, induce mesodermal gene expression, although they potentiate induction of the Erk pathway by FGF [71].

Targeted mutations of murine Shp2 indicate a key role for Shp2 in mammalian development. Homozyotic deletion of either Exon 2 [126] or Exon 3 [127] results in early embryonic lethality. Exon 3 $(Ex3)^{-/-}$ embryos die between E8.5 and E10.5, with a range of abnormalities consistent with defective gastrulation and mesodermal differentiation [127,128]. These defects resemble the effects of dominant-negative Shp2 (and FGFR) mutants in *Xenopus* and the effects of vertebrate FGFR mutations [129]. Chimeric analyses using $Ex3^{-/-}$ embryonal stem (ES) cells reveal an essential role for Shp2 in limb development and branchial arch formation, two other pathways controlled by FGFR signaling [130,131]. Studies of hematopoietic differentiation in $Ex3^{-/-}$ ES cells [132] and in chimeric mice [130] indicate a stringent requirement for Shp2 in the earliest progenitors, consistent with a role for Shp2 in Kit (stem cell factor receptor) signaling [133].

The Ex3 mutation generates a truncated Shp2 protein that lacks part of its N-SH2 domain and is expressed at ≈25% of WT levels in $Ex3^{-/-}$ cells. Due to the N-SH2 deletion, however, the Ex3 mutant is activated markedly; consequently, $Ex3^{-/-}$ cells actually have increased Shp2 activity [127,132], although the mutant protein is defective at localizing to at least some signaling pathways [134]. This finding raised the possibility that some effects of Ex3 deletion might be neomorphic.

Recent studies of other targeted mutations argue against this possibility. $Ex2^{-/-}$ embryos die earlier (≈E6–E6.5) than $Ex3^{-/-}$ embryos. Despite earlier reports (which used antibodies against the N terminus to assess expression), $Ex2^{-/-}$ mice also express an N-terminally truncated protein. However, for reasons that are unclear, this mutant protein is not hyperactivated. More convincingly, a variant Ex2 mutation (Ex2*), in which a strong splice acceptor sequence was introduced into the targeting construct, is, in fact protein null, and $Ex2^{*-/-}$ embryos also die at E6 to E6.5. Total Shp2 deficiency causes defective inner cell mass expansion, due to markedly increased apoptosis (W. Yang and B.G.N., manuscript in preparation). The timing and nature of the lethality of $Ex2^{*-/-}$ embryos are consistent with roles for Shp2 in FGF-4 [135] and/or β1 integrin [136] signaling.

Shp Signaling and Substrates

Shp1

Shp1 is implicated as a regulator of signaling by RTKs, cytokine receptors, multichain immune recognition receptors (MIRRs), chemokine receptors, and integrins. Many of these studies utilized cells from *me/me* or me^{v}/me^{v} mice. Such cells provide the advantage of a genetic model of Shp deficiency, but the reported defects may reflect altered development caused by Shp1 deficiency, rather than the effects of Shp1 on a specific signaling pathway *per se*. Remarkably, despite much progress in defining signaling pathways affected by Shp1, its direct targets remain controversial and/or elusive.

Signaling Pathways in Myeloid Cells

Bone marrow macrophages (BMMs) from **me** mice were reported to show increased proliferation in response to colony-stimulating factor 1 (CSF-1; MCSF) [137]. Subsequent studies found no effect of Shp1 on proliferation *per se* [138,139], although **me** BMMs required less CSF-1 for survival [139]. The target(s) of Shp1 in this pathway also are controversial. In one study, the CSF-1R was found to be hyperphosphorylated, albeit for short times, following stimulation [137]. Others failed to confirm these observations, noting instead that p62Dok (Dok) is the major hyperphosphorylated species [139]. The reason for this discrepancy is not clear. Regardless, because Dok is primarily a negative regulator, acting to recruit RasGap [140–142], it remains unclear how Dok hyperphosphorylation might explain the lower CSF-1 dependency of **me** BMMs. Shp1 does not bind directly to either the CSF-1R or Dok. Instead, two inhibitory receptors, Shps1 (Sirpα/BIT/MFR) and PirB (p91A) are the major Shp1 binding proteins in BMMs [35,143–145]. Both also are Shp1 substrates [35], but it does not appear as if dephosphorylation of these proteins plays a major negative regulatory role. Also, it is not clear if either regulates RTK signaling in BMMs or has another function, such as in integrin signaling [146].

Shp1 also negatively regulates cytokine signaling in BMMs. IFNα/β signaling is dramatically enhanced in **me** BMMs, as shown by increased JAK1 and STAT1 tyrosyl phosphorylation [147]. These data are consistent with other studies implicating Shp1 in Janus PTK dephosphorylation. Others have reported that granulocyte–macrophage colony-stimulating factor (GM-CSF)-evoked proliferation is enhanced in Shp1-deficient BMMs [138]. These workers observed no change in JAK/STAT phosphorylation but did notice a hyperphosphorylated 126-kDa species, which is most likely Shps1 and/or PirB. As indicated previously, hyperphosphorylation of these proteins alone is unlikely to explain increased GM-CSF responsiveness.

Shp1-deficient BMMs are markedly hyperadherent to ligands for both β1 and β2 integrins, suggesting a negative regulatory role for Shp1 in integrin signaling [148]. The direct targets of Shp1 in this pathway also remain unclear, although actions on *src* family PTKs (SFKs) and/or the p85 subunit of phosphatidylinositol 3-kinase (PI3K) have been suggested. If SFKS are, in fact, Shp1 targets, presumably only specific members mediate the increased adhesiveness, because SFK activity also is increased in $CD45^{-/-}$ BMM yet these cells fail to sustain integrin-mediated adhesion [149]. Although p85 is reportedly hyperphosphorylated, and phosphatidylinositol 3,4,5-triphosphate (PIP3) levels are elevated in **me** BMMs [148], a direct stimulatory effect of p85 tyrosyl phosphorylation on PI3K activity has not been demonstrated. Shps1 (and possibly PirB) probably play an important role in mediating Shp1 action in integrin signaling. Shps1 becomes rapidly phosphorylated in response to BMM adhesion, most likely by SFKs [146], and recruits Shp1. Immunostaining experiments suggest targeting of Shps1 to sites of adhesion (K. Swanson and B.G.N, unpublished data). Shps1 also forms complexes with other proteins that probably play important roles in integrin signaling. One contains the adapter proteins Skaphom/R and SLAP130/Fyb (now known as ADAP). ADAP is essential for in inside-out signaling in T cells [150–152], perhaps by virtue of its ability to bind Ena/Vasp family proteins [153]. The other Shps1 complex contains the focal adhesion kinase (FAK)-related PTK Pyk2, and FAK regulates fibroblast adhesion [154]. Skaphom, ADAP, and Pyk2 associate constitutively with Shps1, but all undergo inducible phosphorylation in response to adhesion [146]. It is tempting to speculate that upon recruitment to tyrosine-phosphorylated Shps1, Shp1 dephosphorylates one or more of these associated proteins. Although tyrosyl phosphorylation activates Pyk2, its effect on Skaphom and ADAP remains to be determined, as does whether any of these proteins are direct Shp1 targets. In any case, it is unlikely that all effects of Shp1 on BMMs are mediated via Shps1, as mice lacking the cytoplasmic domain of Shps1 do not exhibit a **me** phenotype, their only obvious defect being mild thrombocytopenia [155]. Interestingly, while haplotaxis is defective in **me** BMMs, probably owing to defective deadhesion, BMMs show markedly increased chemotaxis in response to chemokines [156]. Chemokines signal via G-coupled receptors, so it remains unclear what the targets are for a PTP in chemokine signaling, although Pyk2 is a possibility [157].

Phagocytosis is also regulated by Shp1. BMMs from **me** mice show increased ingestion of IgG-opsonized sheep red blood cells (RBCs), indicating defective negative regulation of FcγR signaling [158]. Complement-mediated phagocytosis, which utilizes β2 integrins, also is enhanced in **me** BMMs. Recent data indicate that both of these negative regulatory pathways involve Shps1/Shp1 complexes. CD47, a ubiquitously expressed glycoprotein [159], is a ligand for Shps1 [160]. CD47 on the RBC surface engages macrophage Shps1, leading to its tyrosyl phosphorylation, Shp1 recruitment, and inhibition of FcγR signaling [161,162]. Interestingly, unopsonized RBC phagocytosis is unaffected in **me** BMMs, indicating that Shps1/Shp1 complexes do not regulate the receptor responsible for phagocytosis of unopsonized RBC.

Neutrophil signaling is also affected by Shp1 deficiency. The number and size of colonies evoked by G-CSF are increased in bone marrow from **me**, compared with WT mice, and also differ qualitatively, containing increased numbers of macrophage-like cells [163]. Increased G-CSF responsiveness also is apparent in short-term proliferation assays and is reflected by an increased magnitude of STAT activity in progenitors [163]. Cell lines expressing dominant negative Shp1 [164] and $Shp1^{-/-}$ DT40 B cells expressing the G-CSFR [165] also show increased G-CSF-evoked STAT activation. Under endogenous conditions, no association between the G-CSFR and Shp1 can be detected, although an unidentified 92-kDa species coprecipitates [163]. When Shp1 and the G-CSFR are massively overexpressed in 293 T cells, a small amount of coprecipitation is detected. Despite the lack of strong association, the G-CSFR C terminus is necessary for Shp1 to mediate its effects on G-CSF signaling, although the receptor tyrosines are dispensable [165]. Thus, the mechanism by which Shp1 regulates GCSF signaling, including its direct targets, remains unclear.

A provocative study indicates that Shp1 also acts as an effector of death receptors, such as the TNF receptor (TNFR) and Fas [52]. These receptors contain a conserved AXYXXL motif in their cytoplasmic domains and undergo tyrosine phosphorylation upon activation. Despite its non-canonical nature, pTyr peptides containing this motif were found to bind to Shp1 from neutrophils. Death receptor activation antagonizes cytokine-evoked neutrophil survival and depends on the presence of the pYXXL motif. Moreover, $me/_{+}$ neutrophils appear relatively resistant to death receptor stimuli. These effects correlated with increased cytokine-evoked Lyn phosphorylation, suggesting that Lyn may be a target for death receptor/Shp-1 complexes.

Neutrophil function is also altered in **me** mice. Oxidant production, surface expression of the integrin subunit CD18 and adhesion to plastic are enhanced in **me** neutrophils, whereas chemotaxis is diminished, perhaps due to increased adhesion [166]. However, it is not certain whether all of these defects reflect direct effects of the absence of Shp1 or altered granulocytic differentiation in **me** mice.

REGULATION OF LYMPHOCYTE AND NK CELL SIGNALING BY SHP1

Multiple studies have shown that Shp1 negatively regulates antigen receptor (TCR and BCR) signaling [167–169]. In these pathways, Shp1 is recruited to specific inhibitory receptors that block cell signaling (Fig. 3A). CD5 [170] and members of the ILT family (LIR, MIR, CD85) [171] probably negatively regulate TCR signaling in different types of T cells. In B cells, knockout studies have established roles for CD22 [172–174], CD72 [175], and CD5 [176]. Other inhibitory receptors, such as PirB/p91A, ILT2, PECAM-1, and CEACAM, are also expressed in B cells and can transduce inhibitory signals [168]. Shp1 initially was suggested to mediate inhibitory signaling by FcγRIIB, the Fc receptor that mediates inhibition of B cell activation by immune complexes [112]. Subsequent studies showed that Shp1 is dispensable for FcγRIIB-mediated inhibition, which instead is transmitted via the inositol phosphatase SHIP [177–180].

There is less agreement over the targets that Shp1 dephosphorylates to mediate inhibition. Lck and Fyn activities are elevated in **me** thymocytes, suggesting that Shp1 might dephosphorylate SFK activation site tyrosyl residues [106]. Transient and stable expression of catalytically impaired Shp1 also causes elevated Lck activity [110,181], and recent studies show that Shp1 has preference for dephosphorylating the activating phosphorylation site (Y394) of Lck [182]. Others have argued that Shp1 dephosphorylates ZAP-70 [183,184] and possibly the TCRζ chain [107]. There also is no agreement over the target of Shp1 in B cells, with suggestions including Syk [185] and more downstream substrates, such as BLNK [186] and Vav [187].

In NK cells, Shp1 mediates inhibitory signaling by killer inhibitory receptors (KIRs; Ly49 family in the mouse) and CD94/NKG2. These receptors bind to host human leukocyte antigen (HLA)/major histocompatibility complex (MHC) and prevent cytolysis by recruiting Shp1 to interrupt signaling by NK cell activating receptors [31,169,188]. Again, the precise targets for Shp1 remain unclear. Initial work indicated that the earliest events in NK cell activation were blocked by KIR engagement, suggesting action of Shp1 on a proximal NK cell PTK [189]. Subsequent studies indicate that SLP-76 may be a direct target [190].

The reasons for these disagreements are unclear. Various groups have studied different lymphocyte or NK cell lines. If Shp1 has several different targets, perhaps reflecting the action of different inhibitory receptor/signaling complexes, the targets in each system might differ. Some studies have compared signaling in cells from **me** and WT mice or WT with inhibitory receptor knockout mice. Because Shp1 and/or inhibitory receptor deficiency can result in altered development, these studies can end up comparing cells at different developmental stages. Such "apples and oranges" comparisons make it difficult to know whether differences are primary or secondary. New approaches such as inducible knockouts and/or RNAi, combined with substrate trapping mutants, may help to resolve these important questions.

The role of Shp1 in other lymphocyte signaling pathways is controversial. Shp1 has been reported to regulate interleukin-2 (IL-2) receptor signaling in human T cells [191]; however, **me** thymocytes show no difference in their IL-2 response [106]. Shp1 reportedly is required for Fas-induced cell death [192,193], analogous to its role in mediating death receptor signaling in neutrophils, but two subsequent reports failed to find such a requirement [106,194].

SHP1 SIGNALING IN ERYTHROID CELLS

Shp1 binds directly to the EPO receptor (EpoR) [195, 196]. Studies with EpoR mutants suggest that upon recruitment, Shp1 dephosphorylates the receptor-associated kinase, Jak2 [196]. This model (Fig. 3B) provides a possible molecular explanation for the phenotype of a family that inherits an EpoR truncation and exhibits erythrocytosis [197]. Knock-in mice bearing a similar truncation do not have erythocytosis but show increased EPO sensitivity [198].

The recent finding that SOCS3 can be recruited to the same region raises the question of whether the effects of these EpoR mutants are due to loss of Shp1 or SOCS binding [59]. Conceivably, Shp1 and SOCS proteins act in combination to negatively regulate the EpoR and possibly other cytokine receptors. Upon activation, JAK2 (and other Janus PTKs) become phosphorylated on two adjacent sites, Y1007 and Y1008. Monophosphorylated Y1007-containing peptides bind SOCS3, but it is unclear (although it is unlikely) if bisphosphorylated (i.e., pY1007/pY1008) peptides can bind. Perhaps Shp1 first dephosphorylates Y1008, thereby allowing a SOCS protein to bind via pY1007 and mediate degradation (Fig. 3B). An analogous model may explain the positive actions of Shp2 in cytokine signaling. Shp1 also binds to [18] and negatively regulates [120,121] Kit. Loss of this regulation may contribute to the enhanced erythogenic potential of progenitors from **me** mice.

SHP1 SIGNALING IN EPITHELIAL CELLS

The few studies that have explored Shp1 actions in epithelial cells have yielded surprising results. First, as indicated above, a significant amount of Shp1 appears to be nuclear in these cell types [24,84]. Also, in at least some cell types and signaling pathways, Shp1 may play a positive signaling role [199], although how it does so remains to be determined. Nevertheless, Shp1 clearly plays a negative regulatory role in other epithelial signaling pathways. For example, it binds directly to and dephosphorylates the RTK Ros. Loss of this regulation may help explain the sterility of **me** mice [200].

Signaling by Shp2 and Its Orthologs

Shp2 is also implicated in a wide variety of signaling pathways, yet, unlike Shp1, in most cases it appears to have a positive role. There are, however, exceptions, as described below.

SHP2 IN RTK SIGNALING

In most, if not all, RTK signaling pathways, Shp2 is required for full activation of the Erk MAP kinase pathway.

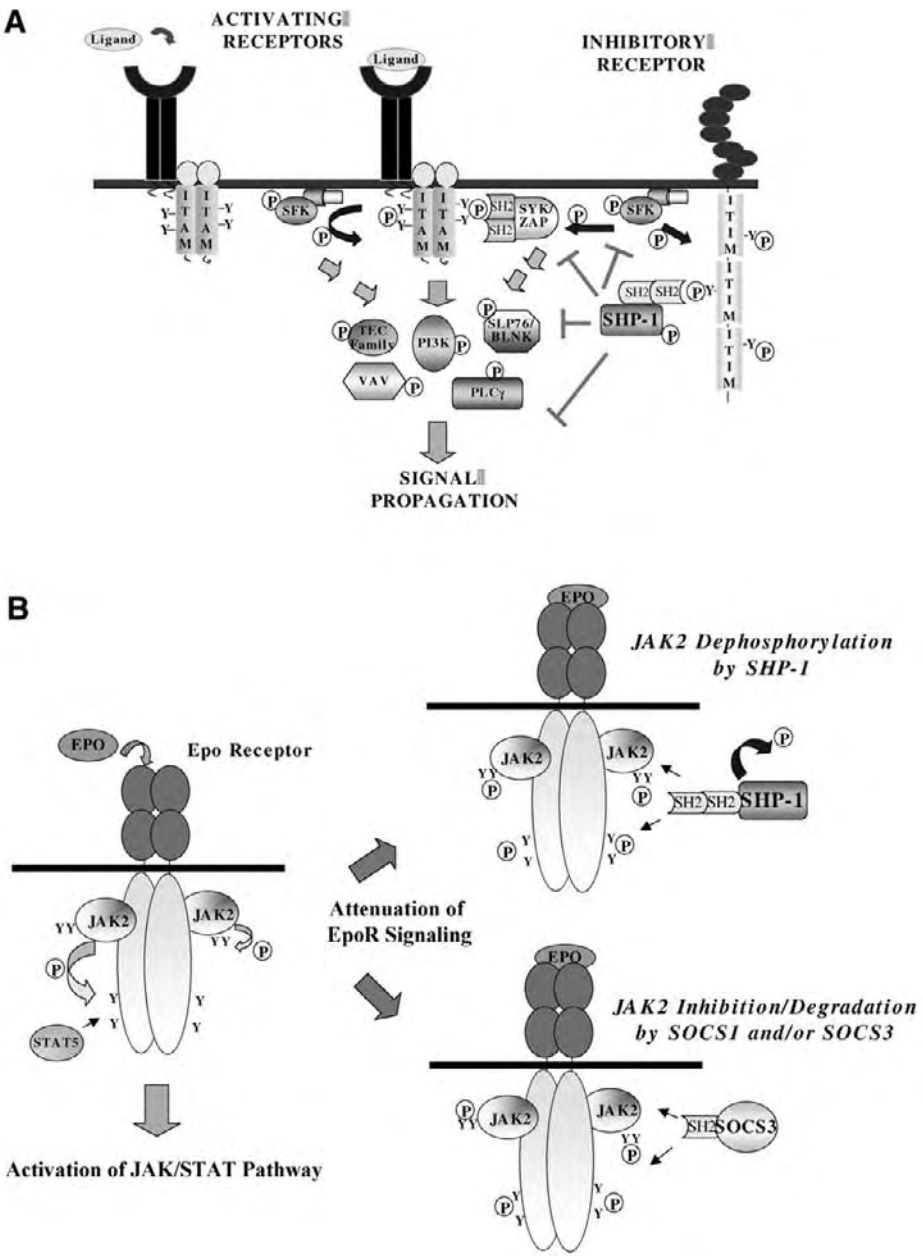


Figure 3 Signaling by Shp1. (A) Regulation of MIRR signaling by inhibitory receptor/Shp complexes. Shown is a typical activating multichain immune recognition receptor (e.g., TCR, BCR, FcR, activating NK receptor) and a typical inhibitory receptor. Upon cell activation, signals from the activating MIRR result in tyrosyl phosphorylation of the inhibitory receptor, which in turn recruits Shp1 (and possibly Shp2 and/or SHIP). Recruitment both localizes and activates the Shp, which then dephosphorylates one or more targets in the vicinity of the activating receptor complex. Possible direct targets are indicated; however, for most pathways, the direct substrates of Shp1 remain unknown or controversial. For many MIRR pathways, cross-linking of inhibitory receptors to activating receptors may be important for inhibitory signaling. (B) Regulation of cytokine signaling. Shown is a model of the EpoR, which couples to JAK2. Upon ligand binding, the receptor-associated PTK becomes activated and phosphorylates the receptor on multiple sites, including the binding site for Shp1. Shp1 may directly dephosphorylate JAK2, leading to its inactivation. However, SOCS proteins also are important for inactivating cytokine receptors. How Shps and SOCS proteins interact to effect negative regulation is unknown. Shown is a highly speculative model in which Shp1 and SOCS proteins might collaborate to negatively regulate cytokine receptor signaling. In this model, Shp1 is responsible for dephosphorylating the adjacent pY1008 on JAK2, thereby allowing an appropriate SOCS protein to bind at pY 1007. For details, see text.

For some RTKs and in some cell types, such as insulin-like growth factor 1 (IGF-1) signaling in fibroblasts, there is virtually no ERK activation in the absence of Shp2; in other RTKs, initial ERK activation is normal, but Shp2 is required for sustained signaling [89,90,127].

Although Shp2 binds directly to some RTKs (e.g., platelet-derived growth factor receptor [PDGFR]), in many other pathways, it binds to one or more scaffolding adapters [201]. For example, Shp2 binds to Gab1 upon EGFR, hepatocyte growth factor (HGF), and FGFR stimulation [202,203], to IRS family proteins following insulin/IGF stimulation [204], and to FRS2 (SUC1-associated neurotrophic factor [SNT]) downstream of the FGFR [205]. Studies using fibroblasts derived from Gab1$^{-/-}$ [206,207] and FRS-2$^{-/-}$ [208] mice and experiments with chimeric and mutant forms of scaffolding adapters [209,210] indicate that these scaffolding adapter/Shp2 complexes mediate the effects of Shp2 on ERK activation, at least in some RTK pathways. Genetic analysis of the DER and EGL-15 pathways suggests similar roles for Dos/Csw [124] and Soc-1/Ptp-2 complexes [125].

Despite intensive study, how Shp2 mediates ERK activation remains incompletely understood (Fig. 4A). Cells expressing dominant-negative Shp2 [211] or Ex3$^{-/-}$ fibroblasts [89,134] show defective Ras activation in response to multiple RTKs, indicating that Shp2 acts upstream of Ras. Initial work suggested an "adapter" model, in which Shp2 becomes tyrosyl phosphorylated in response to RTK stimulation and then binds Grb2/SOS [22,212,213]. Although this mechanism may contribute to Ras activation in some RTK pathways, it is unlikely to be generally required, as Shp2 is not tyrosyl phosphorylated in all RTK signaling pathways. Studies of *Xenopus* [214] and *Drosophila* [215] showed no absolute requirement for the C-terminal tyrosyl residues in Sevenless and XFGFR signaling, respectively; an important caveat is that these studies involved overexpression of mutant forms of Csw/Shp2.

Instead, multiple studies indicate that the PTP activity of Shp2 is vital for its positive signaling function. Shp2 dephosphorylates the RasGap binding site on the PDGFR preferentially, which suggested a model in which RasGap is recruited precociously to the PDGFR in the absence of Shp2, thereby limiting Ras activation [216]. Similar findings were reported in studies of Torso (which is structurally related to the PDGFR) signaling [217]. Although this model and these observations are attractive, they cannot provide the entire explanation. Dos (*daughter of sevenless*), a *Drosophila* Gab ortholog, is required for embryogenesis, presumably in Torso signaling, and Csw binding to Dos appears to be essential for this function [218–220]. Gab1$^{-/-}$ fibroblasts and Shp2 Ex3$^{-/-}$ fibroblasts [89,90] also have defective PDGF-evoked Erk activation [206]. Thus, mere recruitment of Shp2/Csw to PDGFR/Torso (and consequent regulation of RasGap binding) cannot account for the role of Shp2 in Ras activation.

One can imagine three general models by which Shp2, acting via scaffolding adapter/Shp2 complexes, might signal to Ras. Shp2 could regulate the phosphorylation of another site(s) on the scaffolding adapter, controlling binding of an SH2 or PTB domain protein. Alternatively, Shp2 could regulate the phosphorylation of a protein that binds to the scaffolding adapter in either a pTyr-dependent or -independent manner. Finally, the scaffolding adapter could merely target Shp2 to where the substrate resides, presumably a membrane compartment given that scaffolding adapters have membrane-targeting modules such as pleckstrin homology (PH) domains.

Recent data strongly support the third model. The only Tyr residues on Dos required to rescue Dos loss-of-function mutants are the Csw binding sites, thus arguing strongly against Dos itself being the relevant substrate. A Gab1–Shp2 fusion protein can lead to growth-factor-independent ERK activation in mammalian cells and potentiates activation in response to ligand [221]. Fusion of the Gab1 PH domain alone (or several other membrane-targeting sequences) to the PTP domain has a similar effect [221]. Ligation of the N terminus of Src to Csw also produces a gain-of-function mutant [123,124].

What, then, is the key substrate(s) that Shp2 (or its orthologs) must dephosphorylate to mediate Ras (and ultimately, ERK) activation? Cells expressing the Gab1–Shp2 fusion exhibit enhanced Src activity, and Src inhibitors block the ability of the fusion to activate ERK [222]. These findings suggest that Shp2 acts upstream of, and even directly on, Src in the Ras/ERK pathway, an attractive idea as SFKs have negative regulatory C-terminal tyrosyl phosphorylation sites. However, several lines of evidence raise questions about this simple model. First, receptor-like PTP α (RPTPα) is required for most SFK activation, at least in fibroblasts, and PTPα directly dephosphorylates SFK inhibitory Tyr residues [223,224]. It is not clear how Shp2 might fit into this scheme of Src regulation. Conceivably, Shp2 regulates a critical pool of SFKs (or specific SFKs), amounting to only a fraction of total cellular SFK, with PTPα regulating the rest. An intriguing alternative is that Shp2 regulates the recruitment of Csk (the kinase that phosphorylates Y527) to the membrane through PAG/CBP, a transmembrane phosphotyrosyl protein, which can bind Csk upon phosphorylation. The lack of sufficiently sensitive immunoreagents has prevented an adequate test of this possibility. A significant problem with any "Src activation" model of Shp2 action is that several studies have failed to find a role for SFK in RTK-evoked ERK activation [88,225]. Thus, the effects of the Gab–Shp2 fusion on Src activation may not reflect the normal role of Gab1/Shp2 complexes in RTK-evoked ERK activation, but rather an artificial gain of function. It would be interesting to know whether tethering of other PTPs has similar effects on Src and ERK activity in this system.

Although Shp2 clearly regulates Ras/ERK activation, genetic and biochemical evidence indicates other key roles in RTK signaling (Fig. 4A). In the Torso pathway, Csw mutations have far more severe effects than mutation of either Drk (Grb2) or Ras [226]. Epistasis analysis places Csw both upstream and downstream of Raf or in a parallel pathway in Sevenless signaling [123], as well as upstream and downstream of (or parallel to) Ras in the DER pathway [124].

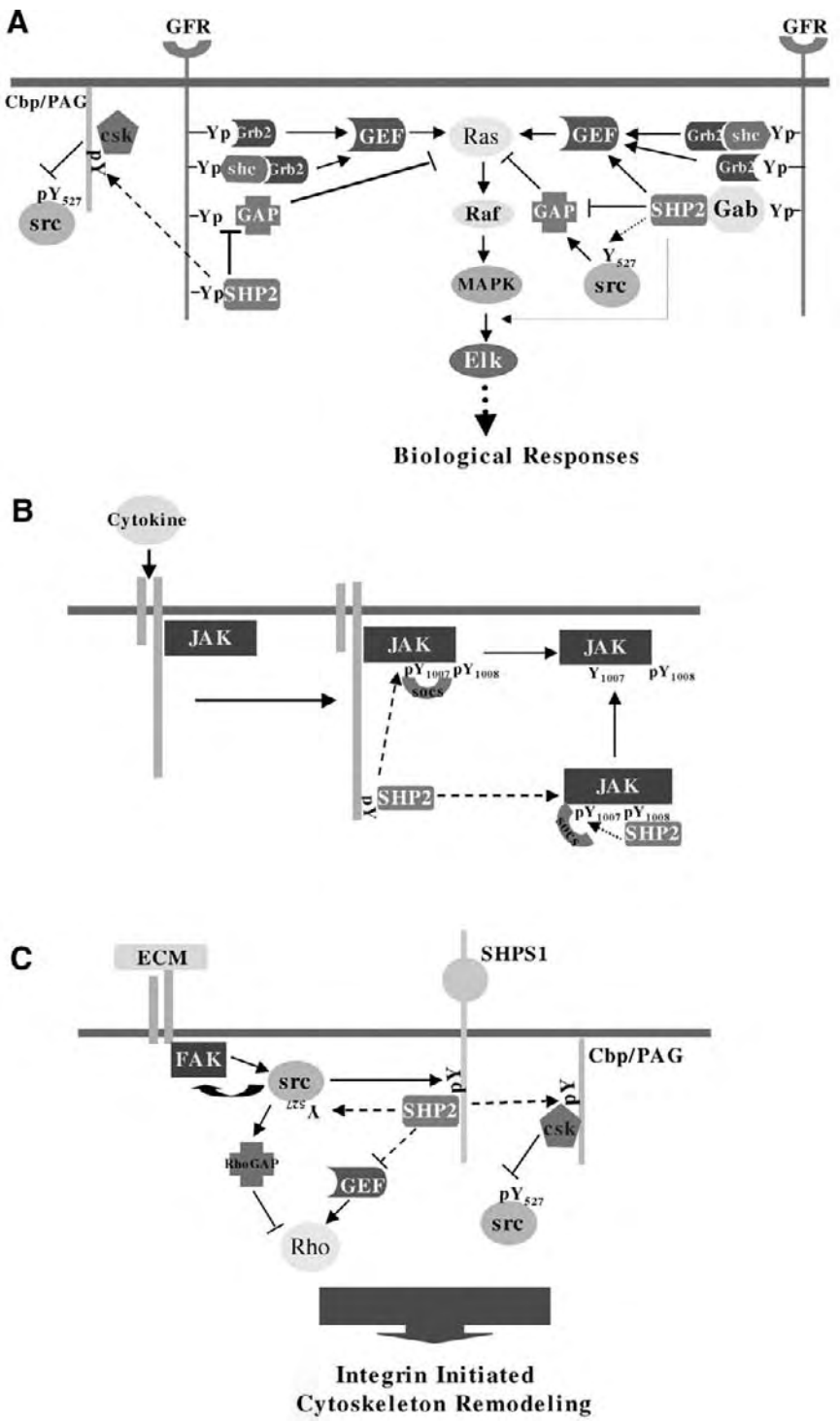


Figure 4 Signaling by Shp2. (A) Role in RTK signaling. Shp2 (and its orthologs) can be recruited directly to some receptors, such as the PDGFR (and Torso). Part of its positive signaling role may involve preventing dephosphorylation of RasGap binding sites until appropriate signaling has occurred. Shp2 also binds to several scaffolding adapters, such as Gab/Dos, IRS, and FRS-2, and genetic and biochemical studies indicate that scaffolding adapter/Shp2 complexes are critical for the ability of Shp2 to fully activate Ras, even in PDGFR and Torso signaling. The precise targets that Shp2 must dephosphorylate to mediate Ras activation are unknown, as is whether adapter/Shp2 complexes act upstream of Ras exchange factors or RasGap, or both. Potential direct targets include SFK inhibitory tyrosyl residues or the Csk binding partner PAG/CBP, but none of these has been shown convincingly to be a target. In addition to its role upstream of Ras activation, substantial evidence suggests that Shp2 also is required for some downstream (or parallel) function needed for transcription factor activation. Studies of Csw suggest that, besides its likely function in Ras activation, it may regulate nuclear transport of ERK via the importin β homolog, Dim-7. For details, see text. (B) Role in cytokine signaling. In cytokine signaling pathways, Shp2 may function analogously to its role in RTK signaling. A highly speculative alternative, however, is that Shp2 acts on pY1007 to regulate SOCS protein recruitment and consequent degradation. Note that this model is the converse of that in Fig. 3B. (C) Role in integrin signaling. Upon adhesion to appropriate ligands, Shp2 is recruited to tyrosyl phosphorylated Shps1. Shp2 is required for normal integrin-evoked ERK activation and for control of the cytoskeleton and cell motility. The latter may be mediated via its ability to regulate the small GTPase Rho. However, the extent to which Shps1 participates in each of these signaling pathways remains unclear. Also, unclear are the direct targets of Shp2 in integrin signaling, although several candidate proteins are shown.

The latter function may involve regulation of nuclear import, as Csw associates with the importin β ortholog Dim-7, which controls nuclear transport of the ERK ortholog Rolled [227]. This notion is consistent with the studies of the role of Gab2/Shp2 complexes in cytokine signaling [36]. In some RTK (e.g., PDGFR, IGFR, FGFR) pathways, Shp2 is required for PI3K activation [90,228]. However, in the EGFR pathway, Shp2 has the converse role, negatively regulating PI3K activation by dephosphorylating the PI3K binding sites on Gab1 [90]. Shp2 mutant fibroblasts also exhibit increased c-Jun N-terminal kinase (JNK) activation in response to a variety of stress stimuli [89]. Further studies are required to unravel the molecular details of these actions of Shp2.

SHP2 IN CYTOKINE SIGNALING

Multiple studies also indicate that Shp2 is important for Erk activation in response to a variety of cytokines. Most workers probably assume that Shp2 has a similar mechanism of action (i.e., acting upstream of Ras) and similar targets in cytokine receptor and RTK signaling. However, the possibility of a unique action in cytokine signaling should not be excluded. For example, as indicated above, Janus PTKs undergo dual phosphorylation within their activation loops, with one site (Y1007 in JAK2) being required for binding to SOCS proteins (and thus for degradation). Conceivably, Shp2 might dephosphorylate Y1007, thereby preventing premature access of SOCS proteins to this site (Fig. 4B).

Although there is virtual agreement that Shp2 is a positive regulator of cytokine-evoked ERK activation, its role in cytokine-evoked STAT activation is controversial. Studies using dominant-negative mutants and STAT-dependent transcriptional reporters indicate that Shp2 is required for STAT activation [36,229,230]. Subsequent studies, most of which examined the effects of mutant cytokine receptors unable to bind Shp2, reached the opposite conclusion; namely, that Shp2 negatively regulates the STAT pathway, either by dephosphorylating or inactivating (not activating, as in the model described earlier) Janus PTKs, STAT proteins, and/or STAT binding sites on the receptors themselves [231–234]. The discovery that Shps and SOCS proteins bind to the same sites on several cytokine receptors complicates interpretation of these mutant receptor studies. Nevertheless, interferon α/β (IFN-α/β)-evoked STAT activation is enhanced in immortalized fibroblasts from $Ex3^{-/-}$ mice, consistent with a negative regulatory role for Shp2 on the JAK/STAT pathway [235]. Conceivably, Shp2 might have positive effects on some Janus PTKs and negative effects on others, or it could act on more than one target in cytokine receptor signaling.

SHP2 IN INTEGRIN SIGNALING

Studies of $Ex3^{-/-}$ fibroblasts [236,237] and with dominant negative mutants [237–239] established a role for Shp2 in integrin signaling. Shp2 function is required for integrin-evoked cell spreading, migration, and ERK activation, and Shp2-deficient cells exhibit increased stress fibers. Consistent with the latter, recent studies indicate that activation of the small G protein Rho, which controls stress fiber formation [240], is enhanced in $Shp2^{-/-}$ fibroblasts [241].

How Shp2 mediates these effects also remains unclear. Shp1 becomes tyrosine phosphorylated following integrin activation, probably by one or more SFKs, and recruits Shp2 [33,237,242], raising the possibility that the effects of Shp2 on adhesion are mediated by Shp1/Shp2 complexes (Fig. 4C). However, studies of fibroblasts from mice lacking the Shps1 cytoplasmic domain reveal a more complicated picture. Whereas Rho activation is enhanced in Shp2 mutant cells, it is inhibited in Shps1 mutants, despite the fact that both types of cells have increased stress fibers and defective migration [155]. The effect of Shp2 on early events in integrin signaling is controversial. Whereas some workers report no effect of dominant-negative Shp2 on FAK tyrosine phosphorylation [239], others have found that phosphorylation is enhanced [238]. Studies on Shp2 mutant fibroblasts also disagree, with one report showing that Shp2 is required for normal integrin-evoked Src and FAK phosphorylation [237], whereas another found no effect on integrin-evoked FAK phosphorylation but a decrease in the rate of FAK dephosphorylation upon de-adhesion [243]. It is unclear whether these discrepancies reflect differences in experimental details and design, reagents, or experimental systems. An interesting possibility is that Shp2 helps to integrate some RTK/integrin signals, as stimulation of the RTK EphA2 was reported to cause Shp2-mediated FAK dephosphorylation and inhibit integrin function [244].

SHP2 IN ANTIGEN RECEPTOR SIGNALING

Upon TCR activation, Shp2 becomes tyrosyl phosphorylated and associates with the scaffolding adapter Gab2 [36,245–247]. Shp2 tyrosyl phosphorylation and association with Gab1 have been reported in B cells [248], although Shp2/Gab2 complexes also form in some B-cell lines [36]. The functional consequences of these interactions remain somewhat unclear. Stable expression of dominant-negative Shp2 in a T-cell line inhibits ERK activation [246]; however, overexpression of Gab2 inhibits TCR-evoked activation of ERK-dependent and nuclear factor of activated T cells (NFAT)-dependent reporters [249,250]. One group found that only the PI3K binding sites were required for this inhibitory effect [249], whereas another observed a requirement for both Shp2 and PI3K binding [250]. Studies of the effects of Gab family-deficient B and T cells should help to resolve these issues.

Shp2 also may mediate some inhibitory receptor signals. Shp2 was reported to bind the CD28 family member CTLA-4 and dampen TCR activation, most likely by dephosphorylating TCR-associated kinases [50]. The purported binding site for Shp2 on CTLA-4 is quite atypical, raising questions as to whether Shp2 binds directly to CTLA-4. Moreover, subsequent studies indicate that the Shp2 binding site is dispensable for CTLA-4-mediated inhibition [251–253]. The inhibitory receptor PD1 contains two potential ITIMs, and

a chimera between the extracellular domain of FcγRIIB and the intracellular domain of PD1 inhibits BCR activation upon co-crosslinking. Shp2, but not Shp1 or SHIP, binds the chimeric receptor, implicating Shp2 as the mediator of inhibitory signaling [254]. Because Shp2 binding sites can overlap with those of other inhibitory molecules, such as SOCS proteins, confirmation using dominant-negative, RNAi, or B-cell-specific knockout approaches is desirable. Shp2 also is implicated in inhibitory signaling by CD31 (PECAM-1) in B cells [255] and platelets [256]. Finally, Shp2 binds to several other inhibitory receptors, most of which also bind Shp1 [31]. Whether or not Shp2 has a role in mediating these inhibitory signals remains unknown.

Determinants of Shp Specificity

Shp1/Shp2 chimeras have been used to probe the determinants of Shp specificity in *Xenopus* [214] and mammalian cell [257] systems. These studies show that both the SH2 and the PTP domains contribute, and, surprisingly, the PTP domain contains the more critical specificity determinants. Even more surprisingly, the C-tails, which differ the most among Shps, do not confer specificity, at least in these systems. This suggests a combinatorial model of Shp specificity, whereby the SH2 domains direct Shps to appropriate cellular locations and the PTP domain then selects appropriate substrates.

Recent work has begun to address how the PTP domain selects substrates. Only the PTP domain of Shp1 has been co-crystallized with relatively "good" and "poor" substrate peptides [258,259]. These structures suggest that the α0 helix and the loop α1/β1, α5–loop–α6, and β5–loop–β6 motifs make contacts with bound peptides. The residues in the α1/β1 motif involved in binding are identical in Shp1 and Shp2, whereas the two Shps have only relatively minor differences in the β5–loop–β6 and α5–loop–α6 motifs. Thus, the more divergent α0 helices may be of greatest importance in determining differences in substrate specificity.

There are several caveats to this interpretation. First, these studies predict that hydrophobic residues should be preferred at the Y−2 position, which conflicts with combinatorial library approaches that assigned acidic amino acids as the preferred Y−2 residues [260]. Second, the WPD loop, which contains the general acid critical to all PTP catalysis [261], remained open in these co-crystals, raising questions as to whether they represent authentic views of enzyme/substrate complexes. Third, the enzyme–peptide contacts extend at least four residues to either side of the pTyr; conceivably, additional interactions important for specificity may extend even further and thus would have been missed. Finally, it remains possible that specificity is not due to *bona fide* differences in substrate preferences between Shp1 and Shp2, but instead reflects the higher intrinsic catalytic rate of the Shp1 PTP domain [262–265]. Additional structural, biological, and biochemical studies will be required to resolve these issues.

Shps and Human Disease

The most exciting recent discovery about Shps has been the finding that Shp2 mutations occur in ≈50% of the cases of Noonan syndrome (NS) [266]. NS is a fairly common (incidence ≈1 : 2000 births), autosomal-dominant disorder characterized by abnormal facies featuring a broad forehead, downward-slanting palbebral (eyelid) fissues, low-set ears, and hypertelorism (abnormal distance between paired organs, such as the eyes); a webbed neck resembling that observed in Turner syndrome; proportionate short stature; and cardiac abnormalities, most frequently pulmonic stenosis and hypertrophic cardiomyopathy. Chest and spine deformities, mental retardation, delayed puberty, cryptorchidism, and bleeding diathesis occur with variable penetrance [267–270]. Also, case reports have noted a possible association between NS and increased incidence of malignancy, notably leukemia [271–274].

Nearly all NS mutations are found in either the N-SH2 or PTP domains and involve residues that participate in basal inhibition of PTP activity (Fig. 2B). For example, one NS mutation is D61G, and another is E76D; by means of biochemical and biological assays, quite similar mutations (D61A, E76K) were shown to be activating mutants [71]. Together with molecular modeling studies [266], and the autosomal dominant inheritance pattern, these results strongly suggest that activating mutations of Shp2 are the cause of a substantial number of NS cases.

Recently, other NS-associated mutations have been identified that fail to conform to this simple model [275]. One (D106A) occurs in the linker between the N-SH2 and C-SH2 domains; this mutation might kink the N-SH2, thereby disrupting its ability to mediate basal repression and resulting in a novel type of activated mutant. Two other mutations—one in the N-SH2 (T42A), the other in the C-SH2 (E139D)—are more difficult to explain. Both involve residues in the SH2 domain pTyr-peptide-binding pocket. Conceivably, T42A mimics N-SH2 domain engagement by a pTyr peptide and thereby prevents binding of the backside of the N-SH2 to the PTP domain. However, the C-SH2 does not make direct contact with the catalytic cleft, and the role of the C-SH2 domain in direct enzyme activation remains controversial. If, as suggested [73], phosphorylated Y580 can activate Shp2 by engaging the C-SH2, E139D might act as if it is similarly engaged. Additional enzymatic and biological studies are required to resolve this issue.

The precise mechanisms and pathways by which Shp2 mutations cause the various NS abnormalities remain to be elucidated, but previous studies yield several clues. The cardiac abnormalities, particularly the valve defects, probably stem from abnormal regulation of the EGFR (and other EGFR family) signaling pathways [276], whereas the facial and skeletal abnormalities are reminiscent of activating mutations in FGFR signaling pathways [277]. Proportionately shortened stature may be due to defective GH and/or IGF-1 signaling. The origins of the cognitive and coagulation abnormalities are less clear and may reflect the role of Shp2

in several signaling pathways. Modeling NS by gene knock-in approaches should help to resolve these issues.

The genetic basis of the other 50% of NS cases remains to be defined. At least some cases of NS exhibit autosomal recessive inheritance [278]. Others are autosomal dominant but unlinked to the Shp2 locus [275]. Clues to the identity of some of these may be provided by rare chromosomal translocations that have been associated with NS [279]. Identifying these genes is of great importance, as they may encode key Shp2 substrates and regulators. However, recent studies indicate differences between the spectrum of abnormalities in NS patients with and without Shp2 mutations [275], so it remains possible that cases of NS caused by mutations in genes other than Shp2 actually represent a different disorder.

Inappropriate activation of Shp2 also appears to be important in bacterial pathogenesis. *Helicobacter pylori*, the major cause of gastric ulcer and carcinoma worldwide, encodes a number of virulence determinants, one of which, CagA, becomes tyrosyl phosphorylated in infected cells. Recent studies indicate that tyrosylphosphorylated CagA recruits and activates Shp2. Shp2 binding is essential for the morphological effects of CagA on gastric epithelial cells, which are similar to the effects of HGF. Remarkably, previous studies established a critical role for Gab1/Shp2 binding in mediating the morphogenetic effects of HGF in epithelial cells [209, 210]. It remains to be seen whether overexpression of scaffolding adapters that bind Shp2 or somatic activated mutants of Shp2 are involved in *H. pylori*-negative gastric cancers and/or other malignancies.

At least one Shp2-binding protein probably does play an important role in human disease; Gab2 is a critical determinant of the lineage (myeloid vs. lymphoid) and severity (latency) of Bcr-Abl-evoked leukemia [280]. Gab2$^{-/-}$ cells expressing Bcr-Abl exhibit defective activation of the PI3K/Akt and ERK pathways; although the former probably reflects Gab2 binding to PI3K, defective ERK activation may be due to failure to recruit Shp2. Because Gab2 is overexpressed in a significant number of breast cancers [281], it may play a wider role in carcinogenesis.

The jury remains out on whether Shp1 deficiency plays a causal role in human disease. EpoR mutations that result in loss of Shp1 binding are associated with familial erythocytosis, but it is unclear whether loss of Shp1 binding (or Shp1 binding alone) is important. Shp1 deficiency was implicated in the pathogenesis of polcythemia vera [282], but these findings are in dispute [283]. Markedly decreased Shp1 expression has been reported in human lymphoma [284], HTLV-1-induced leukemia [191], and Sezary syndrome [285], suggesting a possible role for Shp1 in suppressing these malignancies. The mechanism and physiological consequences of decreased expression are not known.

On the other hand, several human pathogens may hijack Shp1 to help suppress the host immune response and/or to effect tissue damage. Binding of Shp1 to CD46, the measles virus receptor, in macrophages, is associated with the production of IL-12 and nitric oxide [286]. The Opa52 protein produced by *Neisseria gonorrheae*, binds to CEACAM (CD66a) on primary CD4$^+$ T cells. This leads to recruitment of Shp1 and Shp2 and suppression of T-cell activation and proliferation [287]. Finally, elevated levels of Shp1 and Shp2 are associated with congenital neutropenia [288], although whether or not the Shps play a causal role in this disorder remains unclear.

Summary and Future Directions

Substantial progress has been made since the discovery of Shps in the early 1990s. We know many of the pathways in which Shps participate and the biological consequences of loss-of-function and, in the case of Shp2, gain-of-function mutations in Shps. Shp structures have been solved to atomic resolution, and the basics of regulation of Shp activity are well understood. Still, many important questions remain. Chief among these is the identification of the proximate targets of each of the Shps. We also do not understand Shp specificity in detail, and questions remain about the role of phosphorylation and, indeed, the C-tail (and, for Csw, the PTP insert) domain in general. Finally, it remains to be seen whether mutations in human Shps themselves or in Shp binding proteins other than Gab2 play causal roles in diseases such as autoimmunity and cancer. With the availability of powerful technologies such as inducible and tissue-specific knockout mice, RNAi, and substrate-trapping mutants, answers to these and other questions about the Shp subfamily should be found soon.

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CHAPTER 119

Insulin Receptor PTP: PTP1B

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Introduction

The dramatic increase in obesity and diabetes mellitus is reaching epidemic proportions and emphasizes our need to understand the mechanisms that coordinate body composition and energy metabolism. In humans, diabetes is characterized by an inability to maintain glucose homeostasis due to perturbations in insulin secretion (Type I) and/or signaling (Type II). Over 90% of diabetic patients exhibit resistance to insulin action, and it is well accepted that the molecular defect for this occurs at a level of postreceptor signaling [1]. The insulin receptor (IR) is a transmembrane tyrosine kinase that becomes activated upon ligand binding. This leads to subsequent tyrosine phosphorylation of its substrates, which in turn activate downstream signaling cascades [2]. Although several mechanisms for attenuation of insulin signaling are known, it has long been suggested that protein tyrosine phosphatases (PTPs) that dephosphorylate the IR play a key role in this aspect [3]. One such PTP, protein tyrosine phosphatase 1B (PTP1B), is well established in the insulin signaling pathway and has attracted immense interest as a target for pharmaceutical companies. This review focuses on the biological function of PTP1B, its known modulation, the substrates that it recognizes, and the signaling pathways that it controls.

PTP1B as a *Bona Fide* IR Phosphatase

PTP1B is the prototype for the superfamily of PTPs and was initially thought to indiscriminately dephosphorylate phosphorylated tyrosine residues (a housekeeping function) [4]. Although it is widely expressed in most cell types, it is one of the few identified PTPs found in the major tissues controlling insulin-mediated glucose metabolism (liver, muscle, fat). It has long been known that PTP1B can dephosphorylate the activated IR and attenuate insulin signaling and its biological effects [3]. Within the IR, three tyrosine phosphorylation sites (pTyr1146, 1150, 1151) are involved in the binding to PTP1B [5–7], although structural and kinetic studies suggest that PTP1B has a preference for the tandem tyrosine phosphorylated motif (pTyr1150, pTyr1151) [8]. One issue that remains is where the action of PTP1B actually occurs within the cell. Although PTP1B is predominantly localized to the endoplasmic recticulum (ER) and the IR at the plasma membrane, recent data suggest that its action on receptor protein tyrosine kinases requires endocytosis of the receptors to intracellular sites that coincide with ER markers [9].

The *in vivo* confirmation for the role of PTP1B in insulin signaling was first established with knockout mice [10,11]. Evidently, PTP1B is not required for embryonic development, and no gross histological abnormalities have been observed in PTP1B-deficient mice. However, loss of PTP1B results in increased insulin sensitivity. PTP1B-deficient mice maintain moderately lower glucose levels at half the circulating insulin levels, compared to wild-type controls. Loss of PTP1B also results in the ability to maintain lower glucose levels during an insulin or glucose-tolerance test. This increased insulin sensitivity is even more evident when PTP1B-deficient mice are challenged to an insulin-resistant state by a high-fat diet, indicating that PTP1B plays a role in insulin resistance. Thus, PTP1B could be a promising target for the therapeutic treatment of Type II diabetes.

At the molecular level, PTP1B-deficient mice display an enhanced and/or prolonged ligand-dependent IR phosphorylation in liver and muscle tissues. Interestingly, this increased IR phosphorylation has not been observed in fat tissue, suggesting that other PTPs may play a crucial role in IR dephosphorylation there. Perhaps regulation of insulin signaling by PTP1B is tissue specific. For example, in 3T3-L1 adipocytes, over-expression of PTP1B does not affect

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insulin-stimulated Akt activation or glucose uptake [12], whereas over-expression of PTP1B in L6 myocytes and Fao hepatoma cells attenuates insulin-induced Akt activation and glycogen synthesis [13].

Widespread genetic ablation of PTP1B in mice unequivocally demonstrates the importance of this enzyme in insulin signaling and glucose homeostasis. However, an emerging and exciting concept is that central insulin signaling [14], in addition to peripheral insulin signaling, also plays a key role in whole body metabolism. Because PTP1B-deficient mice seem to display tissue-specific insulin specificity, it will be intriguing to see if PTP1B contributes to central insulin signaling as well.

PTP1B Gene Polymorphisms and Insulin Resistance

The human PTP1B locus maps to chromosome 20 in the region q13.1–q13.2 [15] and its mouse ortholog to the syntenic H2–H3 region of chromosome 2 [16]. Interestingly, this region was also identified as a quantitative trait loci (QLT) linked to insulin and obesity [17]. Consistent with a role for PTP1B in insulin resistance, single nucleotide polymorphisms have been found within the coding [18,19] or 3′ UTR region [20] that are associated with diabetic parameters.

Insulin-Mediated Modulation of PTP1B

Not only does PTP1B act on and modulate the IR, but recent evidence suggests that the reverse is also true. Insulin stimulates phosphorylation of PTP1B on both serine and tyrosine residues, although their effects on PTP1B are not entirely clear. In Rat1 fibroblasts over-expressing human IRs, insulin stimulation results in tyrosine phosphorylation of PTP1B on three sites (Tyr66, Tyr152, Tyr153) crucial for its binding to the IR [5]. Furthermore, this also increases the activity of PTP1B [21], thus providing a potential negative-feedback loop to prevent prolonged insulin signaling. On the other hand, insulin stimulation of muscle and fat tissue also results in PTP1B tyrosine phosphorylation; however, this seems to decrease PTP1B activity [22].

An insulin-mediated decrease in PTP1B activity also occurs via Ser50 phosphorylation by Akt and impairs its ability to dephosphorylate the IR [23]. PTP1B is also phosphorylated on other serine residues by enzymes such as protein kinase C (PKC) or protein kinase A (PKA) [22,24], thus it will be intriguing to discover how both tyrosine and serine phosphorylation regulate PTP1B in a temporal fashion.

Reversible oxidation of the invariant cysteine in the catalytic center of PTPs is also becoming an emerging theme for their regulation. In fact, insulin-stimulated hydrogen peroxide production is thought to temporarily inactivate PTP1B to promote insulin signaling in 3T3-L1 adipocytes [25].

Genetic Evidence for Other PTP1B Substrates

From biochemical and cell culture studies, many potential PTP1B substrates have been identified; however, only a few to date have been found to be hyperphosphorylated in PTP1B-deficient cells, suggesting that only a subset may represent important PTP1B substrates. These are described in brief below.

Src

In addition to the IR, the adaptor protein p130Cas was one of the first candidate substrates for PTP1B [26], implicating the phosphatase in both integrin signaling [27] and transformation [28]. However, p130Cas is not hyperphosphorylated in PTP1B-deficient fibroblasts during fibronectin signaling, thus raising the possibility that this function might be redundant or nonphysiological. Nevertheless, at least in immortalized cells, PTP1B does seem to be a positive regulator of fibronectin signaling, possibly via dephosphorylation of the inhibitory site (Tyr527) of Src [29]. This is, in fact, in agreement with the previous studies in L cells [30] and breast cancer cell lines [31].

IGF-IR

Previous studies have implicated PTP1B in insulin-like growth factor I (IGF-I) signaling. By virtue of similarity between the IR and IGF-IR, this seems quite expected. In particular, the tandem-phosphorylated tyrosines found in the IR are also found in the IGF-IR. Indeed, loss of PTP1B in immortalized fibroblast cells leads to increased IGF-I-mediated receptor phosphorylation, Akt activation, and increased cell survival under apoptotic stress [32]. Interestingly, though, IGF-I-mediated Erk activation is significantly diminished in the absence of PTP1B, despite the enhanced IGF-IR phosphorylation and Akt activation. One possibility is that the positive effects of PTP1B on Src may contribute to this impaired Erk activation. It is also possible that PTP1B may regulate other pathways leading to Erk activation that have yet to be identified.

JAK2

PTP1B-deficient mice are resistant to diet-induced obesity and display lower leptin levels than their wild-type counterparts. Leptin is a peptide hormone that regulates adiposity primarily by inhibiting food intake and increasing energy expenditure. Despite the fact that PTP1B-deficient mice exhibit lower leptin levels, their food intake is not dramatically affected, suggesting that perhaps they present enhanced leptin sensitivity. Indeed, preliminary results suggest that PTP1B-deficient mice are more sensitive to the effects of exogenously administered leptin [33,34]. This provides one mechanism for the obesity resistance of the PTP1B knockout mice and suggests that PTP1B would also be a likely target for treating obesity due to leptin resistance.

Based on the findings that PTP1B preferentially acts on the tandem phosphorylated tyrosines on the IR, it was soon realized that there might be additional proteins with a similar PTP1B-binding site. Indeed, both Janus tyrosine kinase 2 (JAK2) and tyrosine kinase 2 (TYK2), which possess tandem tyrosine-phosphorylated residues, were found to be hyperphosphorylated in PTP1B-deficient fibroblasts upon interferon stimulation [35]. At the same time, using the PTP1B knockout mice, we and others independently found that JAK2 is a key substrate whereby PTP1B negatively regulates leptin signaling [33,34].

Concluding Remarks

Several laboratories have now documented multiple physiological PTP1B substrates, from the initial hypothetical housekeeping function in cells to unique IR dephosphorylation. PTP1B appears to modulate a class of substrates that preferentially but not exclusively include tandem tyrosine-phosphorylated proteins. The substrate specificity of PTP1B can therefore be thought of as a combination of its catalytic domain structure and the potential contribution of its proline-rich domains to bind associated-proteins, as well as spatial and temporal regulation. It is intriguing that PTP1B controls signaling pathways downstream of two major regulators of metabolism (i.e., insulin and leptin). Yet, the PTP1B-deficient mice are well and thriving. One potential view could be that this enzyme is part of a core of signaling molecules that are responsible for sensing and responding to basic metabolic survival needs. A potential model could be that, in a stress period, an increase in expression (or activation) of PTP1B would be protective and contribute to survival. For example, it stands to reason that in a period of food deprivation, PTP1B activity would be extremely useful. By its action on the IR, animals decrease their glucose uptake and thus prevent hypoglycemia. Similarly, during starvation conditions, PTP1B action on leptin signaling would reinforce hunger and the need to seek food. These phenomena are reversed in the PTP1B-deficient mice. Together, they provide us with a glimpse of the complex signaling events that must be investigated in order to understand all the prospective benefits of PTP1B inhibitor therapies.

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CHAPTER 120

Low-Molecular-Weight Protein Tyrosine Phosphatases

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Introduction

The eukaryotic low-molecular-weight protein tyrosine phosphatases (LMW PTPases) are cytoplasmic enzymes with molecular weights of approximately 18,000. This family of PTPases shares no sequence identity with the various high-molecular-weight (HMW) families except for the CXXXXXR signature sequence of the phosphate binding site (Fig. 1). In the HMW PTPases, the conserved signature sequence, HCXAGXGR(S/T), is typically found in the C-terminal third of the protein, while in the LMW PTPases the related invariant motif XCXGNXCRS is found close to the N terminus [1,2]. This motif forms the phosphate binding loop (P-loop). The P-loop peptide backbone in LMW PTPase has effectively the same structure as that found in a representative HMW PTPase from *Yersinia*, with Cα positions exhibiting only 0.37-Å root mean square deviation [3,4]. Thus, despite apparent differences in the consensus sequences, the phosphate binding regions of these proteins are surprisingly similar.

Bovine liver and brain and human red cell, liver, and placenta enzymes were among the first to be extensively characterized. A LMW PTPase was partially purified from bovine liver [5] and characterized as an acid phosphatase due to its apparent optimal activity at pH 5 when assayed with *p*-nitrophenyl phosphate (*p*NPP). The LMW enzymes from bovine liver and heart and human placenta and liver were later purified to homogeneity and shown to exhibit catalytic activity *in vitro* toward phosphotyrosyl substrates [6–11]. The bovine enzyme was the first to be cloned and expressed [12]. The use of this clone opened the way to a critical advance when the human placenta and red cell isoenzymes (HPTP-A and -B) were purified, cloned, sequenced, and shown to be identical and expressed in all human tissues [13]. The enzyme was also shown to be active against several phosphotyrosyl substrates [11,14]. Subsequently, many other putative LMW PTPases from eukaryotic and prokaryotic organisms have been identified. The predicted proteins generally share a high level of amino acid sequence identity in addition to the active site consensus sequence that is characteristic of LMW PTPases. However, reflecting recent rapid advances in DNA sequencing, many of the putative gene products have not been expressed, purified, and characterized.

The human red cell enzyme (red cell acid phosphatase) was the first enzyme shown to be genetically polymorphic and, as a result, the associated ACP1 gene and gene products assumed important roles as genetic markers [15,16]. The human enzyme and some other eukaryotic LMW PTPases were known to exist as isoenzymes that could be separated on the basis of their electrophoretic mobility [17]. The ACP1 gene has been localized to chromosome two (2p25) by fluorescence *in situ* hybridization (FISH), and the majority of the gene has been sequenced [18]. Two major human isoenzymes occur, and they differ only in the sequence of 34 amino acids from residues 40 to 73 (Fig. 2). The human LMW phosphatase gene is comprised of seven exons interrupted by six introns [19]. Two alternatively spliced exons, 114 bp in length, encode the 34-amino-acid variable region sequence. These exons are separated by a short 41-bp intron [18,20]. RNA blot hybridization analysis has shown that the human LMW PTPase is present in all human tissues, thus the name "red cell acid phosphatase" is inappropriate [13]. Two isoenzymes are also present in *Drosophila* retina and in chicken and rat brain, where they have been suggested to be associated with

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HPTPA	V	L	F	V	**C**	L	G	N	I	C	**R**	**S**	P
BPTP	V	L	F	V	**C**	L	G	N	I	C	**R**	**S**	P
LTP1	V	A	F	I	**C**	L	G	N	F	C	**R**	**S**	P
Erwinia	I	L	V	V	**C**	I	G	N	I	C	**R**	**S**	P
Human LAR PTPase	M	V	V	H	**C**	S	A	G	V	G	**R**	**T**	G
Human PTPase 1B	V	V	V	H	**C**	S	A	G	I	G	**R**	**S**	G
Human T-cell PTPase	A	V	I	H	**C**	S	A	G	I	G	**R**	**S**	G
Yersinia PTPase	P	V	I	H	**C**	R	A	G	V	G	**R**	**T**	A
Human cdc 25	I	V	F	H	**C**	E	F	S	S	E	**R**	G	P

Figure 1 Phosphate binding loop of several of LMW PTPases compared with other PTPases. The active site motif of the LMW PTPases is V/ICXGNI/FCRSP. The first Cys is the active-site nucleophile, Arg is essential for substrate binding and catalysis, Ser serves a role in stabilizing the thiolate form of the nucleophile, and the side chain of Asn plays a critical structural role in altering the main-chain conformation such that a backbone NH is pointed into the P-loop so that it can more effectively bind substrate and transition-state structures. In other PTPases, this function is served by the presence of a second glycine.

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BPTP        AEQVTKSV-LFV CLGNICRSP IAEAVFRKLVTDQNISDNW-    39
HPTPB       AEQATKSV-LFV CLGNICRSP IAEAVFRKLVTDQNISENW-    39
HPTPA       AEQATKSV-LFV CLGNICRSP IAEAVFRKLVTDQNISENW-    39
RATACP1     AEVGSKSV-LFV CLGNICRSP IAEAVFRKLVTDENVSDNW-    39
RATACP2     AEVGSKSV-LFV CLGNICRSP IAEAVFRKLVTDENVSDNW-    39
LTP1        TIEKPKISVAFI CLGNFCRSP MAEAIFKHEVEKANLENRFN    41
STP1        T---KNIQVLFV CLGNICRSP MAEAVFRNEVEKAGLEARFD    38

BPTP        VIDSGAVSDWNVGRSPDPRAVSCLRNHGINTAHKARQVTKE      80
HPTPB       VIDSGAVSDWNVGRSPDPRAVSCLRNHGIHTAHKARQITKE      80
HPTPA       RVDSAATSGYEIGNPPDYRGQSCMKRHGIPMSHVARQITKE      80
RATACP1     RIDSAATSTYEVGNPPDYRGQNCMKKHGIHMQHIARQITRE      80
RATACP2     AIDSSAVSDWNVGRPPDPRAVNCLRNHGISTAHKARQITRE      80
LTP1        KIDSFGTSNYHVGESPDHRTVSICKQHGVKINHKGKQIKTK      82
STP1        TIDSCGTGAWHVGNRPDPRTLEVLKKNGIHTKHLARKLSTS      79

BPTP        DFVTFDYILCMDESNLRDLNRKSNQVKNCRAKIELLGSYD       120
HPTPB       DFATFDYILCMDESNLRDLNRKSNQVKTCKAKIELLGSYD       120
HPTPA       DFATFDYILCMDESNLRDLNRKSNQVKTCKAKIELLGSYD       120
RATACP1     DFATFDYILCMDESNLRDLNRKSNQVKNCKAKIELLGSYD       120
RATACP2     DFATFDYILCMDESNLRDLNRKSNQVKNCKAKIELLGSYD       120
LTP1        HFDEYDYIIGMDESNINNLKKI--QPEGSKAKVCLFGDWN       120
STP1        DFKNFDYIFAMDSSNLRNINRV--KPQGSRAKVMLFGEYA       117

BPTP        PQK---QLIIEDPYYGNDADFETVYQQCVRCCRAFLEKVR       157
HPTPB       PQK---QLIIEDPYYGNDSDFETVYQQCVRCCRAFLEKAH       157
HPTPA       PQK---QLIIEDPYYGNDSDFETVYQQCVRCCRAFLEKAH       157
RATACP1     PQK---QLIIEDPYYGNDSDFEVVYQQCLRCCKAFLEKTH       157
RATACP2     PQK---QLIIEDPYYGNDSDFEVVYQQCLRCCKAFLEKTH       157
LTP1        TNDGTVQTIIEDPWYGDIQDFEYNFKQITYFSKQFLKKEL       160
STP1        SPG--VSKIVDDPYYGGSDGFGDCYIQLVDFSQNFLKSIA       155
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Figure 2 Amino acid sequence alignments of LMW PTPases from bovine, human (isoenzymes A and B), rat (isoenzymes 1 and 2), *Saccharomyces cerevisiae* (LTP1), and *Schizosaccharomyces pombe* (STP1). The shaded region corresponds to a 34-amino-acid segment that differs in human isoenzymes A and B and is the result of alternative mRNA splicing.

neurogenesis or synaptic function [21–23]. Other clinical and genetic studies suggest that the ACP1 gene locus may be associated with a number of developmental and hemolytic disorders, including hemolytic favism, megaloblastic anemia, Alzheimer's disease, and even malaria, but direct causal relationships have not been demonstrated.

Structures of LMW PTPases

The crystal structure of bovine protein tyrosine phosphatase (BPTP) has been determined to 1.9 Å, and the solution structure was simultaneously established by isotope-edited, multidimensional nuclear magnetic resonance (NMR) [24–27]. Crystal structures of the human isoenzyme A (the fast isoelectric form) and the *Saccharomyces* enzymes have also been published [28,29]. The structure of BPTP consists of a four-stranded central parallel β-sheet with α-helices flanking both sides (Fig. 3). The overall structure of BPTP resembles a classical dinucleotide binding or Rossmann fold, with two right-handed βαβ motifs. The variable region (which distinguishes the two human isoenzymes) exists as two extended loops connected by a short segment of α-helix. These loops wrap around the active site and appear positioned to play a role in substrate specificity.

The phosphate binding site of BPTP exists as a loop connecting the first β-strand and the first turn of the α-helix. The phosphate molecule sits at the center of this loop, positioned near the N-terminal end of α1, and embraced by the side chains of Cys 12 and Arg 18 (Fig. 4). Backbone and side-chain NHs from residues 13 to 18 provide multiple hydrogen bonds to each of the three oxygens of the phosphomonoester. These serve to tightly position the phosphate at the active site while the rigid geometry of the P-loop enforces even stronger hydrogen bonding interactions in the trigonal bipyramidal transition state [30]. Mutagenesis of Arg 18 to Ala completely abolishes catalytic activity, consistent with a critical electrostatic role [31,32]. The side chain of Cys 12 is directly centered between the three oxygens on the tetrahedral face of the phosphate ion, in an optimal position for an S_N2 displacement on phosphorus [12,24,31]. Figure 3 also shows the location of Asp 129, a catalytically essential general acid catalyst, at the entrance to the active site [33]. This group serves to protonate the leaving group oxygen.

The high-resolution structures of human, bovine, and yeast LMW PTPases show several extended hydrogen bond networks that participate in structural roles and in catalysis (Fig. 4). The conserved arginine (Arg 15), histidine (His 72), and two serines (Ser 19 and 43) are at or near the active site. His 72, Ser 19, and Ser 43 interact with the Asn 15 residue of the active-site loop. The interaction of these residues with Asn 15 stabilizes the left-handed α-helical conformation of the latter residue and modifies the structural orientation of the backbone so that the NH groups in the active site P-loop are oriented toward the phosphate ion. Asn 15, Ser 19, His 72, and to a lesser extent Ser 43 serve important structural roles

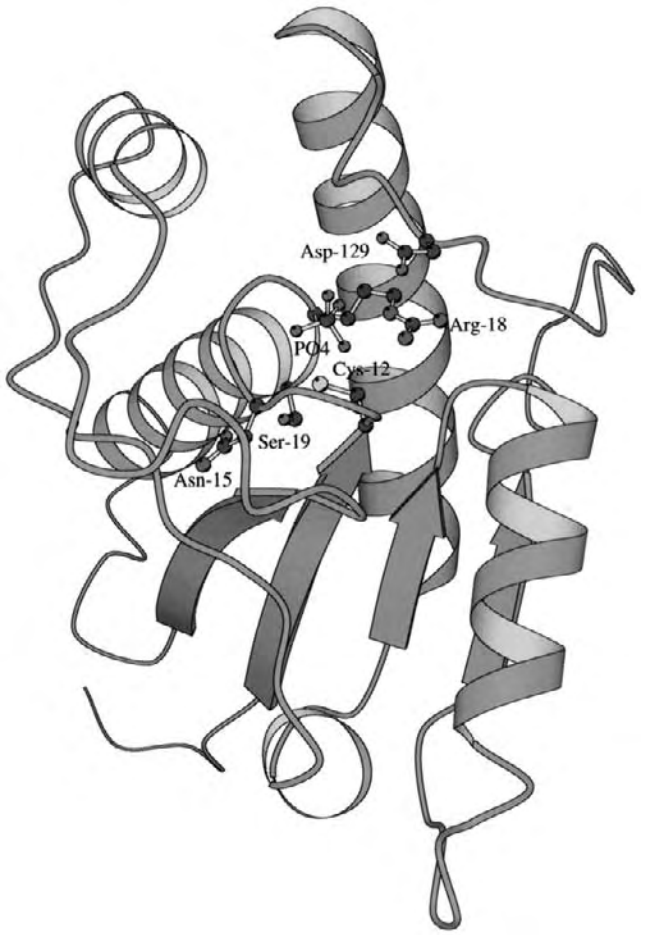


Figure 3 Ribbon diagram of bovine protein tyrosine phosphatase (BPTP). The structure of BPTP consists of a four-stranded central parallel β-sheet with α-helices flanking both sides [24]. A phosphate ion is shown at the active site, together with the locations of the active site general acid (Asp 129) and nucleophile (Cys 12). (Residue numbering is for the human and bovine enzymes.) The phosphate binding site (P-loop) is positioned at the N-terminal end of the prominent αα1-helix.

in stabilizing the geometry of the active-site loop region for optimal substrate binding and catalytic activity [34,35]. The hydroxyl group of Ser 19 is in very close proximity to Cys 12 (2.98 Å) and forms a hydrogen bond with the thiolate anion. Mutagenesis, pH dependence studies, leaving group dependence studies, partition experiments, and computational results all support the conclusion that the hydrogen bond to Cys 12 is a significant factor in causing the unusually low pK_a (<4) of this nucleophilic residue [32,35].

In solution and in certain crystal structures, the LMW PTPases can form dimers involving protein side chains located at the active site region [36,37]. The physiological significance of these interactions remains to be established.

Catalytic Mechanism

The chemical mechanism of catalysis by LMW PTPases has been studied extensively. Early experiments using chiral phenyl-[^{16}O, ^{17}O, ^{18}O] phosphate demonstrated that transfer to

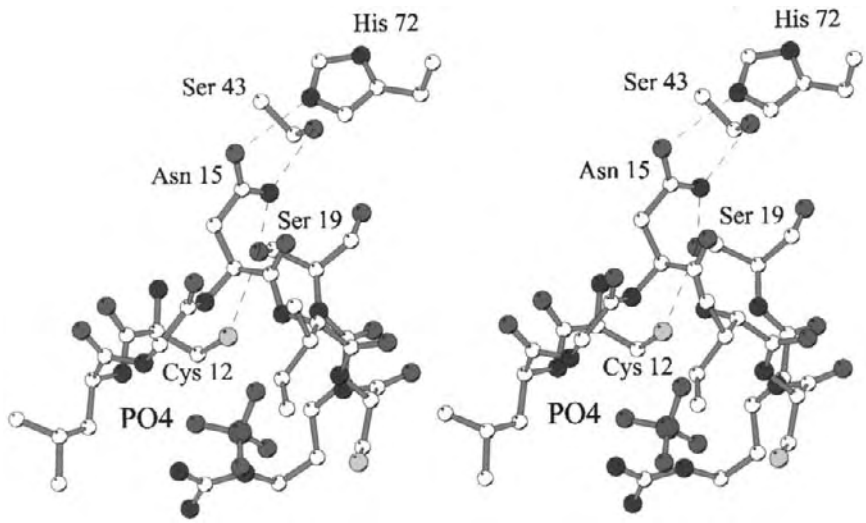


Figure 4 Phosphate binding loop of BPTP. Stereo view of the active site of BPTP shows the position of a bound phosphate ion together with residues Ser 19, Ser 43, and His 72, which interact with Asn 15 to stabilize the conformation of the active-site P-loop. The phosphate binding site is viewed from the rear.

an acceptor alcohol occurs with complete retention of configuration around phosphorus [38]. Thus, the overall catalytic pathway is consistent with a double displacement mechanism in which both phosphorylation and dephosphorylation proceed with inversion of configuration at phosphorus. The existence of a phosphoenzyme intermediate during hydrolysis of phosphate monoesters has been confirmed by a variety of methods such as pre-steady-state and steady-state kinetic measurements as well as ^{18}O-exchange experiments, ^{31}P NMR and direct trapping [12,39]. Burst titration kinetics and leaving group dependence experiments also show that the reaction occurs via a two-step mechanism. The first step is a relatively rapid transfer of the phosphate group from the substrate to form a cysteinyl phosphoenzyme intermediate. Proton inventory experiments and D_2O solvent isotope effects indicate that only one proton is "in flight" in the transition state of the phosphorylation step [40]. This step is followed by a rate-limiting dephosphorylation in which water attacks the phosphoenzyme intermediate with the release of inorganic phosphate [40].

The human and bovine LMW PTPases contain eight cysteine residues, all in the free sulfhydryl form [41]. Cys 12 mutants are completely inactive, consistent with the identification of Cys 12 as the catalytic nucleophile [31]. In contrast, C17S and C17A represent 5 and 30%, respectively, of the activity of wild-type enzyme. Thus, it is clear that although the loss of Cys 17 may cause some reduction in enzyme activity, the residue is not critical for catalysis, in contrast to artifactual results obtained for a maltose-binding fusion protein [42]. However, C17 may play a role in enzyme regulation [43].

Bovine LMW PTPase is inactivated by diethyl pyrocarbonate (DEP), and the inactivation is reduced in the presence of inorganic phosphate [44]. However, neither of the two histidines present in the protein is located at the active site, and they are not essential for catalysis. Indeed, a mutant lacking histidine is still inactivated by DEP, thus showing that DEP is not a histidine-specific reagent [34]. NMR pH titration studies revealed that His 66 and His 72 have unusually high pK_a values of 8.4 and 9.2, respectively [34,45]. The three-dimensional structure of BPTP reveals that two acidic residues (Glu 23 and Asp 42) are found near His 72, and one acidic residue (Glu 139) is located near His 66 [24]. pK_a perturbation studies utilizing site-directed mutants of the acidic residues together with computational approaches confirm that electrostatic interactions are responsible for the unusually elevated pK_a values of the histidine residues [32,46].

Inhibitors and Activators

Low-molecular-weight PTPases are strongly inhibited by common PTPase inhibitors such as vanadate, molybdate, and tungstate, but not tartrate and fluoride [10]. It was previously proposed that the early transition metal oxoanions such as vanadate and molybdate bind to many phosphomonoesterases because they form complexes that resemble the trigonal bipyramidal geometry of the S_N2 transition state [47]. This was confirmed for the LMW PTPases by the crystal structure of the vanadate complex of the bovine enzyme, which showed that vanadate ion forms a covalent linkage with Cys 12 at the active site and exhibits a trigonal bipyramidal geometry [30]. Pyridoxal phosphate (PLP) binds tightly to LMW PTPases and can even act as a competitive inhibitor of a good substrate. However, PLP is in fact a slowly reacting substrate, because the pyridinium nitrogen of PLP forms a salt linkage to Asp-129 and prevents it from acting as a general acid in catalysis [48]. Despite a claim to the contrary, Cys 17 plays no significant role in the binding of PLP to the bovine LMW PTPase [49].

LMW PTPases are activated in an isoenzyme-selective manner by certain purines [50–53]. Structural studies with the homologous *Saccharomyces* enzyme have established that the purine effector binds to the phosphoenzyme covalent intermediate and facilitates dephosphorylation [54]. However, the physiological significance of such purine activation effects is uncertain, because activation is seen at purine concentrations well above normal cellular levels. Compounds such as sodium nitroprusside that generate NO and NO itself are able to inactivate LMW PTPases *in vitro* [55]. Mass spectrometry has confirmed that the NO modifies Cys 12 and Cys 17 at the active site. This result may be of regulatory significance.

Substrate Specificity, Regulation, and Biological Role

A number of synthetic and biological substrates have been used to investigate the catalytic mechanism and substrate specificity of the LMW PTPases. These enzymes demonstrate strong catalytic activity toward phosphotyrosyl but not phosphoseryl or phosphothreonyl substrates [10]. They also show a steric preference with certain aryl phosphates. For example, these enzymes hydrolyze β-naphthyl phosphate at a rate that is comparable to the rate of hydrolysis of *p*NPP but show very low activity toward α-naphthyl phosphate, thus reflecting the sensitivity of these enzymes to steric factors. These enzymes generally do not hydrolyze alkyl phosphates, with the exception of substrates such as flavin mononucleotide (FMN) that have significant hydrophobic substituents as part of their structure [10]. The reason for the latter selectivity is clear from the x-ray crystal structure. The side chains of Trp 49, Tyr 131, and Tyr 132 extend out from either side of the active site, forming two large hydrophobic walls of a deep groove on the surface of the structure [24]. These hydrophobic walls also provide some selectivity against phosphoserine and phosphothreonine peptides.

The discovery of the LMW PTPase in budding and fission yeast appeared to provide an attractive model for exploring the physiological role of this enzyme. However, studies of the *Saccharomyces cereviseae* LTP1 gene have shown that neither a disruption of this gene nor a tenfold overexpression of the product result in any apparent changes in phenotype [56]. The *Schizosaccharomyces pombe* gene STP1 was isolated by screening temperature-sensitive Cdc25 mutants [57]. Here, too, disruption of STP1 resulted in no detectable changes in phenotype in wild-type or mutant $Cdc25^{-}$ strains, indicating that STP1 is not normally involved in mitotic control [57]. However, all such studies are complicated by effects of overlapping phosphatase enzyme activities. LMW PTPases have been isolated from a number of prokaryotes, but their substrate specificities and physiological roles are also uncertain. The genes for *Erwinia amylovora*, *Pseudomonas solanacearum*, and *Klebsiella* LMW PTPases are found clustered with genes associated with exopolysaccharide biosynthesis [58,59], which is necessary for pathogenicity [60,61]. Overexpression of the *ams*I gene, which codes for a LMW PTPase in *E. amylovora*, causes a strong reduction in exopolysaccharide synthesis [62].

The human "red cell" PTPase dephosphorylates a number of phosphotyrosyl substrates including erythrocyte B and 3, angiotensin, and tyrosine kinase P^{40} [8,10,11,14]. The bovine enzyme shows activity toward phosphotyrosyl immunoglobulin G (IgG) and phosphotyrosyl casein, while rat liver PTPase hydrolyzes phosphotyrosyl peptides derived from the insulin receptor, epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, and Band 3 [63]. Both the EGF and PDGF receptor have been reported to be targets of the LMW PTPase. The bovine liver enzyme reportedly dephosphorylates the EGF receptor *in vitro* with a nanomolar K_m value [9]. Other studies suggest that the cytoplasmic domain of activated PDGF receptor is an *in vivo* target of the LMW PTPase, with dephosphorylation leading to receptor inactivation [64]. Overexpression of the bovine PTPase in normal NIH/3T3 cells as well as several oncogene-transformed cells has been reported to result in an inhibition of proliferation [65]. Overexpression of the small enzyme also results in decreased mitogenic activity and decreased autophosphorylation of the PDGF receptor [66]. An increase in PDGF receptor autophosphorylation was seen in PDGF-stimulated cells overexpressing the inactive C12S mutant. Such C12S-transfected cells also show increased proliferation, c-Src activation, and serum-induced mitogenesis compared to control cells overexpressing the wild-type PTPase gene [64,67]. Effects on cell adhesion are reportedly influenced by phosphorylation of LMW PTPase following PDGF stimulation [68,69]. The activity of the LMW PTPase toward artificial substrates reportedly can be increased by phosphorylation on two tyrosine residues (Tyr-131 and -132) that are at the entrance to the active site, but the effects are difficult to control for protein stability [70,71]. Regulatory effects due to reversible oxidation-reduction as well as protein-phosphorylation reactions have been postulated to be involved in PDGF-induced mitogenesis [43,72].

Human LMW PTPase isoenzymes have been shown to have a role in endothelial cell migration and proliferation mediated by vascular endothelial growth factor (VEGF) [73]. Another significant developmental role for LMW PTPase involves the role of the enzyme in assembly and attachment of endothelial capillaries mediated by clustered ephrin-B1 tetramers [74]. Indeed, the interactions of vertebrate LMW PTPases with the ephrin receptor tyrosine kinases offer a particularly promising target for investigation. Human LMW PTPase has recently been demonstrated to be overexpressed in many transformed cell lines, including mammary epithelial cells [75]. Strikingly, the overexpression of LMW PTPase was found to be sufficient to confer transformation upon non-transformed cell lines. The oncogenic potential of LMW PTPase appears to mediated through its interactions with EphA2.

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CHAPTER 121

STYX/Dead-Phosphatases

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Introduction

Protein tyrosine phosphatases (PTPs) catalyze the removal of phosphate from cellular substrates [1]. Individual PTPs exhibit distinct preferences for protein phosphoserine (pS), phosphothreonine (pT), or phosphotyrosine (pY), phosphoinositol phospholipid, and even 5′ triphosphorylated nucleotide moieties [1]. In addition to phosphatases, a number of structurally unique, noncatalytic protein domains recognize the phosphorylated substrates of PTPs. So-called 14-3-3, FHA, and WW domains bind pS/pT sequences [2]; SH2 and PTB domains recognize pY motifs [3]; and PH, PX, FYVE, and ENTH domains interact with specific pools of phosphoinositides [4,5]. These noncatalytic modules serve as focal points for protein interaction and for targeting effectors to specific sites within the cell.

STYX is a modular domain originally identified in a protein that mediates pS, pT, and pY signaling *in vivo* [6]. Although structurally related to phosphatases, the archetype STYX domain contains a glycine in place of the PTP catalytic active-site cysteine. Commensurate to crossing the river Styx in Greek mythology, this single amino acid substitution mechanistically abolishes phosphatase activity and functionally demarcates catalytically dead STYX from active PTPs. Similarly deleterious substitutions have been identified in other proteins that harbor phosphatase-like domains, including Sbf1, 3-PAP, IA-2, receptor-like PTPs (RPTPs) and mitogen-activated protein kinase phosphatases (MKPs) [7]. Rather than simply being the evolutionary relics of active phosphatases, individual dead-phosphatase domains perform noncatalytic roles in substrate recognition and regulation of PTP activity. Moreover, abnormal phenotypes resulting from genetic disruption in mice and yeast, fundamentally establish the importance of STYX/dead-phosphatase expression in complex biological systems. This chapter highlights the diversity of function and mechanism of action for dead-phosphatases in phosphorylation signaling.

Gathering Styx: Structure Implies Function

A summary of proteins with dead-phosphatase domains is provided in Table 1. In practice, dead-phosphatases are identified by similarity in amino acid sequence and predicted secondary structure to active PTPs. Distinguishing dead-phosphatases from PTP pseudogene products requires empirical evidence and an understanding of the components of phosphatase active-site structure.

The superfamily of PTPs is comprised of tyrosine-specific, dual-specific, Cdc25-like, and low-molecular-weight phosphatases [1]. Despite a limited sequence identity within the superfamily, all PTPs contain elements of a conserved structural core and share a catalytic mechanism involving the active-site motif, CXXXXXR (CX_5R) (Table 1). Low-molecular-weight, tyrosine-specific and most dual-specific PTPs also contain a catalytic aspartic acid residue topologically distinct from the CX_5R loop (Table 1) [1]. To catalyze substrate hydrolysis, the active-site cysteine thiolate undertakes nucleophilic attack of the substrate phosphoryl group to form a phospho–enzyme intermediate. Active-site Arg and catalytic Asp play direct roles in the formation, stabilization, and subsequent breakdown of the phospho–enzyme intermediate [1]. Surprisingly, mutations of the catalytic Cys, Arg, or Asp do not abolish substrate interaction, *in vitro* or *in vivo*, and substitution of the active site Cys or Asp has become the basis of PTP–substrate trapping strategies [8]. PTP crystallographic studies also show that catalytically dead Cys mutants bind sulfate and phosphorylated peptides in a manner identical to native enzymes [1], indicating that determinants for substrate specificity and targeting reside outside the active site (Table 1).

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Table I Dead-Phosphatases

Protein name(s)	GenBank No.[a]	Active-site sequences	PTP paralog	Expression	Interaction(s)	Chromosome	Functional attributes and mutant phenotypes
Receptor-like, pY		NR**Y**[c].WP**D** X_{31} **C**SAGVG**R**T	> RPTPγ-D1				
RPTPκ-D2	*Hs*: L77886	NRE..WAS X_{35} **C**LNGGG**R**S		Tandem intracellular	RPTPα-D2:	*Hs*: 6q22.33	RPTPδ-D2 binds and inhibits RPTPσ-D1 [21].
RPTPλ-D2	*Hs*: U60289	NRS..WSA X_{33} **C**LNGGG**R**S		PTP-like domains	calmodulin [17],	*Hs*: 1p35.2	CD45-D2 required for substrate interaction [19].
RPTPμ-D2	*Hs*: X58288	NRC..WPM X_{35} **C**LNGGG**R**S			c-Src [18]	*Hs*: 18p11.22	
RPTPρ-D2	*Hs*: AF043644	NRS..WPA X_{35} **C**LNGGG**R**S				*Hs*: 20q12	Structure of LAR-D2 mimics LAR-D1 [14].
CD45-D2	*Hs*: Y00638	NRN..WSV X_{40} **C**RDGSQQT				*Hs*: 1q32.1	LAR-D2 mediates D1 substrate interaction [20].
RPTPγ-D2	*Hs*: P23470	NRN..WPN X_{39} DEYGAVSA				*Hs*: 3p14.2	
RPTPζ-D2	*Hs*: M93426	NRT..WPN X_{39} DEHGGVTA				*Hs*: 7q31.33	Calmodulin binds catalytic cleft and inhibits
OSTPTP-D2	*Mm*: AAG28768	QNS..FPC X_{37} SSKVTNQL				*Mm*: 1	RPTPα-D2 function [17].
RPTP, single domain		NRS..WY**D** X_{31}**C**SDGAG**R**S	> *Hs*: IA-2β	Dense core secretory vesicles		*Hs*: 2q35	*Mm* null viable, with glucose tolerance
IA-2, ICA512	*Hs*: NP_002837	NRH..WPA X_{31} **C**SDGAG**R**T		*Hs*: neuroendocrine and	β2-syntrophin,	*Mm*: 1	and insulin secretion defects [32].
IA2, CG4355	*Dm*: AF126741	NRP..WPH X_{31} GSAGAG**R**T	> *Dm*: none	pancreatic islets	βIV spectrin	*Dm*: 2L	No effect of *Ce* RNAi ablation on brood size,
Ida-1	*Ce*: CAB52188	NRT..WQK X_{31} SWDGSG**R**T	> *Ce*: none	*Ce*: peptidergic neurons [31]	and nNOS [27,28]	*Ce*: III	embryonic viability or development [32].
Dual-specific, pS, pT, or pY							
Styx	*Hs*: AF085865	D X_{30} GNAGIS**R**S	None	Cytoplasmic and nuclear	Crhsp-24 [33]	*Hs*: 14q21	*Mm* null viable, but males are infertile due to
	Mm: U34973	D X_{30} GNAGIS**R**S		*Hs* & *Mm*: highest in		*Mm*: 12	abnormal spermatid differentiation [33].
	Cn: AC068564	D X_{30} **C**NGGIALS		muscle, heart, testis [6]			
		D X_{30} **C**QAGIS**R**S	> MKP-1				
MK-Styx	*Hs*: NP_057170	D X_{30} STQGIS**R**S		Ubiquitous	Map kinases:	*Hs*: 7q11.23	Also contains a "dead" Cdc25-like domain
	Ci: AV904702	D X_{30} SDNGIS**R**S			JNK & p38		*(below)* [7].
		E X_{28} **C**NRGKH**R**T	> Siw14p		Siw14p and		*Sc* null is viable, but sensitive to diltiazem-HCl
Y-Styx, YNL056w	*Sc*: CAA95929	D X_{25} SNKGKH**R**V		Cytoplasmic	Oca1p [45]	*Sc*: XIV	and hypersensitive to caffeine [47].
Nucleoside triphosphate		D X_{58} **C**THGIN**R**T	> *Ac*-BVP	*Op* single capsid nuclear			
*Op*V-*Styx*, PTP-1	*Op*: NP_046166	D X_{58} WTHGLN**R**S		polyhedrosis virus			*Op* virus encodes a dead *Ac*-BVP [50].
D3–phosphoinositides		D X_{31} **C**KAGKG**R**T	> PTEN				
Tensin1	*Hs*: AAG33700	D X_{31} NKGNRG**R**L		Ubiquitous, focal-adhesions	Actin filaments,	*Hs*: 2q35-q36	Contains SH2 and PTP homology [51].
GAK (Auxilin homolog)	*Hs*: O14976	A X_{31} **C**MDGRAAS		non-neuronal coated vesicles	clathrin	*Hs*: 4p16	

		CSDGWD**R**T	> MTM1[b]				*Mm* null viable, but males are infertile due to
Sbf1, MTMR5	*Hs*: AAC39675	LEDGWDIT		Cytoplasmic and nuclear	SET-domain	*Hs*: 22q	Sertoli cell dysfunction and azoospermia [42].
Sbf, CG6939	*Dm*: AAF54700	LEDGSDVT			proteins [42]	*Dm*: 3R	*Dm* Sbf in chromatin polycomb complex [40].
H28G03.6	*Ce*: AF098501	LEAGRSIT				*Ce*: X	*Dm* Sbf is found in histone acetyltransferase complex [41].
		CSDGWD**R**T	> MTM1[b]				
Lip-Styx, MTMR9	*Hs*: BAA91170	GTEGTDST		Cytoplasmic		*Hs*: 8p23.1	*Hs* protein colocalizes with active
CG5026	*Dm*: AAL39853	GAKGLDST				*Dm*: 3L	PI3-phosphatase, MTM1 [44].
Y39H10A.3	*Ce*: AAK84604	GGDGLDST				*Ce*: V	
		CSDGWD**R**T	> MTM1[b]				
MTMR10, FLJ20313	*Hs*: XP_007769	EEEGRDLS				*Hs*: 15q13.3	Alternatively spliced MTMR11 mRNA are
MTMR11, CRA	*Hs*: XP_001942	ERGDRDLN				*Hs*: 1q21.3	up-regulated in cisplatin-resistant cells [34].
MTMR12, 3-PAP	*Hs*: NP_061934	EENASDLC		3-PAP: heart, lung, liver	3-PAP: PI(3)P	*Hs*: 5p15.33	Cellular immune precipitate of 3-PAP has
CG14411	*Dm*: AAF48390	ESNGRDLC		kidney, brain, placenta	phosphatase	*Dm*: X	PI3-phosphatase activity [44].
Y48G1C.9	*Ce*: NP_490671	EDEGSDMS				*Ce*: I	
D4–phosphoinositide		**C**WDCLD**R**T	> Sac1p[b]				Absence of Sac1-domain reduces Inp51p tandem
Inp51p	*Sc*: NP_012264	AFDSIEKP		Membranes		*Sc*: IX	PI5-P phosphatase function *in vivo* [46].
Rhodanese homology (RH)		**C**LREED**R**S	> CDC25A[b]				RH domains of MKPs bind substrate and
MKP-3 (and paralogs)	*Hs*: Q16828	LRR[d].....DESSSDWN		Cytoplasmic	ERK2	*Hs*: 12q22	enhance carboxyl-PTP domain activity [12].
MK-Styx	*Hs*: NP_057170	KKK.....DNNSSTLE					*Hs* MKP3 NMR structure of RH domain is
PTP3	*Sc*: NP_010998	RRK.....DSTANQTE				*Sc*: V	similar to *Hs* Cdc25A PTP [10].
Other							
PTPLA[a]	*Hs*: XP_011905	**C**LIGIV PT	None			*Hs*: 10p14	
CG6746	*Dm*: XP_079583	ASFGLVKS		*Hs* and *Mm*: highest	*Sc*: Gtt1p and	*Dm*: 2L	*Sc* null mutation is lethal; haploid deletion
YJL097w	*Sc*: P40857	**S**FLGVV**R**S		in heart [48]	YBR061C	*Sc*: X	mutants germinate, then stop growing [49].

[a]*Homo sapiens*, *Hs*; *Mus musculus*, *Mm*; *Drosophila melanogaster*, *Dm*; *Caenorhabditis elegans*, *Ce*; *Orgyia pseudotsugata*, *Op*; *Autographa californica*, *Ac*; *Saccharomyces cerevisiae*, *Sc*; *Ciona intestinalis*, *Ci*; *Cryptococcus neoformans*, *Cn*.

[b]Catalytic acid residues have not been identified for MTM1-, Sac1p-, Cdc25-, and PTPLA-related phosphatases.

[c]pY specificity motif.

[d]MAP kinase interaction motif.

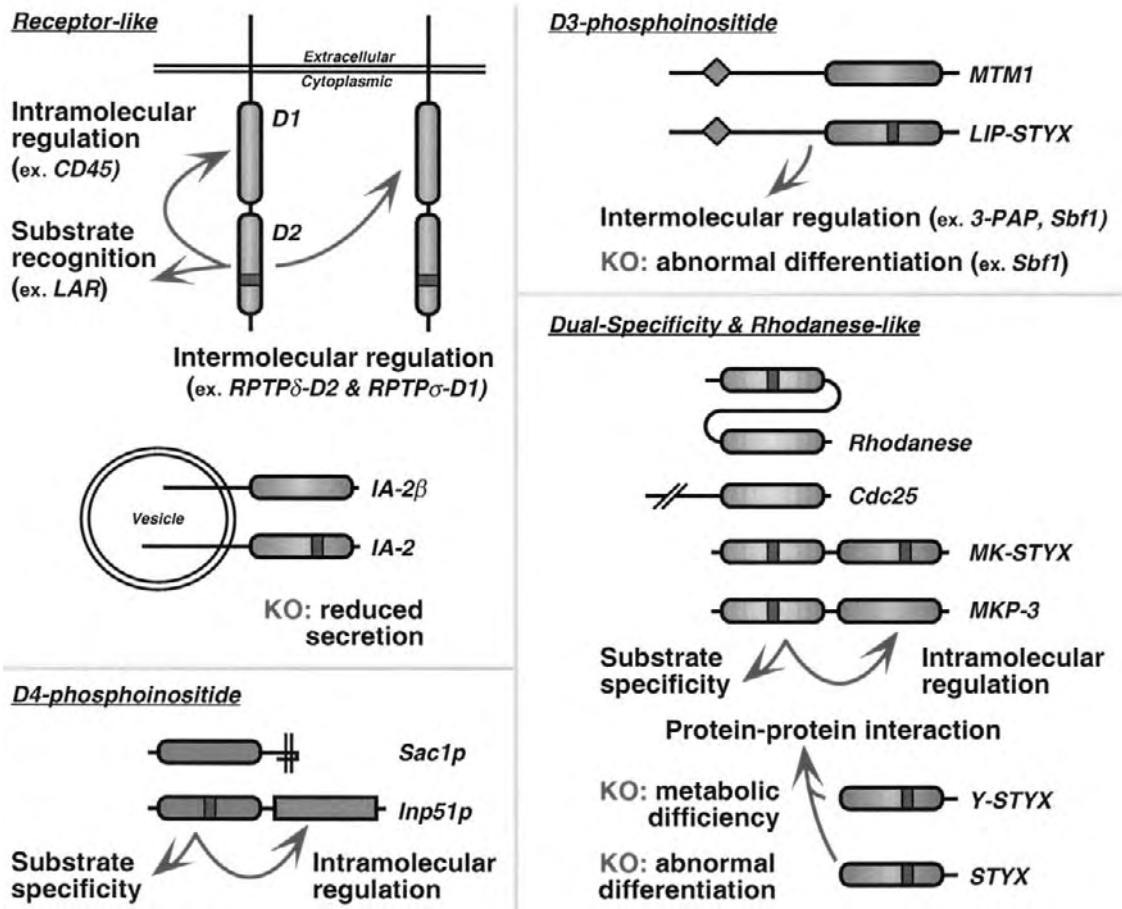


Figure 1 STYX/dead-phosphatases are functionally active proteins. Representative proteins containing catalytically active (open oval) and/or inactive (barred oval) PTP-like domains are depicted for each subclass of dead-phosphatase. Proposed functions and null phenotypes are listed for individual STYX/dead-phosphatases (arrows). Domains involved in protein targeting (diamond) or non-PTP enzymatic activity (rectangle) are provided for MTM1 and Inp51p, respectively.

One form of dead-phosphatase appears to mimic experimental PTP catalytic mutants, in that germline substitution of an active-site residue abolishes activity while preserving affinity toward phosphorylated substrates. In the case of STYX, conversion of the active-site glycine to the corresponding PTP nucleophilic cysteine (Table 1) confers a level of catalytic efficiency to the mutant protein that resembles endogenous phosphatases [6]. This suggests that a subset of STYX/dead-phosphatases possesses the structural components necessary for PTP substrate interaction but not hydrolysis and therefore could act as antagonists of endogenous phosphatase function [9]. In contrast, another subset of dead-phosphatases usurps PTP structure for noncatalytic roles that do not involve the active site. One example is the amino-terminal sequence of MKP3 [10], which adopts a rhodanese-like fold also seen in the Cdc25 family of active phosphatases (Fig. 1) [11]. In all MKPs, the corresponding Cdc25-like catalytic loop bears little sequence similarity to active phosphatases (Table 1) [11] and does not appear to be involved in MKP-3 substrate interaction [10]. Instead, a kinase interaction motif removed from the active site crevice (Table 1) mediates high-affinity binding and specificity for the substrate MAP kinase [10]. Upon substrate binding, the dead-phosphatase also allosterically induces full activation of the constituent MKP dual-specificity phosphatase domain through a process that enhances both PTP–substrate binding and turnover of the catalytic intermediate [12]. Thus, dead-phosphatases with low amino acid identity within the corresponding PTP active-site sequence have evolved functions distinct from mimicking phosphatase–substrate interaction.

The Gratefully Undead: STYX/Dead-Phosphatases Mediate Phosphorylation Signaling

The following sections illustrate the range of functions ascribed to STYX/dead-phosphatase domains. Descriptions of corresponding active PTP subfamilies are found elsewhere in this volume (see Chapters 98, 112, and 116).

Receptor PTP-D2 Domains

The PTP superfamily includes a large subclass of transmembrane receptor proteins that function in cell–cell and cell–matrix adhesion [13]. Intracellular segments of RPTPs contain either one or two tyrosine-specific, phosphatase-like

domains (Fig. 1). In dual-domain phosphatases, the majority of catalytic activity resides in the membrane proximal (D1) region, with little or no intrinsic PTP activity exhibited by the membrane distal (D2) domain. The molecular basis of the relative inactivity of RPTP-D2 sequences likely arises from alterations to the phosphotyrosine specificity motif and catalytic Asp (Table 1), which destabilize formation of the substrate phosphoenzyme intermediate [13]. In addition, the D2 domains of RPTPs β/ζ, γ, CD45 and OSTPTP contain substitutions of catalytic Cys and/or Arg residues that independently abrogate phosphatase activity (Table 1). Despite the sequence divergence of D2 and active phosphatases, crystallographic analysis indicates that the D2 domain of LAR mimics D1 structure [14], and the D2 domains of RPTPε, α, and LAR can be converted into efficient D1-like catalysts by limited amino acid substitution [14–16]. These observations suggest that D2-mediated functions could resemble PTP–substrate binding and are supported by the binding of calmodulin within the active-site cleft of RPTPα-D2 [17]. Alternatively, the RPTPα-D2 domain has also been shown to function as a phosphorylated scaffold for binding the SH2 domain of its D1 substrate, c-Src [18]. The D2 domains of CD45 and LAR are similarly required for D1 recognition of their respective substrate receptors [19,20], although the mechanism of action is unknown. Finally, binding and inhibition of the D1 region of RPTPσ by the D2 domain of RPTPδ, provides a direct role for dead-phosphatase domains in regulating catalytically active phosphatase function [21].

IA-2 (ICA512, PTPN)

IA-2 was first identified as an islet cell autoantigen specifically recognized by insulin-dependent diabetes mellitus (IDDM) sera [22] and subsequently described as a 979-amino-acid transmembrane protein containing a single cytoplasmic PTP-like domain [23]. Autoantibodies to IA-2 recognize the intracellular region and are a major diagnostic predictor for IDDM, although a role in the etiology of disease is unknown [24]. The phosphatase-like domain of IA-2 shares an active-site sequence (Table 1) and ≈80% overall identity with the tyrosine-specific PTP IA2β (Fig. 1) [25]. Unlike A2β, IA-2 contains an Ala in place of the catalytic Asp that is critical for both formation and breakdown of substrate phosphoenzyme intermediates (Table 1) [1]. Neither recombinant nor endogenous IA-2 catalyze the hydrolysis of pY from artificial or cellular substrates; however, restoration of the catalytic Asp increases the maximal rate of pY hydrolysis by mutant IA-2 [26], consistent with its function in PTPs. Thus, the dead-phosphatase domain of native IA-2 is structurally intact, and as such may function *in vivo* by sequestering A2β or related PTP substrates. Binding partners for IA-2 include βIV spectrin and the PDZ domains of β2-syntrophin and neuronal nitric oxide synthase, which likely form a complex that tethers the cytoplasmic IA-2 domain to the actin cytoskeleton [27–29]. Although the mechanism of IA-2 function is not known, its expression on the dense core secretory vesicles of neuronal and neuroendocrine tissues in *Caenorhabditis elegans*, *Drosophila melanogaster*, and vertebrate species [30,31], suggests an important role in vesicle regulation (Fig. 1). In support of this view, endogenous IA-2 is proteolytically cleaved upon insulin stimulation of cultured cells [29], and ablation of IA-2 expression results in abnormalities of glucose tolerance in mice [32]. Likewise, glucose-stimulated insulin release is significantly reduced in islets of nullizygous animals [32], which collectively points to a role for IA-2 in regulating the biogenesis, trafficking, or exocytosis of neurosecretory vesicles.

phosphoSerine, -Threonine, or -tYrosine Interaction Domain Protein

STYX has the distinction of being the first example of a naturally occurring binding domain structurally related to dual-specificity PTPs (dsPTP) [6]. Consisting of 223 amino acids, the 25-kDa cytoplasmic STYX protein represents little more than a phosphatase-like domain (Fig. 1) [6]. In contrast to all PTPs, STYX contains a Gly in place of the active-site Cys which abrogates catalytic activity (Table 1). Restoration of the active-site nucleophile in STYX(G120C) mutants confers potent phosphatase activity toward peptide pS/pT and pY and suggests that the native protein functions by interacting with the substrates of dsPTPs [6]. The importance of STYX structure, including the active-site glycine, is reflected in >90% amino acid similarity among proteins from vertebrate and lower chordate species. The presence of an orthologous gene product in the basidiomycetes fungus, *Cryptococcus neoformans* (Table 1), indicates that a progenitor STYX gene predated the advent of metazoan RPTPs and other dead-phosphatase domains. The physiological importance of STYX expression has been demonstrated by gene disruption in mouse, with pathological consequences to normal spermatogenesis (Fig. 1) [33]. Male mice homozygous for a disrupted *Styx* allele are infertile and devoid of normal epididymal sperm and exhibit a derangement of the orderly differentiation of round spermatids into spermatozoa. Coimmunoprecipitation of endogenous STYX with a multiply phosphorylated RNA-binding protein, Crhsp-24, suggests that they may form a translational checkpoint governing this process [33].

Myotubularin-Like Phosphatases

Myotubularin-related (MTMR) inositol lipid phosphatases comprise the largest known group of dsPTP-like enzymes conserved from yeast to human [34]. Of the 13 MTMR genes in human, eight are predicted to be enzymatically active, while the remaining genes encode proteins with a variety of substitutions to catalytically essential residues (Table 1) [34]. All active myotubularin-related proteins contain the extended PTP catalytic motif, CSDGWDRT (Table 1) and exhibit a marked substrate preference for phosphatidylinositol 3-phosphate, or PI(3)P, *in vitro* and *in vivo* [35,36].

PI(3)P is a lipid second messenger known to function as a targeting motif for various lipid-binding modules, including FYVE, PH, and PX domains [4,5]. Although the specific physiologic roles of myotubularin family phosphatases as regulators of intracellular PI(3)P have yet to be identified, the association of myotubularin and MTMR2 mutations with human neuromuscular diseases underscore their importance in developmental signaling pathways [37].

The most extensively characterized catalytically dead MTMR protein is the cytoplasmic Set-binding factor 1 (Sbf1), also known as MTMR5 (Table 1) [38]. In addition to its dead-phosphatase domain, Sbf1 contains a lipid-targeting PH domain and sequence similarity to a Rab3 guanine nucleotide exchange factor [39]. The function of Sbf1 appears to depend on the noncatalytic state of its phosphatase-like domain, as conversion of active-site residues that abrogate activity, Leu>Cys and Ile>Arg (Table 1), confers hydrolytic activity to mutant Sbf1 toward substrates pY and pS and suppresses the effect of native Sbf1 on differentiation and growth [39]. While the cellular function of Sbf1 is unknown, an ortholog from *D. melanogaster* is found within the polycomb and histone acetyltransferase complexes on nuclear chromatin [40,41], and male mice deficient for Sbf1 protein are infertile and azoospermatic [42]. Unlike the spermatid abnormalities of STYX nullizygous males [33], however, the primary defect caused by the absence of Sbf1 is first observed within the Sertoli cells of the seminiferous tubule [42]. Germ-cell differentiation is also disrupted during the transition from pachytene spermatocytes to round spermatids, possibly as a byproduct of Sertoli cell dysfunction [42].

Perhaps a clue into the molecular function of Sbf1 can be gleaned from the dead-MTMR protein, 3-PAP (Table 1), which stimulates D3-specific lipid phosphatase activity from a cellular complex [43]. Likewise, subcellular colocalization of MTM1 with the dead-MTMR Lip-STYX [34,44] suggests that MTMR-like dead-phosphatases may act as scaffolds for assembling active phosphatase complexes.

Conclusions

This chapter highlights the functional diversity of STYX/dead-phosphatase domains. The emergence of additional catalytically inactive, phosphatase-like proteins from human, yeast, and viral sources (Table 1) [45–51], implies an ancient origin for participation of dead-phosphatases in phosphorylation-dependent signaling. Proposed mechanisms of dead-phosphatase function range from being antagonists of PTP–substrate interaction to allosteric regulators of active PTPs and even structural scaffolds for protein complex formation. Perhaps a diversity of function for dead-phosphatases is expected given the multiplicity of substrates and strategies employed in regulating active PTPs [1]. A crystallographic study of an inactive mutant of the PTP, KAP, and its phosphorylated substrate, CDK2, illustrates how noncatalytic binding at the PTP active site can modulate the conformation and function of its bound substrate molecule [52]. Co-crystallization of STYX/dead-phosphatases with their interacting proteins will likely provide naturally occurring examples of this and other phosphorylation regulatory mechanisms.

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PART II

Transmission: Effectors and Cytosolic Events (Continued)

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SECTION C

Calcium Mobilization

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CHAPTER 122

Phospholipase C

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Introduction

The phospholipase C enzymes that hydrolyze phosphatidylinositol 4,5-bisphosphate in mammalian cells are subdivided into four families, denoted β, γ, δ, and ε, based on sequence similarities. Each family has a unique organization of regulatory sequence motifs or domains that facilitate protein:protein and/or protein:phospholipid interactions. Utilizing these motifs, each family responds to distinct hormonal signals or intracellular cues to produce the second messenger molecules inositol 1,4,5-trisphosphate and diacylglycerol. These metabolites in turn control intracellular levels of free Ca^{2+} and protein kinase C activity, respectively. This review, in addition to discussing molecular structure/function and activation mechanisms for phospholipase C enzymes, presents the physiologic consequences of PLC genetic knockouts.

This review is focused on the phosphoinositide-specific phospholipase C (PLC) isozymes expressed in mammalian cells. This family of isozymes is defined on the basis of sequence similarities and the capacity to mediate the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI 4,5-P_2) to the second messenger molecules inositol 1,4,5-trisphosphate and diacylglycerol. The former provokes mobilization of intracellular Ca^{2+} by regulating the release of stored Ca^{2+} from within intracellular organelles into the cytosol and nucleus. The latter functions as an endogenous and required activator of protein kinase C isozymes. Hence, this enzyme uniquely activates two second messengers, which in turn may control a variety of signaling pathways and thereby influence a panoply of cellular events. This review is constrained by space, and readers are referred to other recent reviews [1–3] for additional information and pertinent references.

PLC Anatomy

The eleven mammalian PI 4,5-P_2 specific PLCs are divided into four subgroups (designated β, γ, δ, ε) based on sequence similarities that produce an organization of structural motifs unique to each subgroup [1]. The organization of these motifs or domains is illustrated in Fig. 1. All PLC isozymes have motifs designated X and Y, which in the native protein are folded together to constitute the catalytic domain. An X-ray structure of PLC-δ1 provides the clearest picture of exactly how this enzymatic center is organized and suggests potential catalytic mechanisms [2]. In addition to conserved catalytic function, each PLC subgroup is characterized by additional motifs that are involved in regulating aspects of enzyme function, such as topological localization within the cell and sensitivity to protein:protein and protein:lipid interactions.

PLC Activation Mechanisms

PLC-β. The activity of four PLC-β isozymes is regulated by hormones that bind to G-protein coupled receptors (GPCRs) [1]. These receptors typically have multiple membrane spanning domains, have no intrinsic catalytic activity, and utilize heterotrimeric G proteins to communicate with downstream second messenger producing enzymes, such as PLC isozymes. When GPCRs are stimulated by hormone binding, G protein complexes containing α, β, γ subunits are activated with the following characteristics: GDP bound to the α subunit is replaced by GTP, dissociating the trimeric complex into two active species–a GTP-bound free α subunit and a βγ dimeric complex. Both of these act as signal transducers to activate PLC-β isoforms in a manner that may depend on both for maximal activation.

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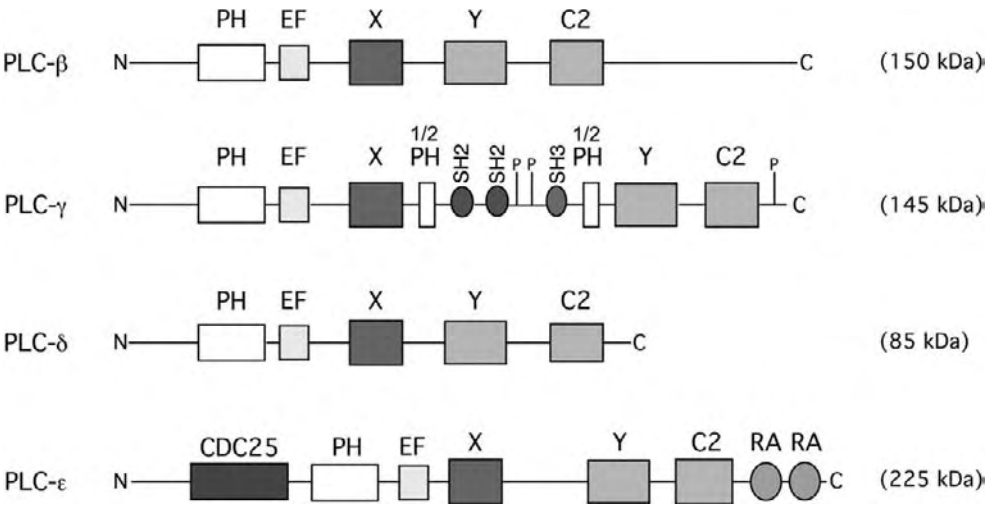


Figure 1 Schematized arrangement of domains within PLC isozymes. Functional domains or motifs (PH, EF, SH2, SH3, C2, RA, CDC25, X, Y) are explained in the text. Tyrosine phosphorylation sites are depicted by Y in the γ isozymes.

The $\alpha \cdot GTP$ subunit interacts with a region in PLC-β that includes a portion of the C2 domain, while the βγ complex interacts with part of the PH domain. The region within PLC-β that binds the $\alpha \cdot GTP$ subunit also appears to function as a dimerization interface, suggesting that PLC-β may function as a dimer [4,5]. Since α subunits and βγ complexes are constitutively anchored by lipid modifications to the cytoplasmic face of the plasma membrane, one consequence of these interactions is to promote a catalytically competent association of PLC-β with the plasma membrane. In unstimulated cells, PLC-βs are usually found loosely associated with the plasma membrane in what can be termed a catalytically incompetent association.

There is evidence for most PLCs that formation of a highly specific plasma membrane association is necessary for hydrolysis of the plasma membrane-localized substrate PI 4,5-P_2. Productive membrane association by PLC-β is further facilitated by interaction of its PH domain with phosphatidylinositol 3-phosphate. Separate regions of the PH domain accommodate this phosphoinositide and βγ complexes. Although the C2 domain in PLC-δ does mediate a Ca^{2+}-dependent phospholipid interaction, there is no evidence for this in PLC-β. While interactions of the C2 and PH domains of PLC-β with membrane-bound molecules might seem sufficient to explain formation of a productive membrane:enzyme complex, it is unclear whether this association *per se* is sufficient for increased catalytic activity or whether these interactions also provoke changes within the X/Y catalytic domain.

Signal transduction mechanisms are, by definition, reactions that are readily reversible. In the case of PLC-β, the most readily reversible component resides in the $\alpha \cdot GTP$ subunit, which can be rapidly converted by intrinsic GTPase activity to $\alpha \cdot GTP$.

PLC-γ. This subgroup contains two members, γ1 and γ2 [1,3]. Initially, the cloning and sequencing of the isozymes indicated significant differences in the COOH terminal sequences; however, more recent data indicate that these apparent differences resulted from sequencing errors for γ2 [6]. As shown in Fig. 1, PLC-γ uniquely contains motifs known as SH2 and SH3 domains in addition to the PH and C2 domains present in other PLC subgroups. The SH2 motifs, in particular, are important to facilitate activation of γ isozymes by growth factor receptor tyrosine kinases (RTKs). RTKs possess a single transmembrane domain separating the ligand-binding ectodomain from a cytoplasmic domain that contains sequences encoding tyrosine kinase activity [7]. Ligand binding facilitates dimerization of RTKs and this in turn facilitates activation of the tyrosine kinase domain. Substrates for the tyrosine kinase include the receptor itself and other proteins, such as PLC-γ.

The initial step in growth factor-dependent activation of PLC-γ involves the recognition of autophosphorylation sites in a RTK by the SH2 domains of PLC-γ1 [1,3]. This recognition event is a prerequisite for tyrosine phosphorylation of PLC-γ by the RTK, which constitutes a major step in the activation mechanism. Receptor association may also relocalize PLC-γ1 from the cytosol to the cytoplasmic face of the plasma membrane, much like the association of PLC-β with membrane-anchored G protein subunits.

Compared to other PLCs, PLC-γ has an elongated linker segment between the X and Y domains. This linker contains not only the SH2 and SH3 domains, but also a split PH domain and at least two important tyrosine phosphorylation sites, which are close together between the C-SH2 and SH3 domains and are conserved in the γ1 and γ2 isoforms. It is possible that modulation of the structure of this linker region, by protein: protein interaction and/or by tyrosine phosphorylation, contributes to activation of the catalytic site [3]. One additional site of tyrosine phosphorylation is located C-terminal to C2 domain in both the γ1 and γ2 isoforms [1,3], while a fourth site in γ2 is located between the Y and C2 domains [8].

Evidence has been presented that in some cell systems PI-3 kinase activity and the formation of phosphatidylinositol 3,4,5-trisphosphate (PI 3,4,5-P_3) is necessary for maximal PLC-γ1 activation [1,3]. The site of action of PI 3,4,5-P_3 is most likely the N-terminal PH domain of PLC-γ1. However, not all reports are in agreement on this and data have been presented to support an interaction of PI 3,4,5-P_3 with the C-SH2 domain of PLC-γ1. The possible contributions of the SH3 and C2 domains of PLC-γ1 to the activation mechanism are unclear. Evidence has been presented to indicate that the SH3 domain of PLC-γ1 can associate with PIKE, a nuclear GTPase, and stimulate its activation [9].

In hematopoietic cells, adaptor proteins, such as LAT in T cells, are localized in specialized membrane microdomains termed lipid rafts and are also tyrosine phosphorylated following antigen activation [1,3]. In T cells, phosphorylated LAT becomes associated with PLC-γ1, and this interaction is necessary for PLC-γ1 activation. Whether similar membrane components are involved in PLC-γ1 activation in non-hematopoietic cells is unknown. However, there is evidence that RTK activation provokes the preferential association of tyrosine phosphorylated PLC-γ with caveolae [10,11], which resemble raft membrane microdomains but contain the protein caveolin.

PLC-δ. The activation mechanisms for this subgroup of PLC isozymes, which are not activated by GPCRs or RTKs, are perhaps least understood. Studies *in vitro* indicate that while all PLCs require free Ca^{2+}, the δ isozymes are the most sensitive to free Ca^{2+} levels [1]. This has led to the notion that this enzymes activity may be enhanced by increased levels of intracellular Ca^{2+}, and there is evidence consistent with this in a few cell-based systems. Available data also indicate that the PH and C2 domains facilitate membrane association of the δ isozymes. The PH domain of PLC-δ recognizes PI 4,5-P2 and may not only tether the enzyme to the plasma membrane but may also facilitate a processive mechanism of hydrolysis. The C2 domain mediates membrane association by recognition of a Ca^{2+}-phospholipid complex in the membrane. It is interesting that the PH domain of PLC-δ also binds inositol 1,4,5-P_3, the product of PI 4,5-P_2 hydrolysis, and this may represent a mechanism to decrease PLC-δ activity when product levels become high.

PLC-ε. This is the most recent addition to the mammalian PLC family and was foreshadowed by the isolation of a *C. elegans* cDNA that has the same organization [1]. Within the PLC family, PLC-ε has novel protein:protein interaction motifs that indicate that its activation is directly controlled by the G protein Ras, which is a particularly important molecule in signal transduction pathways initiated by growth factor RTKs.

Near its N-terminus, PLC-ε contains a sequence identified as a CDC25 or a RasGEF (guanine nucleotide exchange factor) motif, which in other proteins facilitates the activation of Ras by mediating the exchange of GDP for GTP. Evidence indicates that expression of exogenous PLC-ε in cells does promote increased levels of Ras≅GTP. This would place PLC-ε upstream of Ras in a signal transduction pathway. PLC-ε also has RA or Ras association motifs near its C-terminus. This sequence motif allows recognition of PLC-ε by the GTP-bound, or activated, form of Ras. Evidence shows that PLC-ε is indeed recognized with high affinity by Ras·GTP and is not recognized by Ras·GTP. This predicts that Ras is an activator of PLC-ε and would place this PLC isoform downstream of activated Ras. Also, recognition of PLC-ε by activated Ras could also facilitate membrane translocation of PLC-ε, as Ras is constitutively membrane-localized by the presence of covalent lipid constituents. The complex relationship of PLC-ε to Ras is analogous to the reported observation that PLC-β1 acts as a GAP (GTPase activating protein) toward the α·GTP subunit that is its direct activator [12]. Ras is a prototype for a large family of single subunit GTPases, and there are data suggesting that PLC-ε also participates in signaling dependent on Rap 1 [13] and Rap2B [14], members of the Ras superfamily.

It also appears that PLC-ε can be activated by heterotrimeric G protein subunits, including βγ complexes [15] and at least one α·GTP subunit [16]. The former proceeds through recognition of a PH domain in PLC-ε. Hence, PLC-ε may be activated by growth factor RTKs through Ras or by GPCRs through heterotrimeric G proteins. This finding raises the prospect that the PLC activity downstream of these receptors may represent contributions by more than one PLC subgroup.

PLC Physiology

While structural and biochemical questions regarding PLC isozymes have yielded significant information regarding the molecular mechanisms by which these enzymes are activated in cells, there is much less information available regarding the role of these PLC isozymes in physiologic or pathophysiologic processes. Given the ubiquitous occurrence of PLC isozymes and the pleiotropic potential of the second messengers that they generate, this may seem either too obvious or too complex a question to resolve. In view of the multiplicity of isoforms in each PLC subgroup, one might expect substantial functional redundancy to exist, although this expectation is partially offset by the differing patterns of expression for each isoform. Also at play is the extent to which PLC-dependent signal transduction is necessary, sufficient, or dispensable for any given cell response. These issues can to some extent be addressed by selective abrogation of each PLC isozyme through targeted gene disruption technology. The contents of Table I describe results that have been obtained to date by the application of this technology to PLC genes. The results, in some cases, represent phenotypes obtained at the first crucial point in development when a particular PLC isoform becomes required for further development of the organism.

In the case of *Plcb*3 knockouts, discordant results have been reported that may reflect the manner in which the gene was actually disrupted. When *Plcb*3 genomic information corresponding to exons encoding the last one-third of the

Table I Phenotypes Resulting from Targeted Disruption of PLC Genes in Mice

Gene (Protein)	Phenotype	Reference
*Plcb*1 (PLC-β1)	Death 2–6 weeks after birth, increased level of recurrent seizures due to decrease in muscarinic acetylcholine signaling	17
*Plcb*2 (PLC-β2)	Normal life span, increased neutrophil chemotactic response	18
*Plcb*3 (PLC-β3)	Embryonic lethal E2.5	19
	Normal life span, decreased opoid-dependent behavioural responses	20
	Increased skin ulcers	21
*Plcb*4 (PLC-β4)	Normal life span, locomotor ataxia due to metabotropic glutamate receptor signaling	17
	Impaired visual response	22
	Decreased climbing fiber elimination	23
	Decreased long-term depression, decreased conditioned motor learning	24
*Plcg*1 (PLC-γ1)	Embryonic lethal E9.0, impaired erythrogenesis, vasculogenesis	25
	Chimeric mice (plcg1 –/– and +/+) mice, impaired hematopoiesis, polycystic kidney	26
*Plcg*2 (PLC-γ2)	Normal life span, decreased mast cell function, decreased B cell numbers	27
*Plcd*4 (PLC-δ4)	Normal life span, male infertility due to deficiency in acrosome reaction	28

X domain plus the first two-thirds of the Y domain was deleted, embryonic lethality was produced at approximately 2.5 days in gestation [19]. In the second knockout [20], a genomic deletion representing one exon encoding some residues in the X domain was produced and the mice were normal as far as development and growth are concerned. At this time the discrepancy between these two studies has not been resolved. It is possible that in one knockout a mutant protein was produced that acted as a dominant-negative molecule affecting other pathways. Alternatively, one of the knockouts may represent only a partial loss of PLC-β1 function.

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CHAPTER 123

Inositol 1,4,5-trisphosphate 3-kinase and 5-phosphatase

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Introduction

Inositol 1,4,5-trisphosphate ($InsP_3$) 5-phosphatase and 3-kinase are the two major enzyme activities that metabolize $InsP_3$ in mammalian cells. Distinct forms of inositol and phosphatidylinositol 5-phosphatase selectively remove the phosphate from the 5-position of the inositol ring from both soluble and lipid substrates, i.e. $InsP_3$, inositol 1,3,4,5-tetrakisphosphate ($InsP_4$), phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) or phosphatidylinositol 3,4,5-trisphosphate ($PtdInsP_3$). This reaction is often associated with the deactivation pathway of both soluble and lipid second messengers involved in Ca^{2+} signalling, proliferation, growth factor mediated events, and apoptosis [1–3]. Type I $InsP_3$ 5-phosphatase is unable to use the phosphoinositides as substrates and therefore is specific for $InsP_3$ and $InsP_4$. It is a quite different and unique enzyme as compared to the type II isoforms (sometimes also called type II, III, and IV and considered as inositol lipid phosphatases *in vivo,* for review, see [2]).

The $InsP_3$ 3-kinase catalyses the phosphorylation of $InsP_3$ to $InsP_4$. This enzyme is particularly interesting in view of the rapid increase in $InsP_4$ levels in cells upon stimulation by agonists [4] and of several second messenger functions that had been proposed for $InsP_4$ [5]. cDNAs encoding three human isoenzymes of $InsP_3$ 3-kinase (3-kinases A, B, and C) have been reported (Table I). A mammalian inositol polyphosphate multikinase has been shown to phosphorylate inositol 4,5-bisphosphate, thereby providing an alternative biosynthesis for $InsP_3$ [6].

Type I $InsP_3$ 5-phosphatase

Initially detected in human erythrocyte membranes [7], type I $InsP_3$ 5-phosphatase has a 10-fold higher affinity but 100-fold lower Vmax for $InsP_4$ than it does for $InsP_3$ [8]. Molecular cloning revealed that it is a 412 amino acid protein with a C-terminal isoprenylation site CCVVQ (Fig. 1) [9,10]. Type I $InsP_3$ 5-phosphatase is targeted to plasma membranes and perinuclear regions as shown in several transfection studies [12]. The activity of this enzyme could be modified by targeting mechanisms and by phosphorylation. Direct interaction with platelet proteins such as pleckstrin and 14-3-3ξ has been reported [13,14]. In several cell models (e.g. rat cortical astrocytes), this enzyme has been shown to be stimulated by an $InsP_3$ mobilizing agonist such as ATP or carbachol. This effect is triggered by Ca^{2+}/calmodulin kinase II protein phosphorylation, resulting in an inhibition of enzyme activity [15]. Underexpression of type I $InsP_3$ 5-phosphatase in rat kidney cells is associated with increasing levels of $InsP_3$ and $InsP_4$ and increasing levels of intracellular Ca^{2+} [16]. Antisense-transfected cells grow faster as compared to cells transfected with vector alone. Antisense-transfected cells formed colonies in soft agar and tumors in nude mice [17]. In overexpression studies in CHO cells, prenylation of type I $InsP_3$

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Table I Characteristics of Type I $InsP_3$ 5-phosphatase and $InsP_3$ 3-kinase Isoforms

Protein (human)	Accession numbers	Pest regions	Stimulation of activity by Ca^{2+}/CaM	Tissue distribution
Type I $InsP_3$ 5-phosphatase	X77567	—	—	Heart, skeletal muscle, and brain [10]
$InsP_3$ 3-kinase A	X54938	2	2–3 fold	Brain and testis [11]
$InsP_3$ 3-kinase B	Y18024, AJ242780	5	8–10 fold	Ubiquitous [24]
$InsP_3$ 3-kinase C	AJ290975	4	Insensitive	Ubiquitous [25]

The PESTfind program (at *www.embnet.org*) developed by Rogers *et al* [26] was used. The tissue distribution was obtained by Northern blotting.

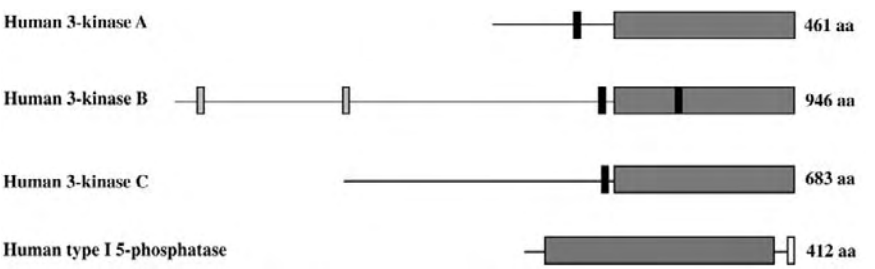


Figure 1 Schematic representation of the enzymes that metabolize $InsP_3$. Dark blue boxes represent the C-terminal catalytic domain of the $InsP_3$ 3-kinase enzymes and the red one of the type I $InsP_3$ 5-phosphatase. Potential phosphorylation sites are represented by black boxes. Two proline rich regions are found in the sequence of the $InsP_3$ 3-kinase B (light blue boxes). The yellow box shows the isoprenylation site of the type I $InsP_3$ 5-phosphatase enzyme.

5-phosphatase appears to be critical in the control of Ca^{2+} oscillations in response to agonists [18].

$InsP_3$ 3-kinase

This enzyme activity that specifically produced $InsP_4$ was initially reported in rat brain and T lymphocytes [19,20]. Purification of the protein and microsequence determination allowed the cloning of a 459 amino acid protein referred to as the A-isoform of 50 kDa. When it was expressed in bacteria, it showed an activity that was stimulated upon the addition of Ca^{2+} and calmodulin as shown for the native enzyme [21]. Transfection studies in HeLa cells show the importance of a 66-aminoacid N-terminal sequence in targeting the $InsP_3$ 3-kinase A to the actin cytoskeleton [22]. The first demonstration of two distinct sequences was reported in 1991 with the isolation of two different cDNAs from a cDNA library. These were referred to as human $InsP_3$ 3-kinases A and B, respectively [23]. The full-length sequence of human $InsP_3$ 3-kinase B has been recently reported and encodes a protein of 946 amino acids [24]. A third human isoenzyme, i.e. $InsP_3$ 3-kinase C, has been isolated following the screening of a human thyroid cDNA library. Its open reading frame encoding 683 amino acids has been expressed in *E. coli* and also in COS-7 cells [25]. Full-length sequences of human $InsP_3$ 3-kinases A, B, and C have been reported in databases (Table I).

The three human isoforms are members of a large family of inositol phosphate kinases present in mammalian but also in yeast that contain a C-terminal catalytic domain and specific residues involved in binding inositide substrates [3]. The mammalian $InsP_3$ 3-kinases A, B, and C show the presence of four conserved motifs in the catalytic C-terminal domain that are not present in inositol hexakisphosphate kinase or in the inositol multikinase [25]. Human $InsP_3$ 3-kinases A, B, and C contain potential PEST-sequences as identified by the PESTfind program (Table I). This suggests that the enzymes are particularly sensitive to proteolysis as noticed during purification of the native enzyme in several tissues.

Several data in the literature suggest that $InsP_4$ by itself shows second messenger function(s) in neurons or in endothelial cells [27,28]. One approach to address the function of $InsP_4$ is to look for the distribution and relative expression of the proteins responsible for its metabolism or action in cells. In this context, a particularly high level of the A isoform is found in the dendritic spines of neurons (Purkinje cells and hippocampal CA1 pyramidal cells) [22,29], thus supporting a role of $InsP_4$ in LTP. The A isoform was reported to associate with F-actin, whereas the B isoform was shown to exist in the cytosol and also to be associated to the cytosolic face of endoplasmic reticulum membranes [22,30]. The localization of the inositol type I 5-phosphatase in plasma membranes was reported to be critical in the control of Ca^{2+} oscillations [18]. The mammalian $InsP_3$ 3-kinases A, B, and C could also control the concentration of several inositol phosphates, $InsP_3$ isomers, and/or higher phosphorylated inositol phosphates such as $InsP_4$ isomers, $InsP_5$, or $InsP_6$. In particular, Ins(1,3,4)P_3, the product of $InsP_4$ dephosphorylation by type I inositol

5-phosphatase, is a potent inhibitor of Ins(3,4,5,6)P_4 1-kinase, resulting in an increase of Ins(3,4,5,6)P_4 and a decrease in chloride efflux [31]. The situation is also complicated by the fact that the three InsP_3 3-kinase isoforms in animals can be distinguished by their N-terminal sequence, presumably targeting sequences and sensitivity to Ca^{2+}/calmodulin [24,25].

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CHAPTER 124

Cyclic ADP-ribose and NAADP

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Introduction

Cyclic adenosine diphosphate ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) are endogenous pyridine nucleotide metabolites with potent Ca^{2+} mobilizing activities. Although the Ca^{2+} mobilizing properties of these two molecules was first discovered in sea urchin eggs, their actions seem to extend to many mammalian, invertebrate, and plant systems where they may function as Ca^{2+} mobilizing intracellular messengers.

In a seminal study, Lee and colleagues reported in 1987 that not only could the established Ca^{2+} mobilizing messenger inositol 1,4,5 trisphosphate (IP_3) release Ca^{2+} from intracellular stores in sea urchin egg homogenates, but so too could the two pyridine nucleotides, NAD and NADP [1]. However, NAD and NADP were not Ca^{2+} mobilizing agents themselves. The active principles were subsequently identified as a cyclic metabolite of NAD, cADPR [2] (Fig. 1A) and a contaminant of commercially available NADP, NAADP [3] (Fig. 1B). Both cADPR and NAADP were shown directly to mobilize Ca^{2+} from intracellular stores by microinjection into intact sea urchin eggs [3,4].

A useful property of the sea urchin homogenate system is that Ca^{2+} release by different Ca^{2+} mobilizing agents displays homologous desensitization. After stimulation of maximal Ca^{2+} release by a given agent, Ca^{2+} stores become refractory to Ca^{2+} release by that same agents. Sequential additions of IP_3, cADPR and NAADP to the same aliquot of egg homogenate all evoked Ca^{2+} releases regardless of the order in which they were added, while a second addition of any of these agents failed to elicit any response. (see [5] for review.) From these data it was proposed that these three Ca^{2+} releasing agents mobilized Ca^{2+} stores by three distinct mechanisms. Further studies with selective pharmacological agents confirmed this view. Heparin, a competitive IP_3 receptor (IP_3R) antagonist, selectively inhibited IP_3-evoked Ca^{2+} release, while cADPR or NAADP-induced Ca^{2+} release were unaffected [4]. Ca^{2+} release by cADPR was found to be selectively blocked by ryanodine receptor (RyR) inhibitors [6] and chemically synthesized 8-substituted analogues of cADPR [7]. In contrast, NAADP-evoked Ca^{2+} release was neither affected by IP_3 or RyR antagonists nor cADPR analogues, but selectively blocked by inhibitors of voltage-gated Ca^{2+} and K^+ channels [8].

cADPR has now been implicated in the regulation of Ca^{2+} release via RyRs in many different cell types, while NAADP, which similarly has also been shown to mobilize Ca^{2+} in a number of cell types from different organisms [9], appears to act on a novel Ca^{2+} release channel. While IP_3Rs and RyRs are well-characterized Ca^{2+} release channels of intracellular organelles, the molecular nature of the NAADP-sensitive Ca^{2+} release channel is unknown. IP_3 and cADPR appear to predominantly mobilize Ca^{2+} from the endoplasmic reticulum [10,11] while NAADP releases Ca^{2+} from a distinct organelle [10,12], possibly an acidic compartment.

A key property of both IP_3Rs and RyRs is that both are modulated by Ca^{2+} itself. This property is responsible for Ca^{2+}-induced Ca^{2+} release (CICR), which serves to amplify normally locally restricted Ca^{2+} transients as global Ca^{2+} signals and is thought to be critical in determining the complex patterns of Ca^{2+} signals widely observed in cells such as repetitive Ca^{2+} spikes and regenerative Ca^{2+} waves [13]. Both IP_3 and cADPR appear to sensitize IP_3Rs and RyRs, respectively, to activation by Ca^{2+}, thereby promoting CICR. The molecular interactions of IP_3 with its receptors have been relatively well defined [14]; however, the molecular mechanisms by which cADPR activates RyRs are not, and the possibility exists that cADPR binds to an accessory protein that in turn modulates RyR openings [15,16]. Indeed, two known RyR associated proteins, calmodulin [17] and FKBP12.6 [18],

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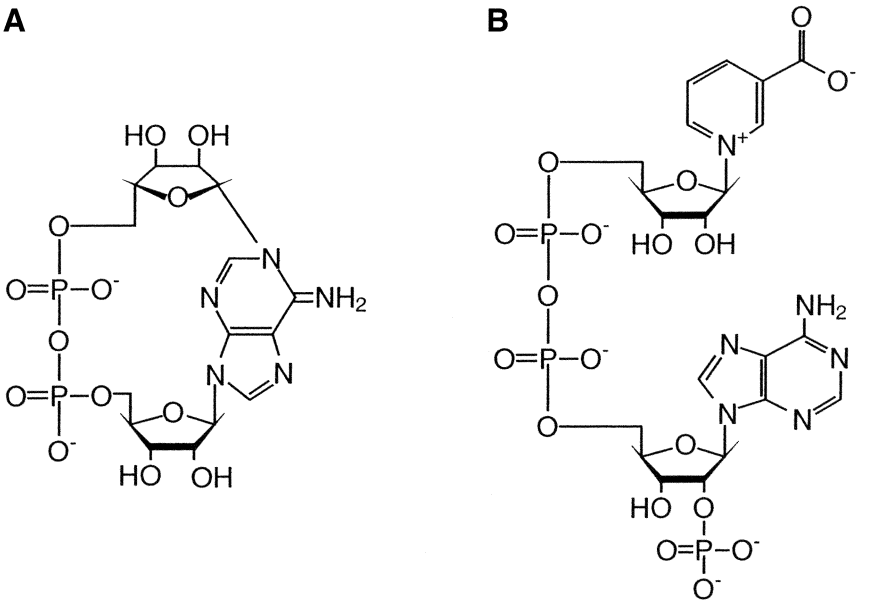


Figure 1 The chemical structures of cyclic adenosine dinucleotide phosphate ribose (cADPR) (A) and nicotinic acid adenine dinucleotide phosphate (NAADP) (B).

have been implicated in cADPR-induced Ca^{2+} release. However, NAADP elicits local Ca^{2+} signals unless amplified by triggering CICR by recruiting IP_3Rs and RyRs [19] or through its own diffusion through cells [20].

Two types of evidence exist to suggest that cADPR or NAADP function as intracellular messengers for Ca^{2+} signaling in cells and tissues. The first is that endogenous levels of these compounds are modulated by cellular stimuli and the second is that the selective block of cADPR or NAADP-sensitive Ca^{2+} release mechanisms can inhibit Ca^{2+} mobilization or functional responses to a range of hormones, transmitters, and other cell regulators. ADP-ribosyl cyclases are a class of enzyme that can synthesise cADPR and NAADP from alternate substrates NAD and NADP, respectively, with pH and phosphorylation state determining which product is produced [21,22]. The best-characterized example of such an enzyme is CD38, which has been implicated in cADPR-based Ca^{2+} signaling in a number of cell types [23]. It was originally thought to be an ectoenzyme, although several reports indicate that it is also present in several intracellular compartments. Studies from tissues and cells derived from CD38 knockout mice implicate CD38 in both cADPR synthesis and Ca^{2+} signaling [24,25], and perhaps also NAADP synthesis as well [21,26], although other synthetic pathways are possible. CD38 is a complex enzyme in that it also catalyzes the hydrolysis of cADPR as well, while NAADP may be metabolized by a calcium-dependent phosphatase [27]. A number of approaches have been employed to determine changes in cADPR levels in cells, including thin layer chromatography, hplc, radioimmunoassay, radioreceptor binding [28], and a new cycling assay [29]. A variety of cell stimuli have been shown to increase cADPR levels in cells [30], including G protein-linked receptors and those linked to tyrosine kinase activities [31], although the precise coupling mechanisms are unknown. There are no data at present on changes in NAADP levels in cells and tissues, although endogenous levels have been reported in plant tissues [32].

The role of cADPR in the generation of Ca^{2+} signals in response to cellular stimuli have been dissected by use of selective cADPR antagonists [30], and for NAADP by injecting high concentrations of NAADP into cells to desensitize NAADP-evoked Ca^{2+} release [19], since no selective antagonists for NAADP exist at present. Such studies have suggested a key role for the use of multiple messengers and multiple Ca^{2+} stores in dictating the complex patterns of Ca^{2+} signals observed in cells, which may be linked differentially to specific cellular responses. In T cells, IP_3 elicits a brief Ca^{2+} transient, while cADPR prolongs the Ca^{2+} signal [31]. In ascidian oocytes, different Ca^{2+} releasing messengers regulate different cell functions with IP_3 inducing Ca^{2+} spiking, cADPR regulating exocytosis, and NAADP modulating plasma membrane Ca^{2+} currents [33]. In pancreatic acinar cells, different Ca^{2+} mobilizing transmitters and hormones appear to be coupled to one or more types of Ca^{2+} mobilizing messengers [34]. The different Ca^{2+} release mechanisms appear also to be coupled in different ways depending on cell phenotype. For example, in pancreatic acinar cells and sea urchin eggs the NAADP mechanism couples to CICR channels to elicit global Ca^{2+} signals, while in T cells and ascidian oocytes, desensitization of NAADP receptors rather puzzlingly renders cells insensitive to IP_3 [9,33,35].

Although much work still remains to be done in elucidating many of the molecular details of the cADPR and NAADP signaling pathways, it is clear that through their involvement along with IP_3 in regulating Ca^{2+} signaling, they provide another layer of regulation in determining

complex Ca^{2+} signaling patterns. They are thus likely to be important components of the Ca^{2+} code whereby a single ion can specifically regulate a diverse array of cellular functions.

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CHAPTER 125

Sphingosine 1-phosphate

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Introduction

Sphingosine 1-phosphate is a putative intracellular second messenger for Ca^{2+} release. The metabolic pathways controlling sphingosine 1-phosphate levels are beginning to be understood, and it is clear that intracellular levels of this molecule can be actively regulated. However, the signaling machinery through which sphingosine 1-phosphate releases Ca^{2+} from intracellular stores remains poorly characterised. This chapter addresses recent issues in this area of Ca^{2+} signaling.

It is well established that sphingolipids, which are ubiquitous structural membrane constituents, are also capable of giving rise to a number of lipid signaling metabolites. In particular, sphingosine 1-phosphate (SPP) (Fig. 1) has been implicated in a variety of processes at both the intracellular and extracellular levels [1–3]. The extracellular targets for SPP, which is present in serum and can be released into the extracellular environment by activated platelets [4], have been clearly identified as members of the endothelial differentiation gene (Edg) family of heptahelical G protein-coupled receptors (GPCRs) [2]. These receptors couple to a variety of heterotrimeric G proteins, and can stimulate intracellular Ca^{2+} release through activation of the inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] signaling pathway. However, SPP has also a number of attributes associated with a role as an intracellular messenger molecule. Thus intracellular levels of SPP can be regulated, often within seconds of an appropriate extracellular stimulus, through the action of the enzyme sphingosine kinase (SPHK). In this case, the resultant increases in cytosolic SPP appear capable of directly activating cell growth and intracellular Ca^{2+} mobilization [1,3,5]; however, unlike the extracellular effects of SPP, the intracellular targets for SPP remain to be defined.

Sphingolipid Metabolism

SPP is formed from the membrane lipid sphingomyelin via a series of enzymatic reactions [see 2,6]. Hydrolysis of sphingomyelin produces ceramide (N-acyl sphingosine), and this appears to be a central molecule in the SPP metabolic pathway. Subsequent removal of the amide-linked fatty acid side chain of ceramide produces sphingosine, which can then be phosphorylated by SPHK to produce SPP. Out of the sphingolipid metabolites, only SPP appears to have any direct Ca^{2+} release activity [7,8], and there is now clear experimental evidence that a variety of extracellular stimuli can activate SPHK and increase intracellular SPP levels (see below). Consistent with a second messenger role, SPP can be rapidly degraded either by dephosphorylation back to sphingosine, or irreversibly removed from the sphingolipid cycle via the enzyme SPP lyase, which hydrolyses a carbon-carbon bond in the sphingosine backbone of the molecule [9]. It is interesting that platelets, a noted source of extracellular SPP, lack SPP lyase [9].

Activation of SPHK

Two distinct forms of human SPHK have been cloned. The type 1 form is a 49 kDa protein that contains putative phosphorylation sites for PKC, PKA, and casein kinase II, as well as Ca^{2+}/calmodulin and SH3 binding domains. The type 2 form is notably larger (65 kDa) and has a different tissue distribution [6, 10,11]. Both these proteins are predominantly cytosolic [5,12] (Fig. 2). SPHK activity has been quantified by measuring the ability of cell lysates (whole cell or fractionated, stimulated vs. unstimulated) to [γ^{32}P]-label added

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Sphingosine 1-phosphate

Figure 1 Structural representation of sphingosine 1-phosphate.

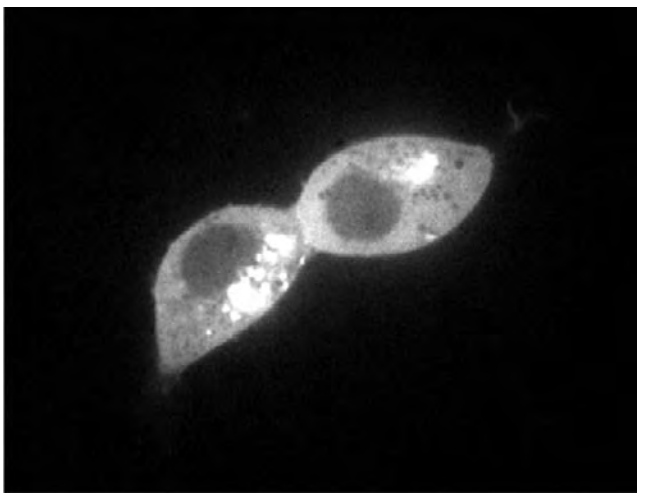


Figure 2 Cellular distribution of SPHK. HEK-293 cells, grown on coverslips, were transiently transfected with SPHK tagged with eGFP (gift from S. Spiegel) and imaged on a confocal microscope (×100 magnification). Similar to studies using HA-tagged SPHK, or involving SPHK1 antisera, SPHK-eGFP was predominantly cytosolic in location.

sphingosine from a pool of [γ^{32}P]-ATP. Such experiments have demonstrated SPHK activity both in cytosolic and membrane fractions [5]. Pretreatment of NIH 3T3 fibroblast cells with PDGF raises the Vmax of SPHK, with no apparent change in Km [13], and both cytosolic and membrane forms of SPHK are similarly activated [5]. SPHK activity can also be investigated indirectly by measuring changes in intracellular SPP. In such experiments, cells are pulse-labeled with [^{3}H]-sphingosine and the resultant [^{3}H]-SPP extracted. A complicating factor with this method is the lack of equilibrium labeling of [^{3}H]-sphingosine, hence making substrate availability a potential issue.

Using these methods, a number of extracellular stimuli, which result in Ca^{2+} mobilizing responses, have been shown to stimulate SPHK, hence supporting the possibility that intracellular SPP functions as a Ca^{2+} release mediator. This list of stimuli include PDGF [13], antigen stimulation [14], and a variety of recombinant and endogenous GPCRs (muscarinic M2 and M3 [15], lysophosphatidic acid (LPA), Edg-4 receptor [16]). Although investigating the role of SPP in Ca^{2+} mobilization is complicated by the fact that many of these extracellular stimuli also utilize the Ins(1,4,5)P_3/Ca^{2+} release pathway, this is not always the case. Of particular note here is the Fcγ receptor-I in U937 monocytes. This receptor, which is an integral membrane glycoprotein, undergoes a molecular switching between Ins(1,4,5)P_3 mediated- and SPHK dependent-Ca^{2+}release according to the differentiation state of the cell [14], thus changing the temporal profile of the Ca^{2+} response. In addition, our own work in the SH-SY5Y human neuroblastoma cell line indicates an Ins(1,4,5)P_3-independent but SPHK-dependent Ca^{2+} mobilizing response to LPA [16,17].

The mechanism by which SPHK, a cytosolic protein, can be activated by cell surface stimuli remains unclear, though there is evidence for a number of possible signaling pathways. PDGF-mediated SPHK activation in TRMP canine kidney epithelial cells (recombinantly expressing PDGF-β receptors) is inhibited by addition of the Ca^{2+} chelator BAPTA [18]. A similar Ca^{2+}-sensitive SPP production has been shown for GPCRs [19]. In addition, receptor-independent increases in intracellular Ca^{2+} levels, either via Ca^{2+} ionophores [18] or voltage-gated Ca^{2+} channels [20], activate SPHK. However, although SPHK binds calmodulin with high affinity in the presence of Ca^{2+} [21], thus supporting a role for Ca^{2+}-mediated SPHK activation, it should be noted that purified SPHK is insensitive to Ca^{2+} in the range 1–100 μM [21]. A second activation pathway involves acidic phospholipids, which despite the lack of a recognized binding domain, increases SPHK activity *in vitro* [22]. In this way, a phospholipase D-mediated increase in phosphatidic acid may be the mechanism through which antigen activation of SPHK and subsequent Ca^{2+} release occurs [14]. Another possibility is that SPHK is activated via protein-protein interactions. Thus, although Ca^{2+} signals were not investigated, activated TNFα-receptors recruit the adaptor protein TRAF2, which in turn binds to and activates SPHK [23]. Such recruitment of SPHK to protein signaling scaffolds near the plasma membrane may have additional important consequences, as this would bring SPHK into contact with its substrate, sphingosine. It is of interest that antigen [24] and GPCR (unpublished data) stimulation of SPHK also involves recruitment of the kinase to the plasma membrane.

Intracellular Target for SPP-mediated Ca^{2+} Release

Although progress is clearly being made on the signaling pathways leading to SPP production, frustratingly little is known about the intracellular targets responsible for Ca^{2+} release. SPP-mediated Ca^{2+} mobilization was first noted in 1990 by Ghosh and co-workers [7]. Using a permeabilized cell preparation, this group demonstrated that sphingosine and the related compound sphingosylphosphorylcholine (SPC) could release $^{45}Ca^{2+}$ from the endoplasmic reticulum (ER). As the sphingosine response involved a short time lag, and required the presence of ATP, it was suggested that sphingosine was being converted to SPP, and it was SPP that possessed Ca^{2+} release activity [7,8]. Direct SPP-mediated Ca^{2+} release has now been shown by a number of groups, either in permeabilized cell preparations [8,16,25] or via microinjection [15]. SPP is active over the concentration range 1–100 μM, and although SPP utilizes ostensibly the same intracellular Ca^{2+} pool as Ins(1,4,5)P_3, there is no requirement for InsP_3- or ryanodine-receptor activation [17]. Functional studies indicate the presence of both SPHK and the putative release

channel for SPP to be present in the ER [7,8]; however, the identity of the intracellular release channel remains elusive. A putative sphingolipid-mediated Ca^{2+} release channel, termed SCaMPER (sphingolipid Ca^{2+}-release mediating protein of the endoplasmic reticulum), has been cloned [26]. However, a more recent study suggests that SCaMPER is not an ion channel and is not associated with the ER [27]. Indeed it is not clear whether SCaMPER has any sort of interaction with SPP.

Due to the complicating effects of cell surface GPCRs for SPP, and because SPP production often occurs alongside increases in Ins(1,4,5)P_3, it has been difficult to look at the single cell and subcellular Ca^{2+} signals to SPP. Thus questions concerning the spatial and temporal aspects of SPP responses remain largely unanswered. It is of interest to note that the SPP dependent-, Ins(1,4,5)P_3 independent-, Ca^{2+} responses to antigen- [15] and LPA- [16] stimulation are similarly transient in nature. To date, there is no evidence that intracellular SPP is capable of producing Ca^{2+} oscillations; however, SPP-mediated Ca^{2+} release can stimulate subcellular Ca^{2+} puffs, presumably by a direct effect of the released Ca^{2+} on $InsP_3$ receptors [16].

Concluding Remarks

The role of SPP as an intracellular messenger will remain controversial until intracellular targets for SPP are clearly identified. Recent elegant work using a fluorescent bioassay for SPP demonstrates that PDGF-mediated increases in SPHK activity produce detectable increases in extracellular SPP that are capable of activating cell surface Edg-receptors [28]. Furthermore, SPP may itself be produced extracellularly via the extracellular export of SPHK [29], and so great care must be taken when investigating supposedly intracellular actions of SPP. As SCaMPER does not appear to be an appropriate intracellular target, attention needs to be focused on the identification and characterization of the intracellular Ca^{2+}-release channel for SPP. In addition, the use of molecular tools such as the recently described catalytically inactive form of SPHK [30] should allow for greater investigation into the role of SPP in mediating Ca^{2+} signals. Such experiments are essential for fully understanding the significance of SPP as an intracellular mediator for Ca^{2+} release.

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CHAPTER 126

Voltage-gated Ca^{2+} Channels

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Introduction

Voltage-gated Ca^{2+} channels mediate calcium entry into cells in response to membrane depolarization. Electrophysiological studies reveal different Ca^{2+} currents designated L-, N-, P-, Q-, R-, and T-type. The high-voltage-activated Ca^{2+} channels that have been characterized biochemically are complexes of a pore-forming $\alpha 1$ subunit of about 190 to 250 kDa, a transmembrane, disulfide-linked complex of $\alpha 2$ and δ subunits, an intracellular β subunit, and in some cases a transmembrane γ subunit. Ten $\alpha 1$ subunits, four $\alpha 2\delta$ complexes, four β subunits, and several γ subunits are known. The Ca_V1 family of $\alpha 1$ subunits conduct L-type Ca^{2+} currents, which initiate muscle contraction, endocrine secretion, sensory transduction, cardiac pacemaking, and gene transcription and are regulated primarily by second messenger-activated protein phosphorylation pathways. The Ca_V2 family of $\alpha 1$ subunits conduct N-type, P/Q-type, and R-type Ca^{2+} currents, which initiate rapid synaptic transmission and are regulated primarily by direct interaction with G proteins and SNARE proteins and secondarily by protein phosphorylation. The Ca_V3 family of $\alpha 1$ subunits conduct T-type Ca^{2+} currents, which are activated and inactivated more rapidly and at more negative membrane potentials than other Ca^{2+} channel types. The distinct structures and patterns of regulation of these three families of Ca^{2+} channels provides a flexible array of Ca^{2+} entry pathways in response to changes in membrane potential and a range of possibilities for regulation of Ca^{2+} entry by second messenger pathways and interacting proteins.

Physiological Roles of Voltage-gated Ca^{2+} Channels

Ca^{2+} channels in many different cell types activate upon membrane depolarization and mediate Ca^{2+} influx in response to action potentials and subthreshold depolarizing signals. Ca^{2+} entering the cell through voltage-gated Ca^{2+} channels serves as the second messenger of electrical signaling, initiating many different cellular events (Fig. 1). In cardiac and smooth muscle cells, activation of Ca^{2+} channels initiates contraction directly by increasing cytosolic Ca^{2+} concentration and indirectly by activating ryanodine-sensitive Ca^{2+} release channels in the sarcoplasmic reticulum [1,2]. In skeletal muscle cells, voltage-gated Ca^{2+} channels in the transverse tubule membranes interact directly with ryanodine-sensitive Ca^{2+} release channels in the sarcoplasmic reticulum and activate them to initiate rapid contraction [3,4]. The same Ca^{2+} channels in the transverse tubules also mediate a slow Ca^{2+} conductance that increases cytosolic concentration and thereby regulates the force of contraction in response to high-frequency trains of nerve impulses. In endocrine cells, voltage-gated Ca^{2+} channels mediate Ca^{2+} entry that initiates secretion of hormones [5]. In neurons, voltage-gated Ca^{2+} channels initiate synaptic transmission [6,7,8]. In many different cell types, Ca^{2+} entering the cytosol via voltage-gated Ca^{2+} channels regulates enzyme activity, gene expression, and other biochemical processes [9,10]. Thus, voltage-gated Ca^{2+} channels are the key signal transducers of electrical excitability, converting the electrical signal of the action potential in the cell surface membrane to an intracellular Ca^{2+} transient. Signal transduction in different cell types involves different molecular subtypes of voltage-gated Ca^{2+} channels, which mediate voltage-gated Ca^{2+} currents with different physiological, pharmacological, and regulatory properties.

Ca^{2+} Current Types Defined by Physiological and Pharmacological Properties

Since the first recordings of Ca^{2+} currents in cardiac myocytes [11,12], it has become apparent that there are multiple types of Ca^{2+} currents as defined by physiological and

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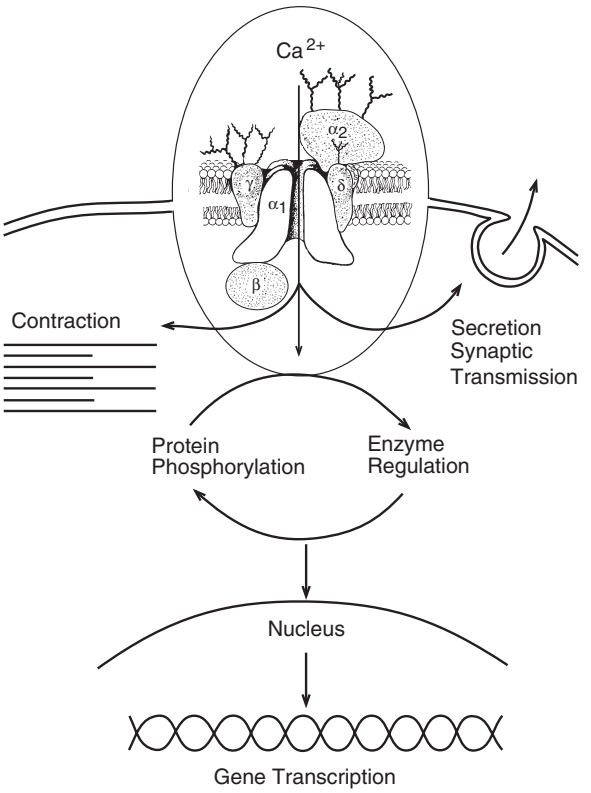


Figure 1 Ca^{2+} channels and signal transduction. Ca^{2+} entering cells initiates numerous intracellular events, including contraction, secretion, synaptic transmission, enzyme regulation, protein phosphorylation-dephosphorylation, and gene transcription. *Inset.* Subunit structure of voltage-gated Ca^{2+} channels. The five-subunit complex that forms high voltage-activated calcium channels is illustrated with a central, pore-forming $\alpha 1$ subunit, a disulfide-linked dimer of α_2 and δ glycoprotein subunits, an intracellular β subunit, and a transmembrane glycoprotein γ subunit.

pharmacological criteria [6,13–15]. In cardiac, smooth, and skeletal muscle, the major Ca^{2+} currents are distinguished by high voltage of activation, large single channel conductance, slow voltage-dependent inactivation, marked regulation by cAMP-dependent protein phosphorylation pathways, and specific inhibition by Ca^{2+} antagonist drugs, including dihydropyridines, phenylalkylamines, and benzothiazepines [16]. These Ca^{2+} currents have been designated L-type, as they are long-lasting when Ba^{2+} is the current carrier [17]. L-type Ca^{2+} currents are also recorded in endocrine cells, where they initiate release of hormones [18], and in neurons, where they are important in regulation of gene expression and in integration of synaptic inputs [9,10,14].

Voltage clamp studies of Ca^{2+} currents in starfish eggs [19] and recordings of Ca^{2+} action potentials in cerebellar Purkinje neurons [20] first revealed Ca^{2+} currents with different properties from L-type, and these were subsequently characterized in detail in voltage-clamped dorsal root ganglion neurons [17,21–23]. In comparison to L-type, these Ca^{2+} currents activate at much more negative membrane potentials, inactivate rapidly, deactivate slowly, have small single channel conductance, and are insensitive to Ca^{2+} antagonist drugs. They are designated low-voltage-activated Ca^{2+} currents for their negative voltage dependence [21] or T-type for their transient kinetics [17].

Whole-cell voltage clamp and single-channel recording from dissociated dorsal root ganglion neurons revealed an additional Ca^{2+} current, N-type [17]. In these initial experiments, N-type Ca^{2+} currents were distinguished by their intermediate voltage dependence and rate of inactivation—more negative and faster than L-type but more positive and slower than T-type [17]. They are insensitive to organic L-type Ca^{2+} channel blockers but blocked by the cone snail peptide ω-conotoxin GVIA [6,24]. This pharmacological profile has been the primary method to distinguish N-type Ca^{2+} currents, because the voltage dependence and kinetics of N-type Ca^{2+} currents in different neurons vary considerably.

Analysis of the effects of other peptide toxins revealed three additional Ca^{2+} current types. P-type Ca^{2+} currents, first recorded in Purkinje neurons [25], are distinguished by high sensitivity to the spider toxin ω-agatoxin IVA [26]. Q-type Ca^{2+} currents, first recorded in cerebellar granule neurons [27], are blocked by ω-agatoxin IVA with lower affinity. R-type Ca^{2+} currents in cerebellar granule neurons are resistant to the subtype-specific organic and peptide Ca^{2+} channel blockers [27] and may include multiple channel subtypes [28]. While L-type and T-type Ca^{2+} currents are recorded in a wide range of cell types, N-, P-, Q-, and R-type Ca^{2+} currents are most prominent in neurons.

Molecular Properties of Ca^{2+} Channels

Subunit Structure. Ca^{2+} channels purified from skeletal muscle transverse tubules are complexes of $\alpha 1$, β, and γ subunits, and the $\alpha 1$ and β subunits are substrates for cAMP-dependent protein phosphorylation [29,30]. More detailed biochemical analyses revealed an additional $\alpha 2\delta$ subunit co-migrating with the $\alpha 1$ subunit [31–34]. Analysis of the biochemical properties, glycosylation, and hydrophobicity of these five subunits led to a model comprising a principal transmembrane α_1 subunit of 190 kDa in association with a disulfide-linked $\alpha_2\delta$ dimer of 170 kDa, an intracellular phosphorylated β subunit of 55 kDa, and a transmembrane γ subunit of 33 kDa (Fig. 1, inset) [31].

The $\alpha 1$ subunit is a protein of about 2000 amino acid residues with an amino acid sequence and predicted transmembrane structure like the previously characterized, pore-forming α subunit of sodium channels [35] (Fig. 2). The amino acid sequence is organized in four repeated domains (I to IV), each of which contains six transmembrane segments (S1 to S6) and a membrane-associated loop between transmembrane segments S5 and S6. As expected from biochemical analysis [31], the intracellular β subunit has predicted alpha helices but no transmembrane segments [36] (Fig. 2), while the γ subunit is a glycoprotein with four transmembrane segments [37] (Fig. 2). The cloned $\alpha 2$ subunit has many glycosylation sites and several hydrophobic sequences [38], but biosynthesis studies indicate that it is an extracellular,

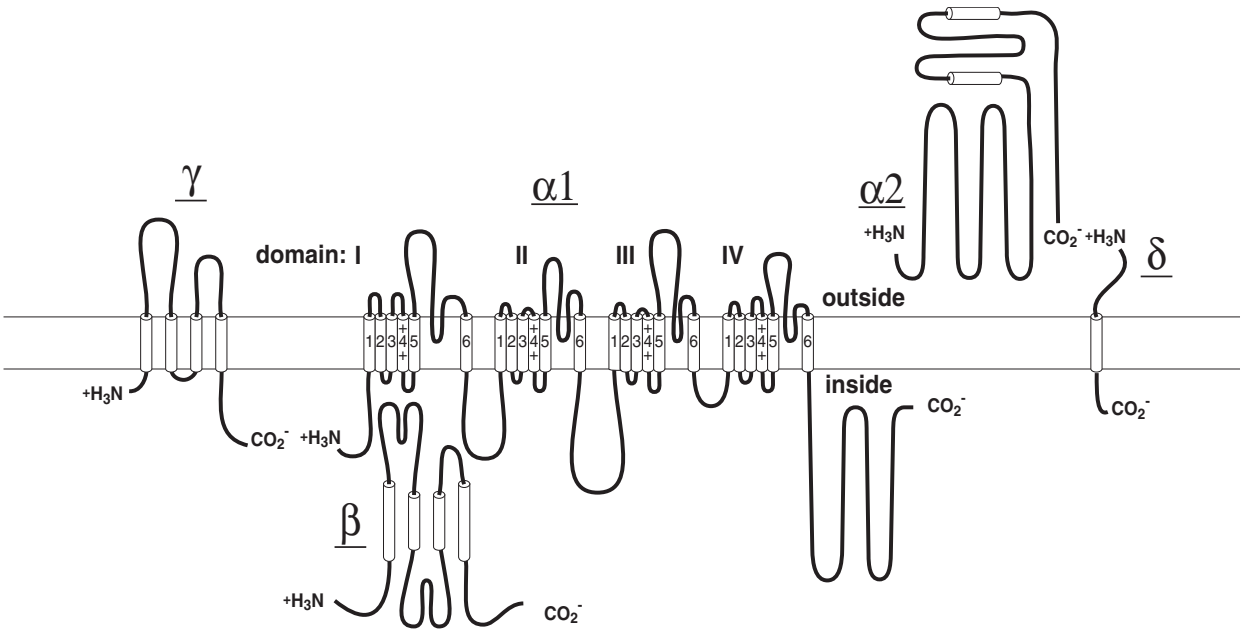


Figure 2 Transmembrane organization of voltage gated Ca^{2+} channels. The primary structures of the subunits of voltage-gated Ca^{2+} are illustrated. *Cylinders* represent probable alpha helical transmembrane segments. *Bold lines* represent the polypeptide chains of each subunit with length approximately proportional to the number of amino acid residues.

extrinsic membrane protein, attached to the membrane through disulfide linkage to the δ subunit [39] (Fig. 2). The δ subunit is encoded by the 3′ end of the coding sequence of the same gene as the α2 subunit, and the mature forms of these two subunits are produced by posttranslational proteolytic processing and disulfide linkage [40,41] (Fig. 2).

Purification of cardiac Ca^{2+} channels revealed subunits of the sizes of the α1, α2δ, β, and γ subunits of skeletal muscle Ca^{2+} channels [42–45], and immunoprecipitation of Ca^{2+} channels from neurons labeled by dihydropyridine Ca^{2+} antagonists revealed α1, α2δ, and β subunits but no γ subunit [46]. Together, these results suggest a similar subunit composition for L-type Ca^{2+} channels in cardiac and skeletal muscle and in neurons.

Purification and immunoprecipitation of N-type Ca^{2+} channels labeled by ω-conotoxin GVIA from brain membrane preparations revealed α1, α2δ, and β subunits [47,48]. Similarly, purified P/Q-type Ca^{2+} channels are composed of α1, α2δ, and β subunits [49–51]. In addition, more recent experiments have unexpectedly revealed a novel γ subunit, which is the target of the *stargazer* mutation in mice [52], and a related series of γ subunits expressed in brain and other tissues [53,54]. These γ-subunit-like proteins can modulate the voltage dependence of P/Q-type Ca^{2+} currents, so they may be associated with these Ca^{2+} channels *in vivo* [52]. If these new γ subunits are indeed associated with all neuronal Ca^{2+} channels, their subunit composition would be identical to that of skeletal muscle Ca^{2+} channels defined in biochemical experiments [31] (Fig. 2).

Functions of Ca^{2+} Channel Subunits. The initial analyses of functional expression of Ca^{2+} channel subunits were carried out with skeletal muscle Ca^{2+} channels. Expression of the α1 subunit is sufficient to produce functional skeletal muscle Ca^{2+} channels, but with low expression level and abnormal kinetics and voltage dependence of the Ca^{2+} current [55]. Co-expression of the α2δ subunit and especially the β subunit enhances the level of expression and confers more normal gating properties [56,57]. As for skeletal muscle Ca^{2+} channels, co-expression of β subunits has a large effect on the level of expression and the voltage dependence and kinetics of gating of cardiac and neuronal Ca^{2+} channels. In general, the level of expression is increased and the voltage dependence of activation and inactivation is shifted to more negative membrane potentials, and the rate of inactivation is increased. However, these effects are different for the individual β subunit isoforms (reviewed in [58,59]). For example, co-expression of the β2a subunit slows inactivation in most subunit combinations. In contrast, co-expression of α2δ subunits [58,59] and γ subunits [52] has much smaller functional effects.

Ca^{2+} Channel Diversity. The different types of Ca^{2+} currents are primarily defined by different α1 subunits. The primary structures of ten distinct Ca^{2+} channel α1 subunits have been defined by homology screening, and their function has been characterized by expression in mammalian cells or Xenopus oocytes. These subunits can be divided into three structurally and functionally related families (Ca_V1, Ca_V2, and Ca_V3) (Table I, [60]). L-type Ca^{2+} currents are mediated by the Ca_V1 type of α1 subunits, which have about 75% amino acid sequence identity among them [35,61,62]. The Ca_V2 type Ca^{2+} channels form a distinct subfamily with less than 40% amino acid sequence identity with Ca_V1 α1 subunits but greater than 70% amino acid sequence identity among themselves. Cloned $Ca_V2.1$ subunits [63,64] form

Table I Subunit Composition and Function of Ca^{2+} Channel Types

Ca^{2+} channel type	α_1 subunits	Specific blocker	Principal physiological functions	Inherited diseases
L	$Ca_v1.1$	DHPs	Excitation-contraction coupling in skeletal muscle Regulation of transcription	Hypokalemic periodic paralysis
	$Ca_v1.2$	DHPs	Excitation-contraction coupling in cardiac and smooth muscle Regulation of enzyme activity Regulation of transcription	
	$Ca_v1.3$	DHPs	Endocrine secretion Cardiac pacemaking Auditory transduction	
	$Ca_v1.4$	DHPs	Visual transduction	Stationary night blindness
N	$Ca_v2.1$	ω-CTx-GVIA	Neurotransmitter release Dendritic Ca^{2+} transients	Migraine Cerebellar ataxia Absence seizures (in mice)
P/Q	$Ca_v2.2$	ω-Agatoxin	Neurotransmitter release Dendritic Ca^{2+} transients	
R	$Ca_v2.3$	SNX-482	Neurotransmitter release Dendritic Ca^{2+} transients	
T	$Ca_v3.1$ $Ca_v3.2$ $Ca_v3.3$	None	Pacemaking and repetitive firing	

Abbreviations: DHP, dihydropyridine; ω-CTx-GVIA, ω-conotoxin GVIA from the cone snail *Conus geographus*; SNX-482, a synthetic version of a peptide toxin from venom of the tarantula *Hysterocrates gigas.*

P- or Q-type Ca^{2+} channels, which are inhibited by ω-agatoxin IVA [65–67]. $Ca_V2.2$ subunits form N-type Ca^{2+} channels with high affinity for ω-conotoxin GVIA [68,69]. Cloned $Ca_V2.3$ subunits form R-type Ca^{2+} channels, which are resistant to both organic Ca^{2+} antagonists specific for L-type Ca^{2+} currents and the peptide toxins specific for N-type or P/Q-type Ca^{2+} currents [27,70,71]. T-type Ca^{2+} currents are mediated by the Ca_V3 channels [72]. These α1 subunits are only distantly related to the other known homologs, with less than 25% amino acid sequence identity. These results reveal a surprising structural dichotomy between the T-type, low-voltage-activated Ca^{2+} channels and the high-voltage-activated Ca^{2+} channels. Evidently, these two lineages of Ca^{2+} channels diverged very early in evolution of multicellular organisms.

The diversity of Ca^{2+} channel structure and function is substantially enhanced by multiple β subunits. Four β subunit genes have been identified, and each is subject to alternative splicing to yield additional isoforms (reviewed in [58,73]). In Ca^{2+} channel preparations isolated from brain, each Ca^{2+} channel α1 subunit that has been investigated is associated with multiple β subunits, although there is a different rank order in each case [74,75]. The different β subunit isoforms cause different shifts in the kinetics and voltage dependence of gating, so association with different β subunits can substantially alter the physiological function of an α1 subunit. Genes encoding four α2δ subunits have been described [76], but the α2δ isoforms produced by these different genes have relatively small functional effects on channel gating and expression. A new family of γ subunits has been recently described [52–54], which has small, but significant effects on the voltage dependence of Ca^{2+} channel gating.

Molecular Basis for Ca^{2+} Channel Function

Intensive studies of the structure and function of the related pore-forming subunits of Na^+, Ca^{2+}, and K^+ channels have led to identification of their principal functional components (reviewed in [77–80]). Each domain of the principal subunits consists of six transmembrane alpha helices (S1 through S6) and a membrane-associated loop between S5 and S6 (Fig. 2). The S4 segments of each homologous domain serve as the voltage sensors for activation, moving outward and rotating under the influence of the electric field and initiating a conformational change that opens the pore. The S5 and S6 segments and the membrane-associated pore loop between them form the pore lining of the voltage-gated ion channels. The narrow external pore is lined by the pore loop, which contains a pair of glutamate residues in each domain that are required for Ca^{2+} selectivity. Remarkably, substitution of only three amino acid residues in the pore loops between the S5 and S6 segments in domains II, III, and IV of sodium channels is sufficient to confer Ca^{2+} selectivity [81]. The inner pore is lined by the S6 segments, which form the receptor sites for the pore-blocking Ca^{2+} antagonist drugs specific for L-type Ca^{2+} channels [82–84]. All Ca^{2+} channels share these general structural features, but the amino acid residues that confer high affinity for the organic Ca^{2+} antagonists used in

therapy of cardiovascular diseases are present only in the Ca_V1 family of Ca^{2+} channels, which conduct L-type Ca^{2+} currents.

Ca^{2+} Channel Regulation

The activity of voltage-gated Ca^{2+} channels is tightly regulated by second messenger signal transduction pathways through direct interactions with G proteins and other intracellular signaling proteins and through protein phosphorylation (reviewed in [85]). Regulation of L-type Ca^{2+} currents in cardiac, skeletal, and smooth muscle cells by the β-adrenergic receptor/cAMP pathway involves a signaling complex of the $Ca_V1.1$ or $Ca_V1.2$ channels, an A kinase anchoring protein designated AKAP-15 or AKAP-18, and PKA targeted to the channel by AKAP binding via a leucine zipper to a site in the C-terminal domain of the α1 subunit [86–89]. A functionally similar signaling complex in the brain includes the β-adrenergic receptor itself, adenylate cyclase, the AKAP MAP 250, and PKA [90]. This signaling pathway is also engaged by other G protein-coupled receptors that activate adenylate cyclase. This pathway regulates beating rate and contractility in the heart, vascular tone, skeletal muscle contractile force, and gene expression in neurons, myocytes, endocrine cells, and other cell types.

The activity of the Ca_V2 family of channels is regulated primarily by direct interaction with G proteins and other signaling proteins and secondarily by protein phosphorylation (reviewed in [8,91]). Many different neurotransmitters and hormones activate G protein-coupled receptors, which release Gβγ subunits that inhibit Ca^{2+} channel activity [92–94]. G protein inhibition can be reversed by strong depolarization, resulting in facilitation of Ca^{2+} channel activity, and by phosphorylation by protein kinase C [8,91,95–98]. In addition, the activity of the Ca_V2 family of channels is regulated by interaction with the SNARE proteins that are required in exocytosis [99–102]. This regulatory mechanism appears designed to focus Ca^{2+} entry on Ca^{2+} channels with exocytotic vesicles docked nearby.

Ca^{2+} itself also regulates the activity of both Ca_V1 and Ca_V2 channels. Low levels of Ca^{2+} entry cause facilitation of Ca^{2+} channel activity, and higher levels cause Ca^{2+}-dependent inactivation [103–107]. Both processes involve binding to calmodulin and interaction with specific calmodulin-binding sites in the C-terminal domain of the Ca^{2+} channel. In repetitively firing neurons and in cardiac myocytes, this mechanism allows integration of Ca^{2+} signals as a function of frequency of action potential generation. This mode of regulation also serves to tune the Ca^{2+} entry to the needs of intracellular regulatory processes and prevent inappropriately wide swings in local Ca^{2+} concentration.

Conclusion

Voltage-gated Ca^{2+} channels are essential signal transducers, converting cell surface electrical signals to intracellular Ca^{2+} transients that initiate many physiological and biochemical events. Recent research has defined their molecular properties, identified many genes that encode their subunits and provide diversity of function, and revealed their complex interaction with cellular regulatory pathways. Further work on this protein family will give essential insights into cellular signaling and its dysfunction in diseases as diverse as epilepsy, migraine, cardiac arrhythmia, hypertension, and diabetes.

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CHAPTER 127

Store-operated Ca^{2+} Channels

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Capacitative Calcium Entry

Many signaling pathways involve the generation of cytoplasmic Ca^{2+} signals. As reviewed elsewhere in this volume, in many instances these Ca^{2+} signals arise as a result of the Ca^{2+}-mobilizing actions of the intracellular messenger, inositol 1,4,5-trisphosphate (IP_3) (see also [1]). IP_3 binds to specific receptor/channels on the endoplasmic reticulum; the binding of IP_3 results in channel opening and release of stored Ca^{2+} to the cytoplasm. In most cell types, this release of Ca^{2+} from intracellular stores is accompanied by an accelerated entry of Ca^{2+} across the plasma membrane. A variety of mechanisms may be responsible for this entry of Ca^{2+} (reviewed in [2,3]). One mechanism that appears to be ubiquitous in nonexcitable cells, and is found in a number of excitable cell types, is *capacitative calcium entry*, also known at *store-operated calcium entry* [4–6]. The signal for capacitative calcium entry appears to be the fall in the concentration of Ca^{2+} in the endoplasmic reticulum or in a specialized subcompartment of it.

While the physiological mechanism for depleting stores and activating capacitative calcium entry generally involves IP_3-mediated discharge of Ca^{2+} stores, a number of experimental manipulations can bypass receptor activation to empty Ca^{2+} stores. Inhibitors of sarcoplasmic endoplasmic reticulum Ca^{2+} ATPases, such as thapsigargin, cause passive depletion of Ca^{2+} stores and are thus efficient activators of capacitative calcium entry. In electrophysiological studies, utilizing the patch clamp technique to examine whole-cell store-operated membrane currents, IP_3 can be included in the patch pipet, or Ca^{2+} stores can be depleted simply by high concentrations of a Ca^{2+} chelator. The first store-operated current to be described was the Ca^{2+} release-activated Ca^{2+} current (I_{crac}) characteristically found in hematopoetic cells [7]. Noise analysis indicates that the unitary conductance of single CRAC channels is likely to be too small to measure [8]. However, in other cell types, the electrophysiological profile of store-operated currents appears to differ significantly from I_{crac}, and in some instances single channels have been observed [9–12]. In these instances, the whole cell current resulting from store depletion is always less Ca^{2+} selective than I_{crac}. In some cases the whole cell currents appear to be nonselective cation currents [12–15]. This finding indicates that the molecular composition of store-operated channels differs among cell types, and it is also possible therefore that multiple mechanisms exist for gating these channels.

Store-operated Channels

The leading contenders for molecular components of store-operated channels are members of the *trp* gene superfamily [16]. In *Drosophila*, the *trp* gene encodes a subunit of a cation channel regulated by a light-sensitive phospholipase C [17]. Seven mammalian *trp* genes with 30–40% sequence similarity to *Drosophila trp* have been cloned, and the proteins they encode have been designated TRPC1 … TRPC7, which fall into four groups based on structural similarities: TRPC1, TRPC2, TRPC3/6/7, TRPC4/5. While the results of transfection experiments sometimes vary from one laboratory to another, there are a gratifying number of instances in which these proteins appear to form or contribute to the formation of store-operated channels (TRPC1 [18,19], TRPC2 [20,21], TRPC3 [22], TRPC4 [23,24]). Generally, the channels formed in these expression studies are not highly calcium selective and thus may be candidates for the less selective channels

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found in nonhematopoetic cells. CaT1 (also known as TRPV6), a member of the *trp* superfamily, which is more distantly related to *trp* than the TRPC proteins, was shown in one study to express as a highly Ca^{2+}-selective store-operated channel [25]; however, these findings are at present controversial [26].

Mechanism of Activation of Store-operated Channels

There are two distinct proposals for the mechanism coupling Ca^{2+} store depletion to activation of capacitative calcium entry. The earliest idea was that a novel messenger molecule might be released from the endoplasmic reticulum, and this messenger would then diffuse to the plasma membrane and activate the store-operated channels [4]. A number of studies have published evidence for such a messenger, although its structure has not been elucidated [27–31]. The alternative proposal is based on analogy with the mechanism of coupling of L-type Ca^{2+} channels to ryanodine receptors in skeletal muscle. Thus, the conformational coupling model [6,32] proposes that IP_3 receptors in the endoplasmic reticulum interact directly with plasma membrane Ca^{2+} channels, perhaps members of the *trp* family. In support of this idea, TRPC3, when expressed in HEK293 cells, forms channels that are activated in a manner dependent on IP_3 and the IP_3 receptor [33–35]. However, in this expression system, TRPC3 is not a store-operated channel, rather its activation requires agonist activation of phospholipase C and production of IP_3. Also, in DT40 B lymphocytes, when IP_3 receptors were eliminated by gene disruption, store-operated Ca^{2+} entry was unaffected [36]. Expression of TRPC3 in these cells produced a store-operated channel whose activity was only partially reduced in the absence of IP_3 receptors [22]. Thus, conformational coupling may play a role in activation of store-operated channels in some situations, while a second messenger mode of signaling may be involved in others.

Summary

Capacitative calcium entry is a process whereby depletion of Ca^{2+} from intracellular stores leads to the activation of Ca^{2+} channels in the plasma membrane and accelerated entry of Ca^{2+} into the cytoplasm of cells. Current research focuses on the molecular nature of the channels and the mechanism of coupling the channels to Ca^{2+} store depletion. Leading contenders for the store-operated channels are members of the *trp* gene family, although no one gene has been definitively linked to a specific store-operated channel. The mechanism of activation of the channels may involve interactions between the plasma membrane channels and endoplasmic reticulum IP_3 receptors in some instances or a diffusible Ca^{2+} influx factor in others. Continued work is needed to clarify and resolve these important issues.

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CHAPTER 128

Arachidonic Acid-regulated Ca^{2+} Channel

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Introduction

The arachidonic acid-regulated Ca^{2+} (ARC) channel is a recently identified Ca^{2+}-selective conductance that is distinct from the store-operated conductances (e.g. CRAC channels) discussed by Putney in the preceding chapter. These ARC channels play a key role in $[Ca^{2+}]_i$ signaling in nonexcitable cells in that they appear to provide the predominant pathway for the receptor-activated entry of Ca^{2+} at low, physiologically relevant, agonist concentrations. Under these conditions, $[Ca^{2+}]_i$ signals typically take the form of repetitive $[Ca^{2+}]_i$ oscillations, and the receptor-activated entry of Ca^{2+} acts to modulate the frequency of these oscillations [1–4]. The realization that during such signals intracellular Ca^{2+} stores are only transiently and/or partially depleted raised the question of whether a sufficient "capacitative signal" to activate the store-operated entry of Ca^{2+} would be generated under these conditions. Subsequent studies revealed that Ca^{2+} entry at these agonist concentrations displayed several features that were inconsistent with the capacitative model [5, 6] (see [6] for details), prompting a search for the basis of this apparent noncapacitative mechanism. The result was that in several different cell types such entry was found to be specifically dependent on the receptor-mediated generation of arachidonic acid [7–10]. Thus, arachidonic acid was shown to be generated by the same low agonist concentrations that induce the noncapacitative entry of Ca^{2+}, and inhibition of this arachidonic acid generation specifically and rapidly blocked the associated entry of Ca^{2+}. Moreover, the direct application of exogenous arachidonic acid activated an entry of Ca^{2+} (typically measured as Mn^{2+} quench rate) that was independent of any depletion of agonist-sensitive stores. Especially significant, the use of blockers of arachidonic acid metabolism and/or nonmetabolizable arachidonic acid analogues (e.g. ETYA) indicated that the observed effects reflected the actions of arachidonic acid itself, rather than any of its metabolites [8].

Identification and Characterization of ARC Channels

Although an agonist-activated, arachidonic acid-dependent entry of Ca^{2+} could be demonstrated in a variety of cells, it was unclear whether this involved some kind of "store-independent" activation of the already well-known capacitative Ca^{2+} entry channels (e.g. CRAC channels) or the activation of a novel channel type. This question was resolved by the identification of a novel Ca^{2+}-selective current that was specifically activated by low concentrations of arachidonic acid and that was entirely distinct from the endogenous "CRAC-like" store-operated Ca^{2+}-selective current recorded in the same cells [11]. This current was named I_{ARC} (for *a*rachidonate-*r*egulated *c*alcium current), and was first described in HEK293 cells [11], but similar currents have since been observed in mouse parotid cells, HeLa cells, and RBL cells (unpublished observations). When measured in either traditional whole-cell or perforated-patch modes, I_{ARC} is seen as a small inward current at negative holding potentials, with a current-voltage relationship displaying marked inward rectification and a reversal potential significantly greater than +30 mV (Fig. 1A) [11]. The current is potently blocked by 50 μM La^{3+} (Fig. 1A) and somewhat less effectively blocked by 50 μM Cd^{2+}. Substitution of

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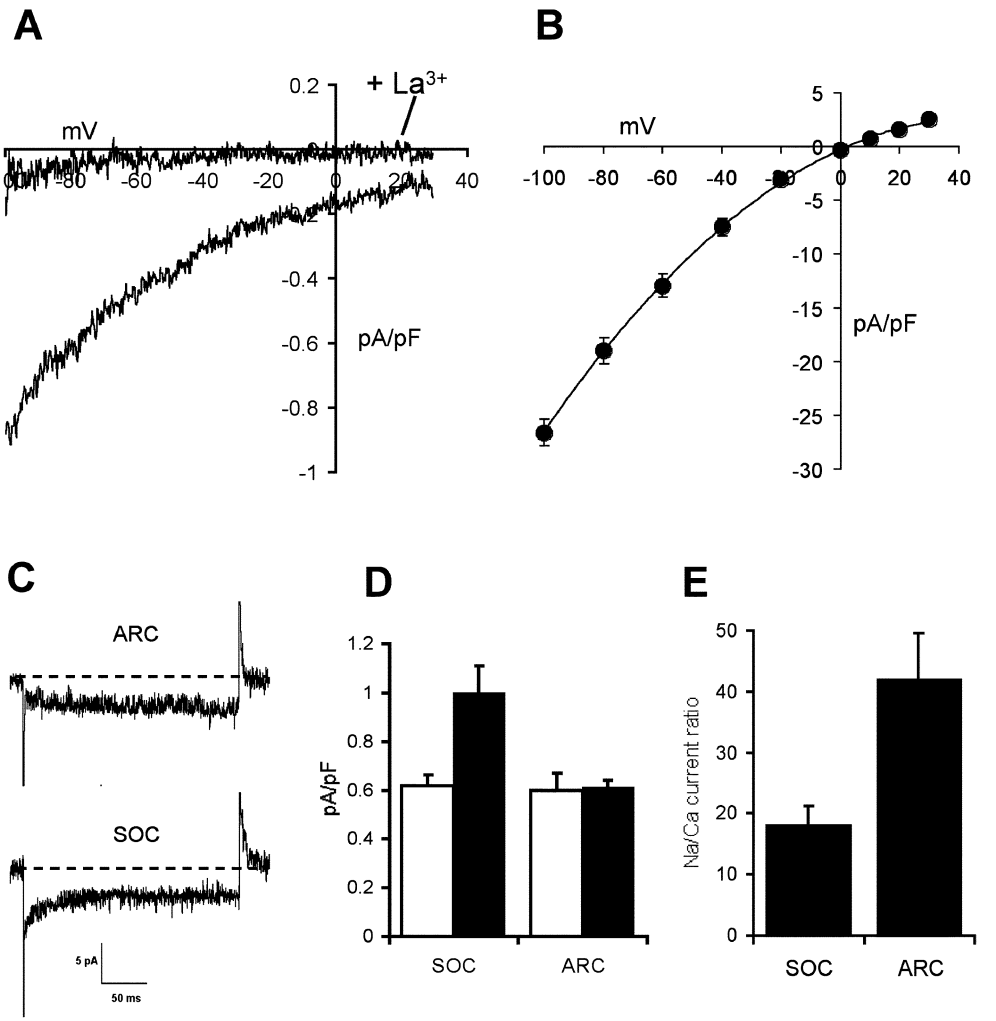


Figure 1 Characteristics of the arachidonic acid-activated current I_{ARC}. (A) Representative I/V curves for macroscopic I_{ARC} in the presence and absence of La^{3+} (50 μM). I_{ARC} was activated by exogenous addition of 8 μM arachidonic acid. Current-voltage relationships were recorded using 150 ms voltage ramps from −100 to +30 mV. External Ca^{2+} concentration was 20 mM. Adapted from [11]. (B) Mean I/V curve for the arachidonate-activated monovalent current recorded in the nominal absence of extracellular divalent cations. Internal $[Mg^{2+}]$ was 8 mm. ARC currents were activated and measured as described in A. (C) Comparison of fast inactivation in I_{ARC} (activated as described in A) and the endogenous CRAC-like store-operated current (I_{SOC}) in HEK293 cells. I_{SOC} was activated by inclusion of 2 μM adenophostin in the pipette solution. Representative recordings of the change in the current measured during a 250 ms pulse to −80 mV. Redrawn from [11]. (D) Effect of substituting internal cesium (open columns) with sodium (filled columns) on the magnitude of the endogenous store-operated currents (SOC) and the arachidonic acid-activated currents (ARC) determined at −80 mV. Store-operated and arachidonic acid-activated currents were determined as described. Data from [13]. (E) Sodium to calcium current ratios for store-operated currents (SOC) and arachidonic acid-activated currents (ARC). Maximal sodium current densities were measured at −80 mV by whole-cell patch clamp in the nominal absence of external divalent cations. SOC and ARC currents were activated as described above. Data from [13].

external Na^+ with $NMDG^+$ has negligible effects on the I/V, thus demonstrating that I_{ARC} is highly Ca^{2+}-selective.

Like other highly Ca^{2+}-selective conductances (including voltage-gated Ca^{2+} channels and CRAC channels), the ARC channels become permeable to monovalent cations on removal of external divalent ions (Fig. 1B). All these features of I_{ARC} are very similar to the archetypal store-operated conductance I_{CRAC} and the endogenous store-operated Ca^{2+}-selective current (I_{SOC}) in HEK293 cells. However, further examination revealed marked differences between I_{ARC} and these store-operated conductances. Unlike I_{CRAC} and I_{SOC}, I_{ARC} shows no Ca^{2+}-dependent fast-inactivation (Fig. 1C) and is largely insensitive to reductions in extracellular pH [11]. I_{ARC} is also insensitive to 2-APB (unpublished observations), which has been shown to potently inhibit I_{CRAC} in a variety of cell types in a manner independent of its originally reported actions on $InsP_3$ receptors [12]. In addition, substitution of the normal Cs^+-based internal (pipette) solution with a corresponding Na^+-based solution results in an approximate 70% increase in the magnitude of I_{SOC} [13], whereas similar substitution is without effect on the magnitude of I_{ARC} (Fig. 1D). Differences are also seen when the conductances are recorded in their monovalent-permeable modes in the nominal absence of external divalent cations [14]. For example, the ratio of the recorded

monovalent (Na^+) currents relative to the normal Ca^{2+}-selective currents range from 5 to 20 for I_{CRAC} and I_{SOC}, whereas the corresponding ratio for I_{ARC} is greater than 40 (Fig. 1E). Additional differences are seen in the rates and apparent nature of the spontaneous decline in the monovalent currents [14]. Of course, the fundamental distinction between I_{ARC} and the store-operated conductances is that activation of I_{ARC} is specifically dependent on the generation or addition of arachidonic acid and is entirely independent of store-depletion. Moreover, I_{ARC} and I_{SOC} are additive in the same cell and I_{ARC} can be readily activated in cells whose Ca^{2+} stores have been maximally depleted, e.g. by treatment with thapsigargin or with the high-affinity $InsP_3$-receptor agonist adenophostin A [11]. Finally, the possibility that arachidonic acid was merely modifying the properties of endogenous store-operated conductances was eliminated by the demonstration that although Ca^{2+}-sensitive adenylyl cyclases are uniquely sensitive to Ca^{2+} entering via the capacitative pathway, they fail to respond to Ca^{2+} entering via the ARC channels [15]. Thus, the ARC channels are entirely distinct, both physically and spatially, from the store-operated channels.

Consistent with the previously observed arachidonic acid-dependent activation of noncapacitative Ca^{2+} entry, significant activation of I_{ARC} is detectible at concentrations of exogenous arachidonic acid as low as 2–3 μM. Such concentrations are likely to be physiologically meaningful as they lie within the typical range of the Km for the intracellular cyclo-oxygenases and lipoxygenases responsible for metabolizing arachidonic acid. It is important that use of higher concentrations be avoided, as it is clear that fatty acids such as arachidonic acid can have a variety of nonspecific effects (e.g. on membrane fluidity) at such concentrations. As demonstrated earlier for the arachidonic acid-dependent noncapacitative entry of Ca^{2+} (see above), experiments using the nonmetabolizable arachidonic acid analogue ETYA indicate that the activation of I_{ARC} is dependent on the fatty acid itself rather than any metabolite. Other poly-unsaturated fatty acids (e.g. linoleic acid) are also able to activate I_{ARC}, but none are as effective as arachidonic acid. Saturated (e.g. palmitic), and mono-unsaturated (e.g. oleic) fatty acids are completely without effect. Finally, the diacylgercerol analogue OAG fails to activate I_{ARC} even at concentrations as high as 100 μM (unpublished observations).

Specific Activation of ARC Channels by Low Agonist Concentrations

Together, the biophysical, biochemical, and pharmacological data demonstrate that the ARC channels represent a novel Ca^{2+} entry pathway entirely distinct from those activated by store depletion, and suggest that they are likely to be responsible for the arachidonic acid-dependent noncapacitative entry of Ca^{2+} seen in a variety of cells at low agonist concentrations. This suggestion was confirmed by the demonstration of the specific activation of I_{ARC} by the same low concentrations of agonists that had been shown to activate the noncapacitative entry of Ca^{2+}. Activation was achieved by using HEK 293 cells stably transfected with the m3 muscarinic receptor and a protocol based on the previous demonstration of the additive nature of the two Ca^{2+}-selective conductances (I_{ARC} and I_{SOC}) in the same cell [13]. Application of a low concentration (0.5 μM) of the muscarinic agonist carbachol to cells in which I_{SOC} had been maximally activated (using adenophostin A in the pipette solution) resulted in the development of an additional inward current at −80 mV (Fig. 2A). The I/V curve of this carbachol-activated current (after subtraction of the underlying I_{SOC}) showed marked inward rectification and a positive reversal potential (> +30 mV) (Fig. 2B). Development of the current was blocked by atropine and reversibly blocked by isotetrandrine, an inhibitor of the receptor-activation of arachidonic acid generation that does not affect the simultaneous stimulation of phospholipase C [8]. Thus, the current activated by carbachol under these conditions was dependent on the muscarinic receptor-mediated generation of arachidonic acid and was therefore likely to be I_{ARC}. This was confirmed by demonstrating that the carbachol-activated current showed no fast-inactivation, was unaffected by substituting Na^+ for Cs^+ in the pipette solution, and displayed a monovalent (Na^+) current in the nominal absence of external divalent ions that was more than 45 times larger than the corresponding normal Ca^{2+}-selective current [13]. As discussed above, these features are uniquely characteristic of I_{ARC}, confirming that the additional current activated by low concentrations of carbachol specifically reflects the activity of the ARC channels.

Activation of I_{ARC} by carbachol was measurable at concentrations that were just sufficient to initiate detectable $[Ca^{2+}]_i$ signals in the same cells (0.2 μM) and reached a maximum at 1μM (Fig. 2C) [13]. $[Ca^{2+}]_i$ signals within this concentration range are typically oscillatory in nature, and previous evidence had indicated that the associated entry of Ca^{2+} is both noncapacitative and entirely dependent on the generation of arachidonic acid [8]. Thus, the data demonstrated that stimulation with low agonist concentrations results in the specific activation of I_{ARC}, which provides the predominant route for Ca^{2+} entry under these conditions.

Roles of ARC Channels and SOC/CRAC Channels in $[Ca^{2+}]_i$ Signals: "Reciprocal Regulation"

Although the ARC channels provide the major route for Ca^{2+} entry at low agonist concentrations, the additive nature of the two conductances I_{ARC} and I_{SOC} would lead to the prediction that entry at high agonist concentrations should be via a combination of both ARC and SOC channels. However, this is inconsistent with earlier evidence indicating that addition of an agonist to cells whose store-operated entry had been maximally activated by treatment with thapsigargin fails to induce any obvious increase in $[Ca^{2+}]_i$ [16]. Indeed, such data were widely used to support the proposition that the capacitative pathway was the sole mechanism responsible for the agonist-induced increase in the entry of Ca^{2+} in nonexcitable cells. Examination of the rate of Ca^{2+} entry at

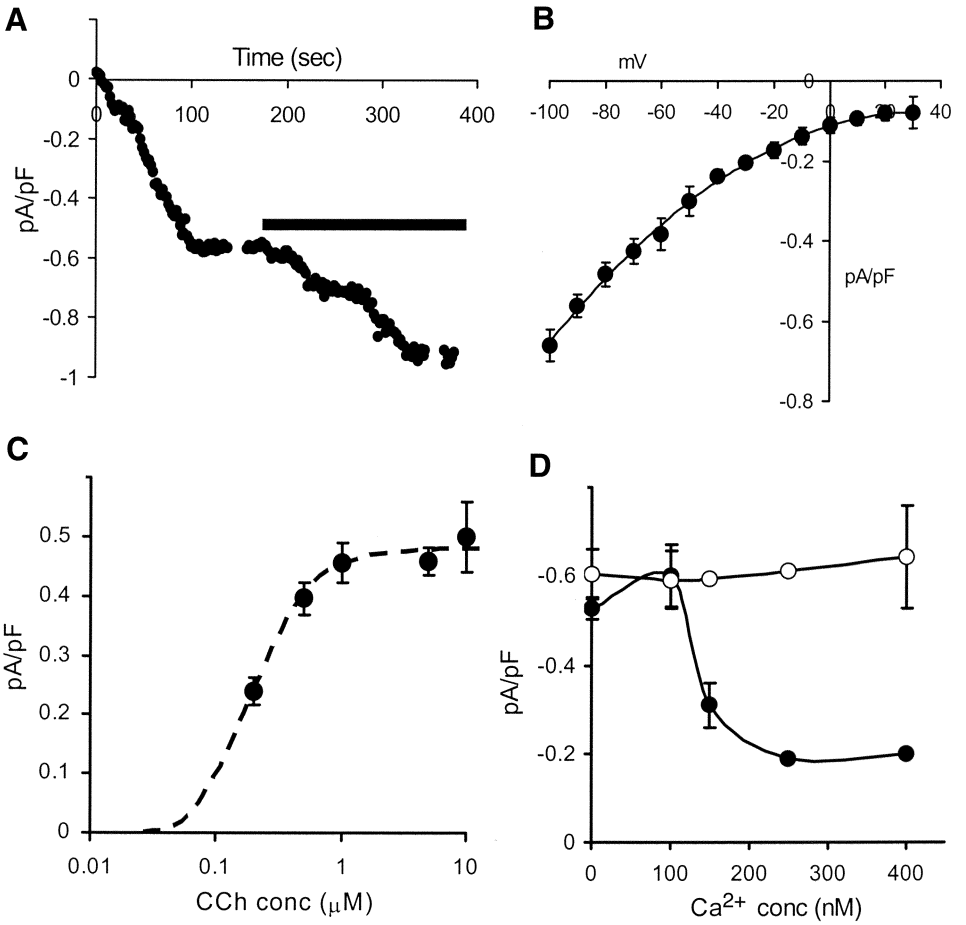


Figure 2 Activation of ARC currents by carbachol in m3-HEK cells. (A) Representative trace showing the activation of an additional inward current measured at −80 mV on addition of low concentrations of carbachol after maximal activation of store-operated currents. On going whole-cell (at time zero), inclusion of adenophostin A (2 μM) in the pipette solution rapidly depletes internal Ca^{2+} stores and maximally activates the endogenous store-operated current, I_{SOC}. Subsequent addition of carbachol (CCh, 0.5 μM, black bar) results in the development of additional inward current. Data taken from [13]. (B) Mean I/V curve for the current activated by carbachol (0.5 μM). Individual curves were obtained from voltage ramps after subtraction of the corresponding I/V curve for maximally activated I_{SOC} in the same cell. Taken from [13]. (C) The magnitude of the ARC current activated by different carbachol concentrations. Carbachol-activated ARC currents were measured after maximal activation of store-operated currents as described in B. Taken from [13]. (D) Effects of different buffered internal Ca^{2+} concentrations on the magnitude of SOC (maximally activated with 2 μM adenophostin A, open circles) and ARC currents (activated by 8 μM arachidonic acid, filled circles). Currents were measured at −80 mV. Taken from [13].

high agonist concentrations (using Mn^{2+} quench) confirmed that this was essentially entirely via the capacitative pathway and no significant arachidonic acid-dependent contribution could be detected, despite an increasing generation of arachidonic acid over the same agonist concentration range [13]. This apparent contradiction was resolved when it was revealed that I_{ARC} is potently inhibited by sustained increases in $[Ca^{2+}]_i$ above resting values [13] such as would be induced in cells stimulated with high agonist concentrations (Fig. 2D). As yet, the precise mechanism for this Ca^{2+}-dependent inhibition of I_{ARC} is unknown, but it is clear that it does not involve any action of Ca^{2+} entering through the channel itself as inhibition is seen even when I_{ARC} is carrying only monovalent cations (i.e. in the nominal absence of external divalent ions) [13]. Instead, it seems to reflect an effect of the general or global cytosolic Ca^{2+} concentration. This inhibition of the ARC channels by elevations in $[Ca^{2+}]_i$ develops only slowly, taking some two minutes to reach completion [13]. This means that the transient increases in $[Ca^{2+}]_i$ associated with oscillatory Ca^{2+} signals (each typically lasting only a few seconds) would have a negligible effect on the activity of I_{ARC} and would not impair the role of the ARC channels in the entry of Ca^{2+} under these conditions. Only with a prolonged elevation of $[Ca^{2+}]_i$, such as seen following the sustained depletion of the intracellular stores and activation of I_{CRAC} (or I_{SOC}), will I_{ARC} be significantly inhibited.

These findings lead to the interesting conclusion that the two coexisting, but independent, modes of receptor-stimulated Ca^{2+} entry, via the ARC channels and the SOC/CRAC channels, are regulated in a unique manner by increasing agonist concentrations—a process we have termed "reciprocal regulation" [13]. This is illustrated in Fig. 3. Low concentrations of

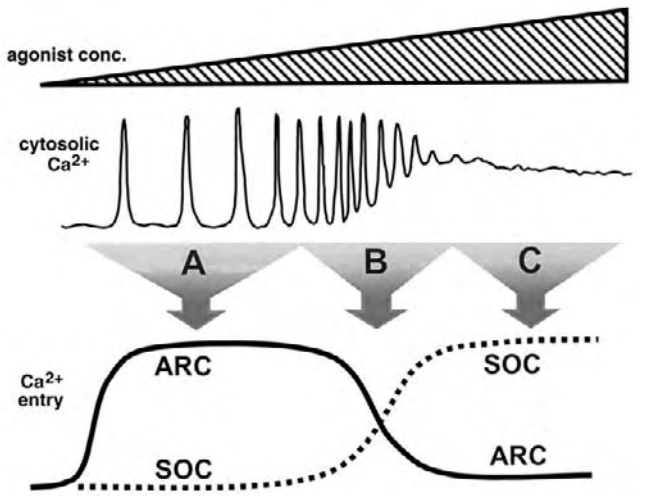


Figure 3 Reciprocal regulation of ARC and SOC channels. Diagram representing the regulation of I_{ARC} and I_{SOC} at different agonist concentrations and the corresponding changes in the nature of the resulting $[Ca^{2+}]_i$ signal (see text for details). Adapted from [13].

agonist result in the specific activation of the ARC channels which provide the main mode of Ca^{2+} entry under these conditions (see "A" in Fig. 3). This, together with the generation of low levels of $InsP_3$, initiates and modulates the cyclical transient discharge and refilling of intracellular Ca^{2+} stores resulting in the generation of the characteristic oscillatory $[Ca^{2+}]_i$ signals. SOC/CRAC channels fail to activate under these conditions because the transient and/or partial discharge of the intracellular Ca^{2+} stores is not able to generate an adequate "capacitative signal". As agonist concentrations increase, increasing levels of $InsP_3$ in the cytosol cause the discharge of the stores to become more complete and sustained, resulting in the activation of the SOC/CRAC channels and the development of a maintained elevated level of $[Ca^{2+}]_i$ ("C" in Fig. 3). This, in turn, inhibits the activity of the ARC channels. Thus, the transition from an oscillatory $[Ca^{2+}]_i$ signal to a sustained $[Ca^{2+}]_i$ signal ("B" in Fig. 3) is associated with a progressive switch in the predominant mode of Ca^{2+} entry from the ARC channels at low agonist concentrations to the SOC/CRAC channels at high ($\approx$ maximal) concentrations.

Conclusions and Implications

The demonstration that ARC channels represent a Ca^{2+} entry pathway that is spatially distinct from those activated by store depletion immediately raises the potential for the specific activation of different targets within the cell, as has already been demonstrated for the SOC channels and certain adenylyl cyclases [15]. Moreover, the fact that these pathways are independently activated at different agonist concentrations adds a new level of complexity to cellular Ca^{2+} signaling. Thus, the appropriate targeting of downstream Ca^{2+}-sensitive effectors to sites in close proximity to either ARC or SOC/CRAC channels may result in the specific selective regulation of these effectors at different agonist concentrations independent of any obvious overall changes in the spatial and/or temporal features of the induced $[Ca^{2+}]_i$ changes.

Obviously, the study of ARC channels is still only in its infancy and much remains to be discovered about their properties, regulation, and functions. Undoubtedly, as this novel Ca^{2+} entry pathway begins to receive more attention from researchers, a more complete picture of its distribution and specific roles will be revealed. Critical to this will be the identification of the molecular identity of the channels, which, to date, remains unknown. However, the demonstration that these channels provide the primary route for the receptor-activated entry of Ca^{2+} at physiologically relevant levels of stimulation [13] is of paramount significance, and the recent identification of currents identical to I_{ARC} in several different cell types suggests that this is a widespread phenomenon. Given this, it seems likely that the identification of this unique and specific function of the ARC channels will result in this channel becoming a prime target for possible pharmacological manipulation in any potential therapeutic strategies aimed at this key signaling system.

Acknowledgments

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CHAPTER 129

IP_3 Receptors

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Introduction

Inositol 1,4,5-trisphosphate (IP_3) receptors are large proteins: the native receptor is some 20 nm across, extends more than 10 nm from the membrane of the endoplasmic reticulum (ER), and each of its four subunits comprises about 2,700 residues. Their close relatives, ryanodine receptors, are even bigger. Size is important for these intracellular Ca^{2+} channels because it allows opening of the channel to be controlled by many different intracellular stimuli and it allows IP_3 and ryanodine receptors to interact directly with proteins in other membranes, including Ca^{2+} channels in the plasma membrane. IP_3 receptors, for example, may interact directly with the trp channels that are thought to mediate store-regulated Ca^{2+} entry [1]. This role for IP_3 receptors remains controversial, but an essential role in linking receptors that stimulate IP_3 formation to release of Ca^{2+} from intracellular stores is accepted [2].

IP_3 receptors are expressed in most eucaryotic cells, with three genes encoding closely related subtypes in mammals and birds, but only a single subtype in each of *Xenopus*, *Drosophila,* and *C. elegans* [3]. At least two of the mammalian subtypes are also alternatively spliced [4]. Because the functional channel is a tetramer, which can assemble from the same or different subunits, and most mammalian cells express more than one receptor subtype, there is considerable scope for IP_3 receptor diversity [3]. The different subtypes and their splice variants are differentially expressed, respond differently to chronic stimulation, and their assembly into heterotetramers is itself regulated, but the physiological significance of IP_3 receptor heterogeneity is unclear. There are subtle differences in the affinities of the subtypes for IP_3 and in their modulation by various intracellular stimuli [5], but more striking than the differences are the properties shared by all IP_3 receptors. All are tetrameric intracellular Ca^{2+} channels with large conductances, they have similar primary structures, Ca^{2+} and IP_3 control their opening, and they are modulated by many additional intracellular signals.

Key structural features of the type 1 IP_3 receptor are shown in Fig. 1. Each subunit has an IP_3-binding site formed by two distinct domains lying close to the amino terminal and linked to each other by a short stretch of residues that includes the S1 splice site. Several conserved, positively charged residues are particularly important for recognition of IP_3; they probably interact with its phosphate groups. The core IP_3-binding region of just 350 residues, which can be expressed as a soluble protein with very high affinity for IP_3, has been used as an "IP_3-sponge" to define the role of IP_3 in intact cells [6]. This is a useful tool because the only other antagonists of IP_3 receptors, heparin, Xestospongin and 2-aminoethyldiphenyborane, are notorious for their side effects.

Toward the carboxy terminal of each subunit there are six membrane-spanning regions, the last two of which together with an intervening loop (the P-loop) line the pore of the channel [7]. In keeping with the similar ion permeation properties of the IP_3 receptor subtypes, the sequences within this pore region are conserved and are also similar in ryanodine receptors [8]. Although almost 1,700 residues separate the IP_3-binding site from the pore, the two regions are associated in the native receptor, with the IP_3-binding region of one subunit perhaps interacting directly with the pore region of a neighboring subunit to control its opening [9]. The long stretch of residues separating the IP_3-binding region from the pore has been described as the "modulatory domain": it certainly includes at least some of the sites through which channel opening is modulated by phosphorylation or binding of small molecules and proteins [4,10] (Fig. 1).

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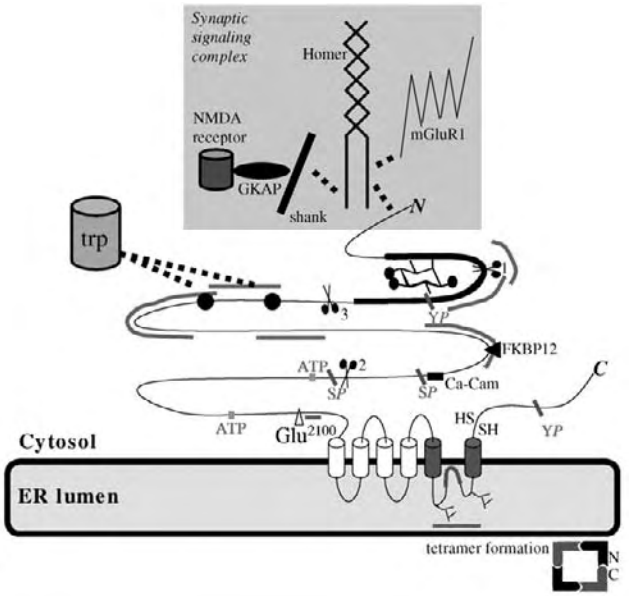


Figure 1 Structure of type 1 IP_3 receptor. The large cytosolic head of the receptor includes the IP_3-binding domain; seven regions (bold lines) to which Ca^{2+} has been shown to bind; the glutamate^{2100} residue shown to affect Ca^{2+}-sensitization; sites phosphoryated by PKA (*SP*) or tyrosine kinases (*YP*); the ATP-binding sites; the sites to which FKBP and trp are proposed to bind; and the three alternative splice sites (scissors). The pore is formed by the last two membrane-spanning regions together with part of the intervening loop. The loop also includes two glycosylation sites and a lumenal Ca^{2+}-binding site. The C-terminal tail includes conserved cysteine residues and another tyrosine kinase phosphorylation site. Assembly of the subunits into tetramers, which places the N-terminal of one subunit in close proximity to the channel region of another, requires residues downstream of the fourth membrane-spanning region. The box illustrates how Homer might function to link IP_3 receptors to various components of the synaptic signaling complex.

Despite controversy, it seems likely that all IP_3 receptors are biphasically regulated by cytosolic Ca^{2+}. Luminal Ca^{2+} has also been proposed to regulate channel opening, but it is difficult, for both ryanodine and IP_3 receptors [8], to resolve whether this really results from Ca^{2+} stimulating the receptor at its luminal surface or at its cytosolic surface after the Ca^{2+} has passed through the channel. There is a Ca^{2+}-binding site within a luminal loop of the type 1 IP_3 receptor (Fig. 1) and Ca^{2+}-binding proteins within the lumen of IP_3-sensitive organelles (calreticulin in ER; chromogranin in secretory vesicles) associate with IP_3 receptors [10], but none of these Ca^{2+}-binding sites has been shown to allow luminal Ca^{2+} to regulate channel opening.

Regulation of IP_3 receptors by luminal Ca^{2+} may be unresolved, but there is no such uncertainty about their regulation by cytosolic Ca^{2+}: all IP_3 receptors are stimulated by cytosolic Ca^{2+} and most (possibly all) can also be inhibited by cytosolic Ca^{2+} [5,11]. The details of how this biphasic regulation by cytosolic Ca^{2+} occurs have not been resolved; they are probably different for different receptor subtypes. It is accepted that IP_3 and Ca^{2+} must both bind to the IP_3 receptor before the channel can open and that binding of IP_3 regulates Ca^{2+} binding. IP_3, in other words, tunes the Ca^{2+} sensitivity of the IP_3 receptor. One scheme suggests that the major effect of IP_3 is to relieve Ca^{2+} inhibition by decreasing the affinity of an inhibitory Ca^{2+}-binding site [12], while another suggests that IP_3 binding reciprocally regulates two Ca^{2+}-binding sites, causing a stimulatory Ca^{2+}-binding site to be exposed and an inhibitory Ca^{2+}-binding site to be concealed [13]. It is not clear whether these Ca^{2+}-binding sites reside on the IP_3 receptor itself—it certainly has many cytosolic Ca^{2+}-binding sites (Fig. 1)—or on proteins associated with the receptor. A glutamate residue lying close to the C-terminal end of the modulatory domain (Fig. 1), and conserved within all IP_3 and ryanodine receptors, may be important in mediating the stimulatory effect of cytosolic Ca^{2+} [14]. The evidence that it is also involved in Ca^{2+} inhibition is less convincing [14] and difficult to reconcile with evidence suggesting that accessory proteins mediate Ca^{2+} inhibition [11]. Whether calmodulin is required for Ca^{2+} inhibition is hotly contested [15]. In summary, it seems likely that both IP_3 and Ca^{2+} must bind directly to the receptor for the channel to open, and that Ca^{2+} inhibition is via an accessory protein, whose binding to the receptor is probably regulated by IP_3. Both the regulation of IP_3 receptors by cytosolic Ca^{2+} and the ways in which different subtypes fine-tune that regulation are important in determining the complex spatio-temporal patterns of IP_3-evoked Ca^{2+} release in intact cells [16].

Besides Ca^{2+} and IP_3, there are many other modulators of IP_3 receptors. ATP binds to sites within the modulatory domain and increases IP_3 sensitivity [5]. Chemicals that modify sulphydryl groups, including reactive oxygen species, also increase IP_3 sensitivity, possibly by modifying conserved cysteine residues toward the C-terminal. These residues may thereby link the redox state of the cell to its IP_3 sensitivity. IP_3 receptors are phosphorylated by PKA, PKC, Ca^{2+}-calmodulin-dependent protein kinase II, and PKG [4,10]. The latter also phosphorylates a protein that is tightly associated with the IP_3 receptor (IRAG), causing inhibition of IP_3-evoked Ca^{2+} release [17]. Phosphorylation of tyrosine residues on IP_3 receptors can also set their sensitivity to IP_3 [18]. The immunophilin FKBP12, which certainly regulates ryanodine receptors, may regulate IP_3 receptors both directly and by anchoring the protein phosphatase, calcineurin, to them.

Most IP_3 receptors are found in the membranes of the ER, but they also occur in the Golgi, secretory vesicles, nuclear envelope, and plasma membrane. Even within the ER, the distribution of IP_3 receptors is far from uniform. On a molecular scale, IP_3 receptors occur in clusters [19], allowing the Ca^{2+} released by one receptor to rapidly influence its neighbors [16]. At a cellular level, they can be concentrated in discrete areas of ER: at the apical pole of pancreatic acinar cells, for example. There are also important functional associations between IP_3 receptors in the ER and other membranes: the possible link between IP_3 receptors and trp channels in the plasma membrane was mentioned earlier (Fig. 1), and there are many examples of IP_3 receptors in close association with mitochondria [20]. We are only just beginning to unravel the mechanisms responsible for putting IP_3 receptors into the right places, but scaffolding proteins are likely to be important.

An example illustrates the likely complexity of the interactions between scaffold proteins and IP_3 receptors. The Homer proteins are a family of dimeric scaffold proteins that assemble signaling proteins at excitatory synapses. The N-terminal of Homer binds to IP_3 receptors, type 1 metabotropic glutamate receptors, and to Shank, another scaffold protein that is targetted by its PDZ domain to the postsynaptic density and that itself binds further signaling proteins [21]. This chain of protein-protein interactions both targets IP_3 receptors to the dendritic spines of hippocampal neurones and brings them into intimate association with other signaling proteins, including receptors that stimulate IP_3 formation and channels that mediate Ca^{2+} entry (Fig. 1).

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CHAPTER 130

Ryanodine Receptors

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Function and Structure

The store from which signal Ca^{2+} is derived is either the extracellular space or the lumenal space of intracellular organelles, the source depending on the specialization of the cell. In muscle, Ca^{2+} is the major signaling molecule for excitation-contraction coupling, the process involving release of Ca^{2+} from the sarcoplasmic reticulum (SR) in response to depolarization of the sarcolemma and transverse tubules, and the subsequent activation of muscle contraction by the binding of Ca^{2+} to troponin, a component of the contractile apparatus. In highly specialized skeletal muscle, signal Ca^{2+} is released almost exclusively from a store located in the lumen of the SR by the activation of a class of Ca^{2+} release channels referred to as ryanodine receptors (RyR). In cardiac muscle, more than two-thirds of signal Ca^{2+} is derived from the SR, the remainder coming from extracellular spaces [1]. Ryanodine receptors are also expressed in other excitable and nonexcitable cells where their contributions to signal transduction may be less pronounced.

Three RyR isoforms have been characterized: RyR1, associated with skeletal muscle; RyR2, associated with cardiac muscle; and RyR3, which is expressed more ubiquitously. Isolated RyR type Ca^{2+} release channels have a high single channel conductance of 80 to 100 pS for Ca^{2+} and 400 to 800 pS for monovalent cations [2,3]. They are activated by micromolar Ca^{2+} and millimolar adenine nucleotides and inhibited by millimolar Ca^{2+} and Mg^{2+}. They are also modulated by calmodulin and cyclic ADP ribose. Pharmaceutical agents that open the channels include caffeine, 4-chloro-*m*-cresol, and halothane, which is a trigger for malignant hyperthermia (MH). Ryanodine binds to the open channel, converting the open state to a partially open subconductance state. Dantrolene, an antidote for MH, blocks the channel.

All three RyR isoforms are homotetramers formed from subunits of about 5000 amino acids, with subunit masses of about 565,000 Da [4,5]. Electron microscopic reconstruction of the tetrameric RyR1 molecule at about 30 Å resolution shows a cytoplasmic component with a square prism shape with dimensions of $28 \times 28 \times 12$ nm and a square transmembrane domain with an edge measuring 12 nm at the point of attachment to the cytoplasmic region and a depth of about 7 nm perpendicular to the membrane [6,7] (Fig. 1). Structures of these dimensions are observed in the junctional terminal cisternae of the SR and in corbular SR in cardiac muscle but are absent from *RYR1* null mice [8]. The N-terminal 85% of the molecule is predicted to form cytosolic domains, while 6 to 8 segments of the remaining C-terminal sequences contribute to the formation of the channel pore [4,5,5a].

The cytoplasmic component appears as a scaffold-like structure composed of at least 10 arbitrarily numbered, interconnected, globular domains, and provides a physical linkage between the SR and the transverse tubule while facilitating flow of Ca^{2+} from a central channel to the periphery (Fig. 1). This structure provides a framework for the identification of binding sites for specific regulatory proteins such as calmodulin (CaM) and FK506 binding protein (FKBP) [9]. CaM binds to an amino acid sequence containing residues 3614–3643 of the ryanodine receptor [10] and FKBP12 to Val^{2461} [11].

The structures of channels, closed in the absence of Ca^{2+} or opened in the presence of Ca^{2+} and other ligands, have been compared [12]. A small, central-axis opening with a diameter of 7 Å is revealed in transiently open channels and this opens to 18 Å in the ryanodine-modified channel. The channel runs through the whole transmembrane structure along a four-fold axis opening into the lumen. The process of opening is comparable to the opening of a camera diaphragm. In open channels, the clamp-shaped subdomains at the four

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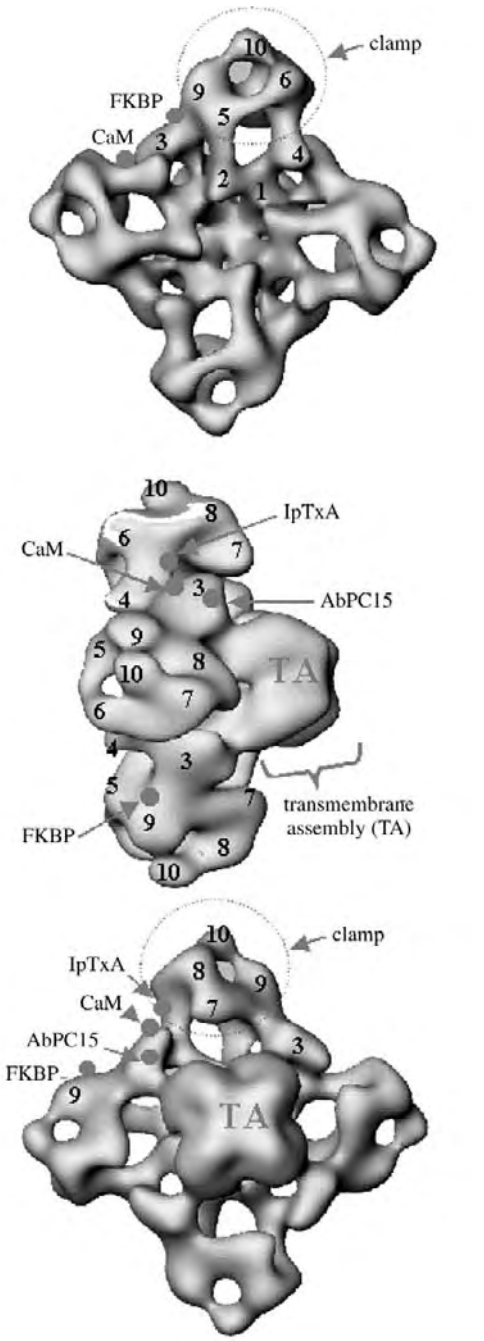


Figure 1 Solid body representation of a 3D reconstruction of RyR1 [7]. The numbers indicate distinct globular structures that correspond to structural domains, all of which are located in cytoplasmic regions of the protein. The filled circles indicate the locations of ligands as determined by reconstruction of RyR-ligand complexes. Abbreviations: CaM, calmodulin; FKBP, FK506-binding protein: IpTxA, imperatoxin A; AbPC15, monoclonal antibody against RyR residues 4425–4621; TA, transmembrane assembly. Reprinted by permission from *Eur. J. Biochem.* **267**, 5274–5279 (2000).

corners most distal from the membrane sector are in an open conformation and are slightly straightened toward the surface of the T-tubule membrane [13]. This feature supports the postulate that the four corners of the molecule interact with a specific protein in the transverse tubule. The N-terminus of RyR3 is located in the clamp region [14].

Activation of Ryanodine Receptor Ca^{2+} Release Channels

The location of RyR1 cytoplasmic domains in the junctional terminal cisternae of skeletal muscle SR suggests that they interact directly with the α1-subunit of the voltage-sensitive, dihydropyridine-modulated, slow or L-type Ca^{2+} channels (DHPR) located in closely apposed transverse tubules or plasma membranes [15]. A cluster of four DHPR molecules in the transverse tubule of skeletal muscle directly apposes every other ryanodine receptor molecule, with an individual DHPR molecule overlying an individual RyR1 subunit. Biochemical, physiological, and molecular genetic studies show that physical interactions occur between skeletal muscle RyR1 and DHPR isoforms, leading to both activation of the Ca^{2+} release channel (orthograde interaction) and modulation of the slow Ca^{2+} channel (retrograde interaction) [16]. The exact site of RyR/DHPR interaction is not clear [17]. Presumably, those Ca^{2+} release channels not opened by direct physical interaction are opened by Ca^{2+}-induced Ca^{2+} release. By contrast, there is no indication that direct physical interactions between cardiac RyR2 and DHPR α1-subunits lead to opening of the cardiac Ca^{2+} release channel [18]. In this case, entry of extracellular Ca^{2+} through the DHPR α1-subunit induces activation of RyR2 by Ca^{2+}-induced Ca^{2+} release.

Since no high-resolution structure is available and since it has proven difficult to carry out structure-function analysis of RyR molecules, evidence for a coherent mechanism of action is sketchy. If the ion pore corresponds to the model for a K^+ channel [19], then each of the four RyR subunits must contribute a hairpin-like structure with two transmembrane helices separated by an ion-selective pore-forming unit. In such a model, M8 and M10 [5] are the best candidates for the hairpin, while M9 is the best candidate for the selectivity filter [20]. M5 and M6 and possibly additional hairpin helices, such as M7a and M7b, may contribute to the periphery of the pore structure [5a]. Although interactions among the triggers that open and close this pore must be very complex [8], two triggers stand out as being of special significance [21]. In skeletal muscle, voltage-induced changes in the conformation of the "voltage sensor" DHPR α1-subunit undoubtedly drive conformational changes in the cytoplasmic segment of RyR1 that are transmitted over long ranges to activate Ca^{2+} release. In most tissues, and even in skeletal muscle, elevations in cytosolic Ca^{2+} trigger Ca^{2+} induced Ca^{2+} release. Indeed, Ca^{2+} may be the master trigger and the ability of all other agents, including protein-protein interactions, to activate the Ca^{2+} release channel may simply reflect an agonist-induced increase in the affinity of an RyR molecule for binding of Ca^{2+} to its trigger sites. A strong candidate for the site for binding of trigger Ca^{2+} is the "Ca^{2+} sensor" amino acid, Glu^{4032} (Glu^{3885} in RyR3), located within a hydrophobic sequence predicted earlier to form transmembrane helix M2 [22]. Other sites for Ca^{2+} binding might be located elsewhere [23].

ATP is a potent activator of the Ca^{2+} release channel in the presence of Ca^{2+} [3], but since cellular ATP concentrations

are rather constant, ATP is not likely to play a major regulatory role. The site of ATP binding is not defined. A transmembrane redox sensor exists within the RyR1 channel complex that confers tight regulation of channel activity in response to changes in transmembrane redox potential [24]. PO2 dynamically controls the redox state of several thiols in each RyR1 subunit and thereby tunes its response to NO [25]. At physiological pO2, nanomolar NO activates the channel by S-nitrosylating a single cysteine residue. S-nitrosylation is specific to RyR1 and its effect on the channel is CaM-dependent.

Caffeine activates Ca^{2+} release but appears to do so by increasing Ca^{2+} sensitivity [26]; ryanodine can drive the channel into an open subconductance state, but this state is Ca^{2+} dependent, with an exceptionally high Ca^{2+} affinity [27,28]. While ryanodine binds to C-terminal sequences [29], the binding site for caffeine is unknown. Most MH mutations alter the apparent affinity of the channel for caffeine and halothane [30], but these mutations are dispersed throughout two "hot spots" in the cytosolic domain [31] and one in the C-terminus [32]. The binding site for dantrolene, which closes the channel, is also not well defined [33].

FKBP [34], triadin [35], junctin[36], CaM [37], sorcin [38], and various protein kinases [39,40] may also regulate the function of ryanodine receptors. The interaction of FKBP with RyR increases channels to full conductance, decreases open probability after caffeine activation, increases mean open time, and coordinates opening of clusters of channels [11]. These observations, together with the 1:1 stoichiometry of FKBP12 with RyR1, suggest that FKBP is an RyR subunit. CaM is both an inhibitor and an activator of Ca^{2+} channel activity [37]. Triadin and junctin, which have single transmembrane sequences and positively charged lumenal sequences, form links to the lumenal, negatively charged Ca^{2+} buffering protein calsequestrin, so that RyR1, triadin, junctin, and calsequestrin form a quaternary complex that may be required for normal Ca^{2+} release. Sorcin acts as an inhibitor of RyR function. Phosphorylation of RyR1 at Ser^{2843} enhances open probability by increasing the sensitivity to Ca^{2+} and ATP [41]. Phosphorylation of Ser^{2809} in RyR2 by CaM kinase II reverses inhibition by CaM and restores prolonged channel opening [42]. Thus the RyR can be viewed as a massive protein with multiple protein and ligand binding sites that is designed to integrate complex signals for activation and inactivation from many different sites in the molecule [8].

Molecular Biology of Ryanodine Receptors

Three ryanodine receptors (*RYR*) genes have been identified: *RYR1* on human chromosome 19q13.1; *RYR2* on 1q42.1-43; and *RYR3* on 15q14-15 [31]. *RYR1* is expressed predominantly in fast and slow-twitch skeletal muscle and also in the esophagus and in cerebellar Purkinje cells in the brain. *RYR2* is the predominant isoform in cardiac muscle and brain. Its expression in the brain, brain stem, and spinal cord is widespread, but it is absent from the pituitary. *RYR3* is differentially expressed in the brain, T-lymphocytes, vas deferens, uterus, and testes [43]. It accounts for a small percentage of total *RYR* expression in mammalian skeletal muscle but is highly expressed in avian and amphibian skeletal muscles. RyR3 may flank RyR1 in the junctional terminal cisternae [44].

The disruption of *RYR1* is neonatally lethal [45]. The mutant mice resemble the mouse mutant *mdg*, which results from disruption in the DHPR α1-subunit gene *CACNA1S*, in that both disruptions lead to failure of excitation-contraction coupling in skeletal muscle [46]. The disruption of *RYR2* is lethal at embryonic day 10, with morphological abnormalities in the heart tube [47]. The disruption of *RYR3* does not cause gross abnormalities in mice, although *RYR3*-null mice have abnormal locomotor activity [48].

Mutations in *RYR1* cause MH, an autosomal dominant genetic abnormality in which susceptible individuals respond to potent inhalational anesthetics and depolarizing skeletal muscle relaxants with hypermetabolism, skeletal muscle rigidity, fever, and muscle cell damage [31]. Mutations in *RYR1* also cause central core disease (CCD), an autosomal dominant myopathy characterized by hypotonia and proximal muscle weakness. Central cores of skeletal muscle fibers lack oxidative or phosphorylase activity, and electron microscopy of the cores shows disintegration of the contractile apparatus and streaming of the Z lines, an increase in content of the sarcotubular system and depletion of mitochondria. CCD is usually closely associated with MH, but an exception has been found [49].

MH and CCD mutations are clustered in *RYR1* exons 2 to 17 (region 1), 34–46 (region 2), and 91–102 (region 3) [31,32]. The ratio of MH to CCD mutations in region 1 is 5 : 1, in region 2, 8 : 1, and in region 3, 1 : 8. MH mutations are more sensitive to caffeine and halothane activation than wild-type and are more "leaky": CCD mutant proteins are even more leaky than MH mutations [50,51]. CCD mutations may cause a more severe imbalance in Ca^{2+} regulation than those that cause MH. Elevation of resting Ca^{2+} by a very leaky CCD mutant channel may trigger the series of degenerative and compensatory events that lead to core formation in the center of the fiber without affecting the periphery of the muscle cell where Ca^{2+} homeostasis can be achieved through the intervention of plasma membrane Ca^{2+} pumps and exchangers. However, at least one CCD mutation is not leaky but, rather, uncouples excitation-contraction coupling by disrupting orthograde signaling between the DHPR and RyR1 proteins without disrupting retrograde signaling between these two proteins [52]. Mutations in the *CACNA1S* gene encoding the α1-subunit of the skeletal muscle DHPR have also been linked to MH, providing further support for strong functional interactions between these two proteins [53].

Mutations in *RYR2* have been linked to two autosomal dominant cardiac diseases [54,55]: catecholaminergic polymorphic ventricular tachycardia (CPVT), which occurs in response to stress and in the absence of either structural heart disease or prolonged QT interval; and arrhythmogenic right ventricular cardiomyopathy type 2 (ARVD2), which is

characterized by partial degeneration of the myocardium of the right ventricle, electrical instability, and sudden death. The mutations are located in regions of the gene that correspond to MH regions 1 and 2 in *RYR1*. Since *RYR2* is not expressed in skeletal muscle, neither MH nor CCD manifest in these diseases. As a corollary, cardiac disease is not associated with MH or CCD mutations, since *RYR1* is not expressed in the heart. It is probable that CPVT and ARVD2, like MH and CCD, are differentiated on the basis of the severity of the alteration in RyR2 channel function.

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CHAPTER 131

Intracellular Calcium Signaling

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The "Calcium Signaling Toolkit" and Calcium Homeostasis

Calcium (Ca^{2+}) is a ubiquitous intracellular messenger that controls a diverse range of cellular processes, such as gene transcription, muscle contraction, and cell proliferation. The ability of Ca^{2+} to play a pivotal role in cell biology results from the facility that cells have to shape Ca^{2+} signals in the dimensions of space, time, and amplitude. To generate and interpret the variety of observed Ca^{2+} signals, different cell types employ components selected from a "Ca^{2+} signaling toolkit," which comprises an array of homeostatic and sensory mechanisms (reviewed in [1]). Since many of the molecular components of this toolkit have multiple isoforms with subtly different properties, each specific cell type can exploit this large repertoire to construct highly versatile Ca^{2+} signaling networks. Thus by mixing and matching components from the toolkit, cells can obtain Ca^{2+} signals that suit their physiology.

In most cells, Ca^{2+} has its major signaling function when it is elevated in the cytosolic compartment. From there it can also diffuse into organelles such mitochondria and the nucleus. The Ca^{2+} concentration inside cells is regulated by the simultaneous interplay of multiple counteracting processes, which can be divided into Ca^{2+} "on" and "off" mechanisms depending on whether they serve to increase or decrease cytosolic Ca^{2+} (reviewed in [2–4]) (Fig. 1).

The Ca^{2+} "on" mechanisms include channels located at the plasma membrane that regulate the supply of Ca^{2+} from the extracellular space, and channels on the endoplasmic reticulum/sarcoplasmic reticulum (ER/SR, respectively), Golgi, secretory granules, and acidic stores (e.g. lysosomes), which release the finite intracellular Ca^{2+} stores. The "off" mechanisms include Ca^{2+}ATPases on the plasma membrane and ER/SR, and exchangers that utilize the electrochemical Na^+ gradient to provide the energy to transport Ca^{2+} out of the cell. Occasionally, some of the "off" mechanisms contribute to cytosolic Ca^{2+} increases; examples are "slippage" of Ca^{2+} through Ca^{2+}ATPases and reverse-mode Na^+/Ca^{2+} exchange.

When cells are at rest, the balance lies in favour of the 'off' mechanisms, thus yielding an intracellular Ca^{2+} concentration of ~100 nM. However, when cells are stimulated by various means, e.g. depolarisation, mechanical deformation or hormones, the 'on' mechanisms are activated and the cytosolic Ca^{2+} concentration increases to levels of 1 μM or more.

As mentioned above, Ca^{2+} signals can be modulated in their temporal, amplitude, and spatial dimensions. Furthermore, Ca^{2+} signals can arise from different cellular sources, which appear to be regulated by a growing number of messengers (reviewed in [5]). The following sections describe the currently known messengers and channels and present examples of the versatility of Ca^{2+} signals.

Multiple Channels and Messengers Underlie Ca^{2+} Increases

Ca^{2+} Influx Channels

Cells utilize several different types of Ca^{2+} influx channels, which can be grouped on the basis of their activation mechanisms (reviewed in [2]). *Voltage-operated Ca^{2+} channels* (VOCs) are employed largely by excitable cell types such as muscle and neuronal cells, where they are activated by depolarization of the plasma membrane. Different types of VOCs,

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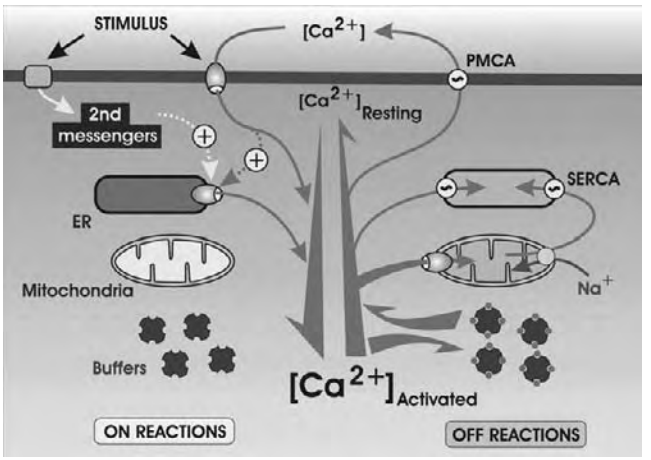


Figure 1 **Calcium "on" and "off" mechanisms in cellular signaling and homeostasis.** The figure illustrates various pathways and mechanisms by which cytosolic Ca^{2+} levels can increase or decline. At rest, cells generally have a free cytosolic Ca^{2+} concentration of around 100 nM. This can be increased by activation of channels at the plasma membrane and release from internal stores (denoted "ER"). Cytosolic Ca^{2+} signals are attenuated by passive Ca^{2+} buffering and actively reversed by mitochondria and Ca^{2+} ATPases on the ER and plasma membrane.

which are expressed in a tissue-specific manner, have been characterized on the basis of their gating characteristics and pharmacology. *Receptor-operated Ca^{2+} channels* (ROCs) comprise a range of structurally and functionally diverse channels that are particularly prevalent on secretory cells and at nerve terminals. Well-known ROCs include the nicotinic acetylcholine receptor and the N-methyl-D-aspartate (NMDA) receptor. ROCs are activated by the binding of an agonist to the extracellular domain of the channel. The different ROCs are activated by a wide variety of agonists, e.g. ATP, serotonin, glutamate, and acetylcholine. *Mechanically activated Ca^{2+} channels* are present on many cell types and respond to cell deformation. Such channels convey information into the cell concerning the stress/shape changes that a cell is experiencing. A particularly nice example of mechanically induced Ca^{2+} signaling was observed in epithelial cells from the trachea, where deformation of a single cell led to a radial Ca^{2+} wave that synchronized the Ca^{2+}-sensitive beating of cilia on many neighboring cells [6], which may serve to aid clearance of mucous or particles from the lungs. *Store-operated Ca^{2+} channels* (SOCs) are activated in response to depletion of the intracellular Ca^{2+} store. The mechanism by which the SOCs "sense" the filling status of the intracellular pool is unknown. At present, the best candidate for the molecular identity of SOCs are homologues of a protein named TRP (*t*ransient *r*eceptor *p*otential) that functions in *Drosophila* photoreception. Several mammalian TRP homologues have been identified and found to be expressed in almost all tissues (reviewed in [7]).

Ca^{2+} Release Channels

Inositol 1,4,5-trisphosphate Receptors ($InsP_3Rs$). The binding of many hormones and growth factors to specific receptors on the plasma membrane leads to the activation of an enzyme that catalyzes the hydrolysis of phospholipids to produce the intracellular messenger inositol 1,4,5-trisphosphate ($InsP_3$). $InsP_3$ is water-soluble and diffuses into the cell interior where it can engage $InsP_3Rs$ on the ER/SR, allowing the Ca^{2+} stored at high concentrations to enter the cytoplasm. Three different isoforms of $InsP_3Rs$ have been found, which appear to subtly differ in their characteristics, such as affinity for $InsP_3$. An important feature of $InsP_3Rs$ is that they are actually co-regulated by $InsP_3$ and Ca^{2+}. Indeed, it seems that $InsP_3$ may simply serve to make $InsP_3Rs$ responsive to an activating Ca^{2+} signal. $InsP_3R$ opening is biphasically regulated by Ca^{2+}; 0.1–0.5 μM Ca^{2+} increases channel activity, whereas greater Ca^{2+} concentrations inhibit their gating (reviewed in [8,9]). This dependence of $InsP_3R$ activity on cytosolic Ca^{2+} is crucial in the generation of the complex patterns of Ca^{2+} signals seen in many cells.

Ryanodine Receptors (RyRs) These receptors are structurally and functionally analogous to $InsP_3Rs$, although they have approximately twice the conductance and molecular mass of $InsP_3Rs$. Another property that RyRs share with $InsP_3Rs$ is their sensitivity to cytosolic Ca^{2+} concentrations, although they are generally activated and inhibited by higher concentrations (activation at 1–10 μM; inhibition at >10 μM). In contrast to $InsP_3Rs$, which are almost ubiquitously expressed in mammalian tissues, RyRs are largely present in excitable cell types, such as muscle and neurons. As with $InsP_3Rs$, RyR subunits are encoded by three genes. However, these genes do not appear to have the same functional redundancy as observed with the $InsP_3R$ isoforms. Instead, the different RyR proteins are often used for specific functions. For example, only type-1 RyRs are employed in triggering excitation of skeletal muscle, whereas only type-2 RyRs fulfil this role in cardiac muscle (reviewed in [1,10]).

Multiple Messengers

A wide range of messengers has been shown to mediate the activation of Ca^{2+} entry and Ca^{2+} release channels (reviewed in [5]). These messengers include $InsP_3$, cyclic adenosine 5′-diphosphoribose (cADPR), nitric oxide (NO), H_2O_2/O_2^-, nicotinic acid adenine dinucleotide phosphate (NAADP), diacylglycerol, arachidonic acid, sphingosine, sphingosine-1-phosphate (S-1-P), leukotrienes, and Ca^{2+} itself. From the specificities of the Ca^{2+}-releasing messengers we know that there must be several different types of intracellular Ca^{2+} release channel, although at present only $InsP_3$ receptors ($InsP_3Rs$) and ryanodine receptors (RyRs) have been characterized in detail. Exactly which messengers act in particular cells is far from clear. However, it is becoming apparent that Ca^{2+} signals can be activated by the simultaneous interplay of several factors. In pancreatic acinar cells, for example, the combined action of $InsP_3$, cADPR, and NAADP underlies the Ca^{2+} signals generated by physiological stimuli [11].

Temporal Regulation of Ca^{2+} Signals

Since prolonged elevation of cytoplasmic Ca^{2+} levels can be toxic, most cells do not usually respond with sustained Ca^{2+} signals. Rather, Ca^{2+} is commonly presented in a pulsatile manner [12,13]. A well-known example of such repetitive Ca^{2+} increases is the response of hepatocytes to stimulation with various hormones that, for example, regulate glycogen metabolism and mitochondrial respiration [14]. The Ca^{2+} increases that occur in hepatocytes during such stimulations are transient spikes, which arise from the cyclical activation of $InsP_3Rs$ (see below). The frequency of the Ca^{2+} spikes is directly proportional to the concentration of hormone applied to the cells, and they can persist for the duration of agonist application. These Ca^{2+} spikes are therefore essentially a frequency-modulated digital read-out of cell stimulation.

Another system in which pulsatile Ca^{2+} increases are critical is excitation-contraction coupling in striated muscle cells. The mechanism and channels underlying these signals are very different from those in hepatocytes. In cardiac muscle, for example, type 2 RyRs are activated following depolarization of the sarcolemma. The Ca^{2+} that enters the cell following voltage-operated Ca^{2+} channel activation triggers release of Ca^{2+} from ryanodine receptors (RyRs) by a process known as Ca^{2+}-induced Ca^{2+} release (CICR) [1]. This Ca^{2+} can then globally diffuse to the myofibrils and promote the interaction between actin and myosin that leads to contraction.

Although the Ca^{2+} signals observed in both hormonally stimulated hepatocytes and cardiomyocytes are repetitive Ca^{2+} transients, the periodicity and kinetics of these signals are very different. The Ca^{2+} spikes in hepatocytes (and many other nonelectrically excitable cells) typically have frequencies in the range of 0.1–0.01 Hz, a time-to-peak amplitude of several seconds, and a recovery phase lasting tens of seconds [15]. In contrast, cardiac Ca^{2+} signals are triggered at frequencies in the 1–10 Hz range (depending on the species of animal), reach peak within a few tens of milliseconds, and persist for only a few hundred milliseconds [16]. The distinct timescales of these Ca^{2+} responses reflect the very different mechanisms by which they are generated.

Pulsatile Ca^{2+} increases, such as those observed in hepatocytes, are generally considered to have a much higher fidelity of information transfer than simple tonic changes in Ca^{2+} concentrations, since they are much less prone to noisy fluctuations. The major sensors for these Ca^{2+} spikes are Ca^{2+} binding proteins such as calmodulin (reviewed in [17]). This ubiquitous protein is one of a family of proteins bearing structural Ca^{2+}-binding motifs known as EF-hands. The binding of Ca^{2+} to calmodulin has a K_d around 1 μM, making it an ideal receiver for the rapid transient Ca^{2+} increases seen with each spike. One of the best-known enzymes that uses calmodulin to help it "count" Ca^{2+} spikes is calmodulin-dependent protein kinase II, which can activate other proteins via phosphorylation. This enzyme is composed of many subunits that undergo variable degrees of activation depending on the frequency of Ca^{2+} spikes. Essentially, increasing the frequency or duration of Ca^{2+} spikes maintains this enzyme in an active state by trapping calmodulin and causing autophosphorylation [18,19].

There are many examples of cellular activities being modulated by the frequency of Ca^{2+} signals. The transcription of Ca^{2+}-regulated genes was sensitive to the frequency at which Ca^{2+} spikes occurred. Indeed, it appears that alternative transcription factors are tuned to distinct frequencies of Ca^{2+} spikes. Thus temporal modulation of Ca^{2+} signaling can underlie differential gene transcription [20].

Spatial Regulation of Ca^{2+} Signals

All Ca^{2+} signals derive initially from local sources such as the activation of Ca^{2+} channels. Both Ca^{2+} entry and Ca^{2+} release channels can give rise to brief pulses of Ca^{2+} that form a small plume around the mouth of the channel before diffusing into the cytoplasm (reviewed in [21]). In many situations, these local sources provoke global signals through regenerative CICR as described for cardiomyocytes above. However, there are numerous instances in which the spread of Ca^{2+} is constrained to a specific subcellular region. Such spatial regulation of Ca^{2+} provides perhaps the most elegant examples of how cells subtly modulate Ca^{2+} signals to control multiple, sometimes opposing, processes.

Nonelectrically Excitable Cells

A few different types of localized Ca^{2+} signals have been observed in various nonelectrically excitable cell types [22]. Probably the best know examples are "Ca^{2+} puffs" and the apical Ca^{2+} signals that occur in secretory cells (reviewed in [3]). Ca^{2+} puffs are local signals that derive from the activation of a cluster of $InsP_3Rs$. Typically Ca^{2+} puffs give a modest elevation of cytosolic Ca^{2+} (~50–600 nM) with a limited spatial spread (~2–6 μm) and are transient (duration of ~1 second) [23]. Such events were first observed in *Xenopus* oocytes [e.g. 24] but have subsequently been observed in many other cell types. The temporally and spatially coordinated recruitment of Ca^{2+} puffs is responsible for the generation of repetitive Ca^{2+} waves and oscillations observed during hormonal stimulation. Essentially Ca^{2+} waves reflect the progressive release of Ca^{2+} by Ca^{2+} puff sites distributed along the ER/SR. Ca^{2+} released by one puff site can diffuse to a neighboring site and activate it (providing $InsP_3$ is bound to the channels). Successive rounds of Ca^{2+} release and diffusion allow the initially local Ca^{2+} puffs to trigger global Ca^{2+} waves and oscillations (reviewed in [3,25]).

It is interesting that in HeLa cells [26] and *Xenopus* oocytes [27], it has been demonstrated that Ca^{2+} puff sites expressing a higher sensitivity to $InsP_3$ consistently trigger Ca^{2+} waves. What gives these pacemaking Ca^{2+} puff sites their enhanced sensitivity is unclear. In the case of somatic cells, the pacemaker sites tend to be distributed in a perinuclear region [28], thus raising the possibility that they can

send signals specifically into the nucleus. In *Xenopus* oocytes, it has been shown that mitochondria can constrain the activity of Ca^{2+} puffs, and locations lacking these organelles may thus define pacemaking sites [29].

Another well-known local Ca^{2+} signal occurs in the apical region of secretory cells such as pancreatic acinar cells (reviewed in [30]). Similar to the pacemaker Ca^{2+} puffs (see above), the $InsP_3Rs$ that underlie the apical Ca^{2+} spikes are distinguished by a heightened sensitivity to $InsP_3$. Also like Ca^{2+} puffs, such apical Ca^{2+} spikes probably arise from the coordinated Ca^{2+} release from multiple Ca^{2+} release channels. Recent evidence has pointed to the apical spikes arising from a stimulus-dependent hierarchical activation of different types of Ca^{2+} release channel [11]. With low levels of cell stimulation, the Ca^{2+} spikes stay restricted to the apical pole of the acinar cells, where they can activate ion channels and trigger limited secretion. Greater stimulation causes the Ca^{2+} spikes to trigger Ca^{2+} waves that propagate toward the basal pole. It appears that the restriction of the Ca^{2+} signal in the apical pole is due in part to a "firewall" of mitochondria that buffer Ca^{2+} as it diffuses from the apical pole and prevent the activation of RyRs in the basal pole [31,32].

Electrically Excitable Cells

Spatial regulation of Ca^{2+} signaling is the forte of electrically excitable cells. For more detailed discussions, the reader is referred to recent reviews [3,33–35].

One of the best-known examples in which spatial regulation of Ca^{2+} signals can have diametrically opposing effects in the same cell is in the regulation of smooth muscle tone (reviewed in [35]). In these cells, global responses induce contraction by the activation of Ca^{2+}/calmodulin-dependent enzymes, whereas local subsarcolemmal Ca^{2+} signals promote relaxation by activating Ca^{2+}-dependent plasma membrane ion channels [36]. The subsarcolemmal Ca^{2+} signals are known as Ca^{2+} sparks; they are analogous to the Ca^{2+} puffs observed in nonelectrically excitable cells, but they arise from the activation of a cluster of RyRs. Ca^{2+} sparks are generally faster in onset and decline than Ca^{2+} puffs and have usually a more restricted spread (~1–3 μm).

In smooth muscle, the subsarcolemmal Ca^{2+} sparks activate K^+ and Cl^- conductances, giving rise to brief currents known as STOCs (spontaneous transient outward currents; K^+ current), STICs (spontaneous transient inward current; Cl^- current) and STOICs (mixed K^+ and Cl^- currents). STOCs have been measured in a wide variety of smooth muscle cell types and serve to hyperpolarize the cell membrane by ~20 mV, thus causing the muscle to relax. STOCs primarily arise due to the activation of large conductance Ca^{2+}-activated K^+ channels (BK channels). These Ca^{2+}-activated channels have a low sensitivity to cytosolic Ca^{2+}, requiring concentrations >1 μM for significant activity. It has been proposed that the BK channels sit in close apposition to Ca^{2+} spark sites and sense rapid step-like Ca^{2+} changes during RyR activation [37].

Spatio-temporal recruitment of Ca^{2+} sparks also underlies the global Ca^{2+} signals that activate skeletal and cardiac myocyte contraction. When the sarcolemma of these cells is depolarized by an action potential, VOCs open and allow a small influx of Ca^{2+} [38]. Through the process of CICR, this trigger Ca^{2+} signal is greatly amplified by clusters of closely apposed RyRs, thereby activating Ca^{2+} spark sites throughout the cell. The spatial overlap and temporal summation of the Ca^{2+} sparks gives rise to the global responses that ensure synchronized contraction in the muscle (reviewed in [3,34]).

The intricate morphologies of neurons means that these cells are well-suited to producing spatially regulated Ca^{2+} signals. Local Ca^{2+} changes in dendritic spines can underlie processes such as synaptic plasticity and neurite outgrowth, whereas more global Ca^{2+} signals can cause gene transcription and neuronal maturation within the brain (reviewed in [39–41]). An example of the necessity for precise spatial regulation of neuronal Ca^{2+} signals can be seen in the effects of activating synaptic versus nonsynaptic glutamate receptors on hippocampal neurons. At synaptic junctions, hippocampal neurons respond to the release of the neurotransmitter glutamate by activation of NMDA receptors. These ROCs are ligand-gated ion channels that allow the influx of Ca^{2+}. The Ca^{2+} that enters neurons through the synaptic NMDARs causes local activation of ERK1/2 [42] and promotes cell survival. When neurons and glia become anoxic, the glutamate released at synaptic terminals can diffuse to nonsynaptic NMDARs and cause Ca^{2+} signals that lead to cell death [43]. The drastically different effects of stimulating synaptic or nonsynaptic NMDARs explains the paradox that glutamate can be a physiological neurotransmitter, yet bath application of glutamate kills cultured neurons. Essentially, the spatial location of the Ca^{2+} signals determines which biochemical pathways will become activated and can switch cells from life to death.

Modulation of Ca^{2+} Signal Amplitude

Although many cell types can grade the amplitude of their Ca^{2+} signals, most control of Ca^{2+} signaling occurs through the types of spatial and temporal regulation described above. Consequently, there are only a few situations in which such modulation has been shown to have a physiological relevance. One well-known example is in muscle, where the amplitude of Ca^{2+} signals governs the force of contraction. In the case of cardiac muscle, inotropic agents (e.g. adrenaline) can alter the influx of Ca^{2+} through VOCs or Ca^{2+} release from RyRs, thus altering the capacity of the heart for pumping blood.

Since large, rapid increases in Ca^{2+} are easier to detect than small, graded changes, Ca^{2+} signals based on frequency modulation are believed to have greater fidelity than those occurring through amplitude modulation. However, it has been shown that cells may interpret modest changes in cytoplasmic concentration. For example, differential gene activation may occur by varying the amplitude of Ca^{2+} signals [44].

Ca^{2+} as a Signal within Organelles and in the Extracellular Space

The discussion above has largely considered the regulation of Ca^{2+} signals within the cytoplasm. However, it is important to point out that Ca^{2+} has crucial functions within organelles. Mitochondrial Ca^{2+} signals can enhance mitochondria respiration by activation enzymes of the citric acid cycle to stimulate production of NADH [45]. Elevation of nuclear Ca^{2+} appears to be important for transcription of specific genes [46]. Ca^{2+} has a diverse range of functions within the lumen of the ER. Depletion of ER Ca^{2+} leads to incorrect folding of nascent proteins and a stress response culminating in cell death [47].

Many cell types express receptors for Ca^{2+} on their surface, allowing them to sense changes in extracellular Ca^{2+} concentration (reviewed in [48]). These receptors can activate $InsP_3$ production to evoke intracellular Ca^{2+} changes. Through the action of such Ca^{2+}-sensing receptors, the Ca^{2+} that is extruded from a cell at the termination of a cytosolic signal can become an agonist for its neighbors [49], perhaps serving to coordinate the activity of adjacent cells.

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CHAPTER 132

Calcium Pumps

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Introduction

The plasma membrane controls the exchange of calcium between the intracellular and extracellular environments [1,2]. A limited and strictly controlled amount of Ca^{2+} is allowed to penetrate into the cells through a number of specific channels to trigger important cellular events, including the massive liberation of Ca^{2+} from membrane enclosed stores. An equivalent amount of Ca^{2+} must then be ejected to the extracellular spaces. Two systems preside over this function in animal cells: a large system that is particularly active in excitable cells exchanges electrogenically Na^+ for Ca^{2+} interacting with Ca^{2+} with low affinity. The other system is an ATPase (the PMCA pump [3]), which interacts instead with Ca^{2+} with high affinity but has low Ca^{2+} ejecting capacity: it is thus normally considered as the fine-tuner of cellular Ca^{2+}. Calcium is also exchanged between the cytoplasm and the internal space of the organelles, chiefly the mitochondria and the endo(sarco) plasmic reticulum (ER/SR). The latter contains an ATPase (the SERCA pump, [4]), which is similar in mechanism to the PMCA pump. The total Ca^{2+} transporting capacity of the reticulum depends on the amount of pump it contains, which is high in heart and skeletal muscle and low in nonmuscle tissues. The SERCA pump works in concert with channels in the ER/SR membrane that are activated by second messengers and return to the cytoplasm the calcium that the pump had transported to the ER/SR lumen. Since the ER/SR is located in close proximity to the mitochondria, the released calcium creates an ambient of high calcium concentration adequate to activate the low affinity electrophoretic uptake uniporter of the inner mitochondrial membrane [5]. Ca^{2+} accumulated in the mitochondrial matrix is released to the cytoplasm via two systems, a well-characterized a Na^+/Ca^{2+} exchanger [6] and a less well-characterized Ca^{2+}/H^+ antiporter.

Ca^{2+} pumps have also been described in lower eukaryotes. In yeasts, two pumps termed PMR1 and PMC1 [7–9] have been described in the Golgi complex [10] and the vacuoles [9], respectively. Their degree of sequence homology to the SERCA and PMCA pump does not exceed 40–50%, and in particular, the PMC1 pump does not contain the calmodulin binding domain that characterizes the PMCA pumps. Most bacteria extrude calcium via Ca^{2+}/H^+ or Ca^{2+}/Na^+ antiporters [11], but bona fide Ca^{2+} ATPases have also been described, e.g. in *Flavobacterium odoratum* [12] and in a cyanobacterium [13].

Reaction Cycle of the SERCA and PMCA Pumps

The basic enzyme cycle of the two calcium pumps is essentially the same [14] (Fig. 1). Ca^{2+} is bound on one side of the membrane in a reaction that does not require ATP, since Ca^{2+} binding can be measured in its absence. ATP is then bound and split to form an acyl-phosphate intermediate on an aspartic residue [15]. The formation of a phosphorylated intermediate has suggested the nomenclature of "P type" pumps [16,17]. After phosphorylation, the pump undergoes a conformational transition from a state termed E1 to one termed E2. In the E1 conformation the pump binds Ca^{2+} with high affinity to sites exposed to the cytosolic site, whereas in the E2 conformation the Ca^{2+} binding sites have lower affinity and are exposed to the ER/SR lumen or to the extracellular space. Ca^{2+} can thus be released. After releasing ATP and Ca^{2+} the enzyme becomes slowly dephosphorylated and returns to the E1 state. The SERCA and PMCA pumps differ in the Ca^{2+}/ATP transport stoichiometry, which is 2 in the former and 1 in the latter. Powerful inhibitors have been described. Lanthanum inhibits both pumps but with interesting differences. In the SERCA pump it decreases the steady state level of

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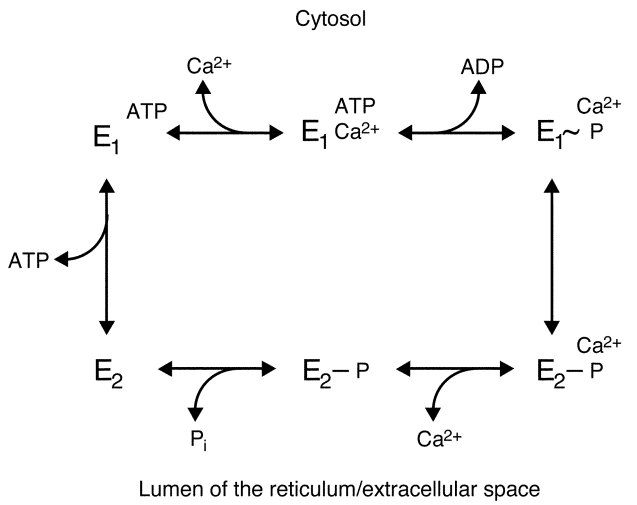


Figure 1 A simplified scheme of the reaction mechanism of calcium pumps. The pump, symbolized by E, is assumed to exist in two different conformations, E1 and E2. E1 binds calcium with high affinity at the cytoplasmic site of the membrane; E2 has lower affinity for calcium and releases it to the opposite site. Molecular details on the uptake and release path for calcium are discussed in the text. The energy of ATP is momentarily conserved in the enzyme as a phosphorylated intermediate (an aspartyl phosphate) that is formed prior to the translocation of calcium. The scheme shows a 1 to 1 stoichiometry between hydrolyzed ATP and transported calcium, which is that of the PMCA pump. The SERCA pump transports instead two Ca^{2+} per ATP hydrolyzed. See text for details.

the phosphorylated intermediate, whereas in the PMCA pump it greatly stimulates it. The phosphate analogue orthovanadate ($[VO_3(OH)]^{2-}$) inhibits both pumps with presumably identical mechanisms, whereas the inhibitors thapsigargin and thapsigarcin [18] and cyclopiazonic acid [19] only act on the SERCA pump by interacting with it with high affinity (K_d in the sub-nM range) [20].

The SERCA Pump

An enzyme that couples the hydrolysis of ATP to the transport of Ca^{2+} across the membrane of SR had been postulated about 40 years ago by Ebashi and Lipmann [21]and Hasselbach and Makinose [22]. Later work has identified the pump in the ER of nonmuscle cells as well. The ATPase, later termed the SERCA pump, was purified by MacLennan in 1970 [4] as a protein of about 100 kDa and cloned 15 years later [23]. The enzyme was predicted to be organized in the membrane of the reticulum with ten transmembrane domains and to protrude into the cytosol with three large units. The ATP binding domain and the catalytic aspartic acid are located in the cytosolic unit that protrudes between the fourth and the fifth transmembrane domains. The pump is the product of a multigene family: three basic gene products have so far been described with peculiar tissue distribution, additional isoform diversity being generated by alternative splicing of primary transcripts. SERCA1a is the major isoform of adult fast twitch muscle, whereas the transcripts of SERCA1b are detected in large amounts in neonatal fast twitch muscle. The SERCA2 gene transcript is spliced to generate SERCA2a, which is found in slow twitch and heart muscles, whereas SERCA2b is found in smooth muscle and most nonmuscle cells. The SERCA2b protein is of particular interest because it replaces the last four residues of the SERCA2a isoform with a 49 amino acid stretch [24] that contains a hydrophobic sequence predicted to be the eleventh transmembrane domain [25]. Thus, the C-terminus of the SERCA2b isoform protrudes into the ER lumen. SERCA3 is only expressed in a limited range of nonmuscle cells [26].

Striking advances on the structure of the SERCA pump have recently extended our understanding of the molecular mechanism by which the enzyme couples the hydrolysis of ATP to the transport of Ca^{2+} across the protein. The pump has been crystallized in the Ca^{2+} bound E1 state by Toyoshima *et al*. [27] (Fig. 2). Its structure has been solved at 2.6 Å resolution, validating a number of previous suggestions on membrane topography and Ca^{2+} binding and transport. Specifically, the structure has confirmed that the number of transmembrane domains is 10 and has shown that the three large cytosolic domains (N for nucleotide binding; P, which contains the catalytic aspartic acid; and A, termed actuator or N anchoring domain) undergo large movements during ATP energized Ca^{2+} translocation. The movement of the three cytosolic units has been predicted by fitting the atomic structure to a low resolution structure (8 Å) derived from tubular crystals of the pump in the vanadate inhibited Ca^{2+} free (E2) conformation. The cytoplasmic portion of the E2 pump is more compact, suggesting that Ca^{2+} loosens the interactions between the cytosolic units. The N and P domains come close to each other whereas the A domain rotates by about 90° to bring a conserved, critically important sequence (TGES) next to the catalytic aspartic acid. The structure has also validated previous mutagenesis experiments [28] that had led to the conclusion that a number of residues in transmembrane domains 4, 5, 6, and 8 would form the two Ca^{2+} binding sites and the path of Ca^{2+} across the protein. The atomic structure has shown that transmembrane domain 5 is straight and extends to the center of the P domain, whereas transmembrane domains 4 and 6 are unwound in the middle to optimize Ca^{2+} coordination geometry. The two Ca^{2+} binding sites are separated by a distance of 5.7 Å, site I being formed essentially by transmembrane domains 5 and 6 (with a contribution of transmembrane domain 8) and site II by transmembrane domains 4 and 6. The two Ca^{2+} binding sites are stabilized by H bridges between coordinating residues and to residues on other transmembrane helices. The structure has also suggested the path for Ca^{2+} to the binding sites and from them to the lumenal space. The path to the sites may be a cavity opened to the cytoplasm formed by transmembrane domains 2, 4, and 6. Ca^{2+} would move along a row of hydrophilic carbonyl oxygens and would exit to the lumen of the ER through a zone ringed by hydrophilic oxygens surrounded by transmembrane domains 3, 4, and 5.

The SERCA pump is regulated by interaction with phospholamban (PLN, [29]), a small hydrophobic protein that has a strong tendency to form pentamers but that is active in

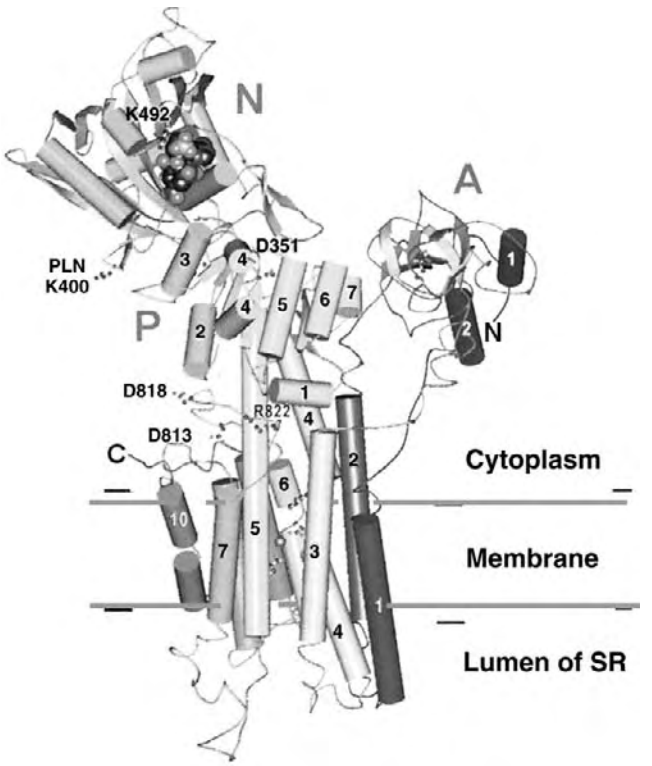


Figure 2 Crystal structure of the calcium bound (E1 form) of the SERCA pump [27]. The structure shows the predicted ten transmembrane domains and three units protruding into the cytoplasm, termed N (nucleotide binding), P (phosphorylation), and A (actuator, or N anchoring domain). Some residues important to the function of the pump are indicated, including K400, which is part of a loop that binds the cytosolic portion of phospholamban. Additional details of the structure and on the predicted motions of the cytosolic domains of the pump in the E1 to E2 conformational transition are discussed in the text.

the monomeric state [30]. Since PLN is only expressed in slow-twitch, heart, and smooth muscles, it only regulates the activity of the SERCA pump in these tissues. PLN is the substrate of two protein kinases, protein kinase A and a calmodulin-dependent kinase (protein kinase G may also phosphorylate it). In the unphosphorylated state it interacts with a cytosolic loop around Lys 400 [31], maintaining the pump inhibited. When phosphorylated on Ser16 and/or Thr17, PLN becomes detached from the binding loop freeing the pump from inhibition. Mutagenesis studies [32] have indicated that PLN also interacts with the intramembrane sector of the pump, specifically, with transmembrane domain 6. This indication has been recently supported by molecular modeling studies of the interaction of PLN [33] with the pump, based on the structure of the latter in the vanadate inhibited state and on the recently solved tertiary structure of PLN [34].

The PMCA Pump

The PMCA pump had been discovered by Schatzmann in 1966 as a system that ejected calcium from erythrocytes. The pump was purified in 1979 as a protein of about 135 kDa by Niggli *et al*. [35], and was cloned ten years later by Shull and Greb [36] and Verma *et al*. [37]. The membrane architecture of the protein resembles that of the SERCA pump, i.e., it is predicted to contain ten transmembrane domains and three large hydrophilic units protruding into the cytoplasm. One important difference with respect to the SERCA pump is the long C-terminal tail, which contains a calmodulin-binding domain [38]. Calmodulin is the most important regulator of the PMCA pump, although polyunsaturated fatty acids, acidic phospholipids, phosphorylation steps involving the C-terminal tail by protein kinase A, or protein kinase C may also activate the pump by lowering its K_m for calcium. Activation is also brought about by a dimerization process that occurs through the calmodulin-binding domain and by the proteolytic removal (e.g. by calpain) of most of the C-terminal tail of the pump [39]. At variance with the SERCA pump, the reaction cycle of the PMCA pump is not regulated by PLN but by a mechanism that has striking similarities to that of the SERCA pump. Specifically, the calmodulin-binding domain interacts in the resting state with two sites in the cytoplasmic portion of the pump, keeping it inhibited [40,41]. Calmodulin removes the binding domain from its "receptors" in the cytosolic portion of the pump, relieving the inhibition. Although in this case phosphorylation is not involved, the similarity to the reversible mechanism of inhibition of the SERCA pump by PLN is even more striking. The phosphorylation of the calmodulin-binding domain of the PMCA pump by protein kinase C impairs its ability to bind to the cytosolic portion of the pump [42,43].

The PMCA pump is the product of a multigene family, with four basic gene products. As in the case for the SERCA pump the number of isoforms is increased by the alternative splicing of primary transcripts. Two of the four basic isoforms (PMCA1 and 4) are expressed in all tissues, whereas PMCA2 and 3 are expressed in significant amounts only in neurons and in cells somehow related to them, e.g. the outer hair cells of the organ of Corti. Alternative splicing occurs at two sites. Site A is located upstream of the third transmembrane domain, next to a site that mediates the sensitivity of the pump to acidic phospholipids, site C within the calmodulin-binding domain itself. Information on the differential functional properties of the PMCA isoforms is very scarce, but it is known that PMCA2 has the highest sensitivity to calmodulin. The proximity of the splicing sites to domains that are important in regulation suggests different regulatory properties of the spliced isoforms. C-spliced variants of the pump may indeed interact with calmodulin with peculiar pH sensitivity [44], whereas a variant of the pump truncated C-terminally as a result of the insertion of a 154 bp hexon at site C [45] has decreased affinity for calmodulin.

An interesting development in the regulation of the PMCA pump has been the finding that its genes are transcriptionally regulated by Ca^{2+} itself [46,47]. The discovery has been made on maturing cultured cerebellar granular neurons, and reflect the regulation of PMCA gene expression within the cerebellum. The cultured granular neurons require a modest

increase in cytosolic calcium (about three-fold) to switch off the apoptotic programs that would otherwise kill them in 3–5 days, and do so by rearranging the expression of PMCA isoforms to accommodate the changing requirements of calcium homeostasis necessary to set cell calcium at a higher level. Under these conditions, PMCA2 and 3 become strongly upregulated within days after the beginning of culture; PMCA1 experiences instead a splicing switch that favors a C-terminally truncated variant. PMCA4, by contrast, becomes rapidly and dramatically downregulated in a process that is mediated by the Ca^{2+}-dependent protein phosphatase calcineurin.

Genetic Diseases Evolving Defects of Calcium Pumps

Pathological phenotypes linked to genetic defects in the genes of both the SERCA and PMCA pumps have been described. In agreement with the distinct brain distribution of PMCA2, which appears to be specifically expressed in cerebellar Purkinje cells and in the outer hair cells of the inner ear, mice with defects in the gene of PMCA2 have been described that display vestibular/motor imbalance and are deaf [48,49]. A similar phenotype has also been described in PMCA2 knockout mice [50].

Pathological phenotypes have also been described as a result of inactivating mutations in the SERCA pump genes. Brody's disease, an autosomal recessive disorder of skeletal muscle characterized by muscle cramping and exercise-induced impairment of relaxation, has been traced back to three different mutations in the SERCA1 gene [51,52] that lead to a loss of SERCA1 activity (although not all cases of Brody's disease are linked to SERCA1 gene defects). Darier's disease, an autosomal dominant skin disorder, has been traced back to mutations in the SERCA2a gene. It has been suggested that the SERCA2 pump influences the adhesion between keratinocites and thus cellular differentiation in the epidermis [53].

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CHAPTER 133

Sodium/Calcium Exchange

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Introduction

The plasma membrane (PM) Na^+/Ca^{2+} exchanger (NCX) is one of the critical mechanisms involved in Ca^{2+} homeostasis and the regulation of Ca^{2+} signaling in most cells. The PM NCX was discovered about 35 years ago in mammalian cardiac muscle [1] and squid neurons [2]. It uses energy from the Na^+ electrochemical gradient, and not directly from ATP, to transport Ca^{2+}. Therefore, as we shall see, a critical aspect of the exchanger's function is that it may either export or import Ca^{2+}, depending upon the NCX coupling ratio and the prevailing membrane potential and Na^+ concentration gradient. The Na^+ gradient and membrane potential are maintained by the ATP-dependent, ouabain-sensitive Na^+ pump (Na^+, K^+-ATPase). A mitochondrial membrane Na^+/Ca^{2+} exchanger has also been identified [3] but has been less well characterized than the PM NCX; the mitochondrial exchanger will not be discussed here. Recent, more extensive reviews of NCX structure and function [3,4] should be consulted for details.

Two Families of PM Na^+/Ca^{2+} Exchangers

Early measurements suggested that the cardiac and neuronal exchangers both had coupling ratios of 3 Na^+: 1 Ca^{2+}, and that these two ion species were the only ones translocated by the exchanger [3,5]. Subsequently, a Na^+/Ca^{2+} exchanger was identified in the PM of photoreceptor cells. The photoreceptor exchanger was also dependent upon K^+ and appeared to have a coupling ratio of $4Na^+:(1Ca^{2+}+1K^+)$ [6,7]. This latter exchanger is therefore designated as the Na/(Ca, K) exchanger or NCKX.

Two families of Na^+/Ca^{2+} exchanger molecules have been cloned and sequenced [8,9]. One corresponds to the cardiac/neuronal NCX [8]; three members of this family, designated NCX1, NXC2, and NCX3, have been identified in mammals [10]. Each of these isoforms is the product of a different gene. NCX1 is the most prevalent, but they all have different tissue distributions. The functional significance of these different isoforms is unclear. In addition, there are several tissue-specific splice variants of NCX1; these, too, exhibit different tissue expression [11], but the functional significance has not been resolved.

The membrane topology of NCX1 is illustrated in Fig. 1. NCX has a molecular weight of 108 kDa (excluding glycosylation) and appears to have nine membrane-spanning segments [12]. A large cytoplasmic loop is located between the 5 N-terminal and 4 C-terminal transmembrane segments. This loop includes a calmodulin-like "exchanger inhibitory peptide" (XIP) binding site, a Ca^{2+} binding site that is involved in internal Ca^{2+}-dependent Ca^{2+} entry, and a peptide region that is alternatively spliced in different tissues (Fig. 1). A site that participates in intracellular Na^+-dependent inactivation may be included within the XIP region. The alpha helix repeat that occurs in helices 2–3 and 7 (gray regions in Fig. 1) has been postulated to participate in the binding and translocation of Na^+ and Ca^{2+}, but the evidence is inconclusive. Part of the second alpha repeat is a P loop-like region between transmembrane segments 7 and 8 that dips into the membrane from the cytoplasmic side but does not traverse the membrane.

Three mammalian members of the second exchanger family, the NCKX family, also have been cloned: NCKX1 is found in rod photoreceptors, NCKX2 is expressed in cones and neurons, and NCKX3 is expressed in the brain and smooth muscles [13,14]. The topology of the deduced NCKX proteins is similar to that of NCX. Nevertheless, the sequence homology of the two families of expressed proteins is limited to two of the putative membrane-spanning domains that

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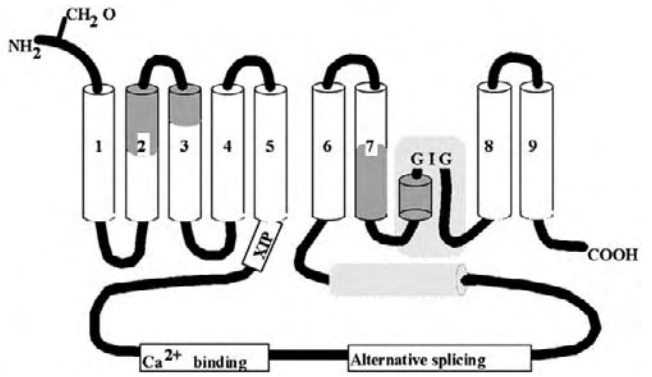


Figure 1 Diagram of Na^+/Ca^{2+} exchanger topology. The model shows the dog cardiac NCX1 (938 amino acids), which apparently has 9 transmembrane segments. The glycosylated N terminus is extracellular. The region between transmembrane segments 7 and 8 apparently forms a "P-type" loop that dips into the membrane (shaded area). The grey portions of segments 2–3 and 7 (and part of the P-type loop) are the alpha repeats. The large cytoplasmic loop (which actually contains nearly 550 amino acids) includes the XIP binding region (and internal Na^+-dependent inactivation site), the Ca^{2+} regulatory site, and the alternative splice site. This cytoplasmic loop also apparently includes a hydrophobic alpha helix region (shaded segment). Reproduced from Philipson and Nicoll [4] with permission.

may be involved in ion binding and translocation. Thus, the NCX and NCKX genes evolved independently.

Modes of Operation of the Na^+/Ca^{2+} Exchangers

As diagramed in Fig. 2, the NCX can mediate electroneutral Na^+/Na^+ exchange and electroneutral Ca^{2+}/Ca^{2+} exchange, as well as the Na^+ entry/Ca^{2+} exit and Na^+exit/Ca^{2+} entry exchange modes. The Ca^{2+}/Ca^{2+} exchange mode is activated by nontransported alkali metal ions. These partial reactions are consistent with a sequential transport mechanism (Fig. 2) [3] in which either one Ca^{2+} ion or three Na^+ ions are bound at one side of the membrane, translocated to the other side, and dissociated before the ion(s) from that side are bound. The reversal potential, $E_{Na/Ca}$, for an NCX with a coupling ratio of $3Na^+ : 1Ca^{2+}$ is given by the equation [3]:

$$E_{Na/Ca} = 3E_{Na} - 2E_{Ca}$$

where $E_{Na} = (RT/F)\ \ln([Na^+]_o/[Na^+]_i)$ and $E_{Ca} = (RT/2F)\ \ln([Ca^{2+}]_o/[Ca^{2+}]_i)$, and the subscripts "o" and "i" refer to the extracellular and intracellular ion concentrations, respectively; R, T, and F have their usual meanings. If the membrane potential is more negative than $E_{Na/Ca}$, the NCX will extrude Ca^{2+}, and if more positive, the NCX will move Ca^{2+} into the cell.

The Na^+ entry/Ca^{2+} exit and Na^+exit/Ca^{2+} entry exchange modes are both rheogenic (i.e., they are associated with net current flow). The exchange of $3Na^+$ for $1Ca^{2+}$ in both of these modes means that one positive charge enters the cells during each Ca^{2+} exit exchange and one positive charge exits the cells during Ca^{2+} entry exchange. Net Ca^{2+} transport mediated by the NCKX also is rheogenic, with one net charge transported per cycle. Consequently, NCX- and NCKX-mediated Ca^{2+} transport can both be measured electrically as ionic current flow across the plasma membrane in the direction opposite the net Ca^{2+} flux. Furthermore, the coupling ratio indicates that Na^+/Ca^{2+} exchange is voltage-sensitive: membrane hyperpolarization promotes Ca^{2+} exit via the exchanger, while depolarization promotes exchanger-mediated Ca^{2+} entry. This is counterintuitive, because hyperpolarization is normally expected to drive Ca^{2+} into cells, while depolarization should slow Ca^{2+} entry.

Regulation of NCX

Several regulatory sites have been identified in the large cytoplasmic loop of NCX. These sites play critical roles in exchanger function. When the cytoplasmic Na^+ concentration ($[Na^+]_i$) is increased, exchanger-mediated Ca^{2+} entry is increased almost instantly, but only transiently; the exchange then declines in a time- and $[Na^+]_i$-dependent manner [15]. This phenomenon, known as Na_i-dependent inactivation, might be expected to limit exchanger-mediated Ca^{2+} entry. However, binding of cytosolic Ca^{2+} to the activation site on the cytoplasmic loop not only is required to activate exchanger-mediated Ca^{2+} entry, but it also reduces Na^+-dependent inactivation [16].

Cardiac NCX is activated by phosphatidylinositol-4, 5-bisphosphate (PIP_2), which is generated from membrane-bound phosphatidylinositol by a mechanism that involves ATP hydrolysis [17]. The PIP_2 apparently binds to the XIP binding region of the large cytoplasmic loop [16]. This not only activates the NCX, but also eliminates Na^+-dependent inactivation.

Inhibition of NCX

NCX is highly selective for Na^+; other monovalent cations cannot substitute for Na^+. While Sr^{2+} and Ba^{2+} can be transported by the NCX, they are very poor substitutes for Ca^{2+} (i.e. maximium transport rates are much lower). Other divalent cations including Ni^{2+} and Cd^{2+}, and La^{3+} and some other lanthanides, inhibit NCX but are nonselective.

NCX inhibitory activity is displayed by various organic molecules. These include some hydrophobic amiloride analogs (e.g. 3,4-dichlorobenzamil), some antiarrhythmic agents (e.g. quinacrine and bepridil), and an isothiourea derivative ("compound 7943"). Unfortunately, none of these molecules is completely selective.

XIP is a synthetic calmodulin-like peptide that can be used as an experimental tool [16]. When introduced into the cytosol, it binds to the "XIP region" of the large cytoplasmic loop (Fig. 1) and inhibits NCX activity.

Localization of the NCX

The PM NCX functions in parallel with the ATP-driven PM Ca^{2+} pump (PMCA), and both transport systems are

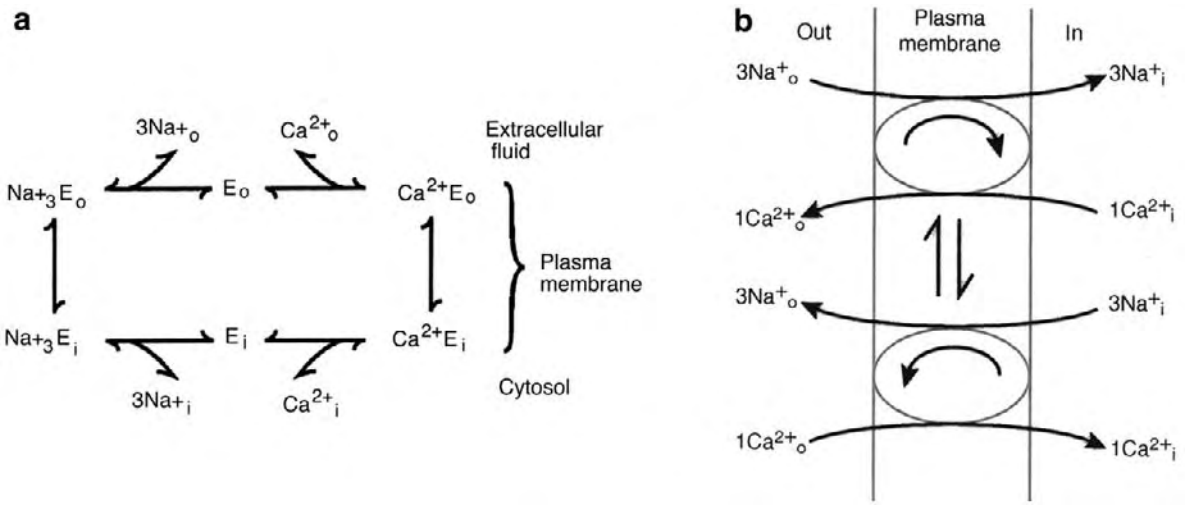


Figure 2 (a) State diagram illustrating the transport reactions mediated by the NCX ("E"). Subscripts "o" and "i" refer to the extracellular fluid or exofacial configuration of the carrier and cytosol or endofacial configuration of the carrer, respectively. Note that the carrier can switch between exofacial and endofacial conformations only when the carrier is loaded ($Na^+{}_3E_o$, $Na^+{}_3E_i$, $Ca^{2+}E_o$, or $Ca^{2+}E_i$); the unloaded carrier does not undergo conformational change (i.e. between E_o and E_i). (b). Diagram of net transport reactions mediated by the Na^+/Ca^{2+} exchanger. The exchanger can either move $3Na^+$ ions into the cell in exchange for one exiting Ca^{2+} ion (top) or move $3Na^+$ ions out of the cell in exchange for one entering Ca^{2+} ion (bottom). Reproduced from Blaustein *et al*. [33] with permission.

present in the PM of most cells. Moreover, the PMCA and NCX have very different kinetic properties—most notably, their affinities for cytosolic Ca^{2+} ($K_{Ca(cyt)} \approx 0.1\,\mu M$ for PMCA and $\approx 1.0\,\mu M$ for NCX1) and their turnover numbers (≈ 30 sec^{-1} for PMCA and $\approx 5{,}000$ sec^{-1} for NCX1). This implies that they have very different functions.

A further clue to their relative functions is their different distributions in the PM. The PMCA is very widely (uniformly?) distributed in the PM of several cell types, including astrogial cells, neurons, and smooth muscle cells [18,19]. In contrast, the NCX has a very much more limited distribution; indeed, in these same three cell types, NCX1 appears to be confined to microdomains of PM that overlie sub-PM ("junctional") elements of the endoplasmic or sarcoplasmic reticulum (jER or jSR) [18]. In skeletal muscle, NCX is localized primarily in T-tubule membranes. In cardiac muscle, too, the NCX is concentrated in T-tubule membranes [20,21]. However, there also is evidence (albeit controversial) of high levels of NCX expression in the peripheral PM [22] and some evidence that the NCX does not reside in the PM overlying jSR [21]. NCX is prevalent at presynaptic nerve terminals, but it appears to be excluded from transmitter release sites ("active zones") where the PMCA is concentrated [23].

Physiological Roles of the NCX

NCX (and NCKX) are expressed at high levels in cells with a large traffic of Ca^{2+} across the PM. Important examples are cardiac myocytes, neurons (especially nerve terminals), photoreceptor cells, and renal distal tubule epithelial cells [3,8,24]. The high level of activity in cardiac myocytes and neurons is consistent with the major role of the NCX in Ca^{2+} extrusion following periods of activity in these cells, and with the >100-fold difference in turnover number between NCX and PMCA. In the heart, the plateau of the action potential may help to maintain a high $[Ca^{2+}]_{CYT}$ during systole by temporarily reducing NCX-mediated Ca^{2+} extrusion. The possibility that NCX-mediated Ca^{2+} entry may contribute to cardiac excitation-contraction coupling has long intrigued investigators but is still controversial.

In the nervous system, the relative distribution of NCX has not yet been directly compared to that of NCKX. The specific roles of these two types of exchangers are not known, nor is it known whether members of both families are expressed in the same cells, but it is noteworthy that the two transporters have different coupling ratios and different reversal potentials.

The NCX is expressed in many epithelia, including gastrointestinal and renal epithelia, and in various endocrine and endocrine secretory cells. In renal distal tubules, the NCX is a key player in the reabsorption of Ca^{2+} and control of Ca^{2+} homeostasis.

NCX plays a role in the modulation of Ca^{2+} signaling in many types of cells. Indeed, this is the basis of the cardiotonic and vasotonic action of cardiotonic steroids [2,25–27]. In some cells, the NCX co-localizes with Na^+ pumps containing $\alpha 2$ or $\alpha 3$ subunits in PM microdomains [18] that are functionally coupled to the underlying jSR or jER [19,24,26]. These units ("PLasmERosomes"), which apparently help regulate Ca^{2+} signaling, contain a tiny diffusion-restricted volume of cytosol wedged between the PM and jSR or jER. Therefore, modulation of the Na^+ pump activity within the PM microdomains by hormones [27] or neurotransmitters [29,30] can alter the local (sub-PM) Na^+ and, via NCX, local Ca^{2+} concentrations. In this way, the Ca^{2+} content of the jSR or jER can be increased or decreased and can thus influence global Ca^{2+} signaling despite minimal change in the bulk $[Na^+]_i$. This resolves a long-standing dilemma about how low-dose

cardiotonic steroids can exert their cardiotonic effect without altering bulk $[Na^+]_i$ [31]. Inhibition (by ouabain, for example) of just a small fraction of the total Na^+ pump molecules [26,32] should raise the local (sub-PM) $[Na^+]_i$. The PLasmERosome structure/function relationships then, in effect, enable the NCX to help translate and amplify the local $[Na^+]_I$ rise into an augmented global Ca^{2+} signal [26]. In other words, the NCX is not simply a "second" Ca^{2+} extrusion mechanism, even though Ca^{2+} extrusion may be a very important part of its function. In addition, the Na^+ pumps and NCX in the PLasmERosome work together to influence jSR/jER Ca^{2+} content; they thereby modulate Ca^{2+} signaling and all of the downstream consequences.

Acknowledgments

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CHAPTER 134

Ca^{2+} Buffers

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Introduction

In principal, any molecule with several negatively charged groups can act as a chelator for Ca^{2+} ions if the negative charges are spatially distributed in such a manner as to satisfy the necessary geometrical considerations for coordination. In biological systems, these requirements are fulfilled by the carboxylic groups of small molecules such as citrate and more especially by the acidic side chain residues (e.g. glutamate, aspartate) or carbonyl groups of proteins. The possibilities for forming Ca^{2+}-binding sites are clearly numerous, and several protein families have been identified that contain different, evolutionarily well-conserved Ca^{2+}-binding domains. These include the EF-hand proteins [1], annexins, and C_2 domain proteins, each of which is described in a separate chapter (see Chapters 136, 140, and 141 of this volume). Almost all known proteins described as "Ca^{2+} buffers" belong to the family of EF-hand proteins [1,2]. An analysis of the human genome has revealed 242 proteins with EF-hand domains, which renders this one of the largest groups of proteins sharing a common motif [3]. EF-hand proteins have been somewhat arbitrarily designated as either "buffers" or "sensors" [4], the distinction being made on the basis that "sensors" undergo Ca^{2+}-dependant conformational changes, which permit them to interact with specific targets in a Ca^{2+}-regulated manner. Typical sensor proteins include calmodulin (see Chapter 137 by Means), some S100 proteins (see Chapter 138 by Heizmann *et al.*), and several others (see Chapter 136 by Bourgoyne and Weiss). Some so-called EF-hand "buffers" [e.g. calretinin (CR) and calbindin D-28k (CB28k)] also display Ca^{2+}-dependent conformational changes, but since no specific targets have as yet been identified, they are currently viewed as buffers. Whether an EF-hand protein can contribute to Ca^{2+}-buffering in a given cell depends largely upon its intracellular concentration. Thus, all proteins classified as "sensors" could essentially act as "buffers" if present at sufficiently high levels.

Relevant Parameters for Ca^{2+} Buffers

In order to understand how a buffer will affect Ca^{2+} homeostasis within a cell, one first needs to consider the relevant parameters. These include (a) its cytosolic concentration, (b) its affinity for Ca^{2+} and possibly also for other metal ions, (c) the kinetics of Ca^{2+} binding and release, and (d) its mobility. But for no single protein have all of these parameters been determined with precision *in vivo*. During the past few years, most studies dealing with EF-hand Ca^{2+}-binding proteins have focused either on their metal-binding affinities (K_D values) or on elucidating their intracellular localization within specific cell types in a given tissue [1]. Proteins that will be discussed here include CB28k, CR, parvalbumin (PV), calbindin D-9k (CB9k; an S100-family protein), visinin-like protein III, and calmodulin (CaM).

Intracellular Concentration

With the exception of the ubiquitously expressed CaM, each of the aforementioned proteins is characterized by a very restricted pattern of expression within a given tissue, which renders an accurate determination of their intracellular concentrations extremely difficult. The proteins are frequently found in excitable cells (e.g. neurons), whose complex morphologies are prone to yield erroneous estimations of volume. Predictions of concentration in specific neurons usually fall within the range 1–50 μM, but the levels of PV in fast-twitch muscles and of CB9k or CB28k in specific cells of the kidney attain millimolar concentrations.

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Metal-Binding Affinities

Two types of Ca^{2+}-binding sites in EF-hand proteins have been identified on the basis of differences in their selectivity and affinity for Ca^{2+} and Mg^{2+} ions [5]. The so-called Ca^{2+}-specific sites predominate, the affinity for this cation being much higher ($K_{Ca} = 10^{-3}$–10^{-7} M) than those for Mg^{2+} ($K_{Mg} = 10^{-1}$–10^{-2} M). Under basal conditions (free intracellular Ca^{2+} concentrations $[Ca^{2+}]_i = 40$–100 nM), the Ca^{2+}-specific sites of most of these proteins are assumed to be essentially vacant of metal ions and thus capable of binding Ca^{2+} rapidly, when $[Ca^{2+}]_i$ is raised. The second type, the mixed Ca^{2+}/Mg^{2+} site binds Ca^{2+} with high and Mg^{2+} with moderate affinity in a competitive manner (dissociation constants: $K_{Ca} = 10^{-7}$–10^{-9} M; $K_{Mg} = 10^{-3}$–10^{-5} M). Under basal conditions, these sites are occupied principally by Mg^{2+} ions, which must dissociate before Ca^{2+} binding can occur. EF-hand proteins have also been shown to contain allosteric effector, Mg^{2+}-specific binding sites, which can influence the affinities of the EF-hand Ca^{2+} binding sites [6]. Most EF-hand domains are paired to form a tandem domain consisting of two helix-loop-helix regions linked by a short stretch of 5–10 amino acid residues. Hence, the majority of these proteins have an even number of EF-hand domains (2, 4, or 6; for details, see Chapter 136). Not only are the tandem domains important for the structural stability of the individual EF-hand domains, but binding of Ca^{2+} ions to one site allosterically affects the affinity and probably also the binding kinetics of the second.

Metal-Binding Kinetics

Under physiological conditions, Ca^{2+}-binding kinetics (on-rates) can vary from $>10^8$ $M^{-1}s^{-1}$ for proteins with Ca^{2+}-specific sites implicated in very fast biological processes, such as muscle contraction (e.g. troponin C; TnC), down to an apparent on-rate of approximately 3×10^6 $M^{-1}s^{-1}$ for the slow-onset buffer PV. In the absence of Mg^{2+} ions, the on-rate of Ca^{2+}-binding to PV is very rapid (1.08×10^8 $M^{-1}s^{-1}$) [7]. But at the free intracellular concentration of magnesium ions $[Mg^{2+}]_i$, pertaining within neurons (0.3–0.6 mM) and rat myocytes (0.9 mM) [8,9], the rate for Ca^{2+}-binding, being determined by the rather slow Mg^{2+} off-rate [7,10] (Table I), will consequently be significantly slower.

During muscle contraction, PV does not compete with TnC for the binding of Ca^{2+} but helps increase the initial rate of $[Ca^{2+}]_i$ decay [11], thereby shortening the relaxation phase following very brief contractions. In this case, Mg^{2+} plays a role of almost equal importance to that of Ca^{2+}; it not only lowers Ca^{2+} affinity to within the physiological range but also exerts a considerable influence on the kinetics of Ca^{2+} binding to, and its release from, PV. The kinetics of Ca^{2+} binding for "fast" and "slow" buffer proteins are similar to those characterizing the synthetic chelators BAPTA and EGTA (Table I), respectively, which are thus often used experimentally to mimic endogenous buffer proteins. Owing to differences in their Mg^{2+}-buffering capacities, PV and EGTA are comparable only so far as their Ca^{2+}-binding kinetics are concerned—not with respect to the Ca^{2+}/Mg^{2+} antagonism (Table I).

Protein Mobility

In the cytosol of *Xenopus laevis* oocytes, only slowly mobile or immobile Ca^{2+} buffers exist. Accordingly, the rate of diffusion for Ca^{2+} ions under basal conditions ($D^* = 13\ \mu m^2/s$) is much slower than that of another small molecule involved in cellular signaling, IP_3 (283 $\mu m^2/s$) [12]. Even when $[Ca^{2+}]_i$ is raised to 1 μM, with a view of saturating the immobile buffer sites, the diffusion coefficient remains relatively low ($65\ \mu m^2/s$). The manner in which a Ca^{2+} transient is affected by the presence of a buffer is also linked to its intracellular localization, that is, whether the buffer is freely

Table I Properties of Ca^{2+}-binding Proteins and Artificial Ca^{2+} Buffers (Adapted from [24])

Buffer	K_D value(s)	k^+_{Ca2+} $M^{-1}s^{-1}$	Ca^{2+}/Mg^{2+} antagonism	No. of EF-hands (functional)	Refs.
PV	4–9 nM [1] $K_{D,app}$ 50 nM [2] 1 nM–100 nM [3]	1.1×10^8 1–2×10^7	strong	3 (2)	[7] [25]
CB	$K_{D1} \approx 180$–240 nM [4] $K_{D2} \approx 410$–510 nM	$\approx 1.2 \times 10^7$ $\approx 8.2 \times 10^7$	weak	6 (4)	[26]
CR	380–1500 nM	$\geq 10^8$ [5]	weak	6 (5)	[27,28]
BAPTA	130–800 nM	10^8–10^9	weak		[29,30]
EGTA	≈ 70 nM	3×10^6–1×10^7	weak		[26]

[1] This represents the K_D value in the absence of Mg^{2+}.

[2] The apparent dissociation constant ($K_{D,app}$) for Ca^{2+} depends heavily upon $[Mg^{2+}]_i$. The K_D value of 50 nM was obtained at a $[Mg^{2+}]_i$ of 0.16 mM. But at a $[Mg^{2+}]_i$ of 0.3–0.6 mM, which corresponds to the range encountered in neurons, $K_{D,app}$ lies around 80–150 nM. This will affect the on-rate of Ca^{2+} binding, lowering it to values of approximately 3–6×10^6 $M^{-1}s^{-1}$ (similar to that for EGTA).

[3] K_D values ranging from 1–100 nM were obtained under different experimental conditions (pH, ionic strength, etc.).

[4] CB contains two types of binding sites, which differ in their affinity for Ca^{2+} and their Ca^{2+}-binding on-rates.

[5] This is an approximation proposed by Edmonds *et al.* [31]. With its five Ca^{2+}-binding sites, CR would be expected to have different K_D and k^+_{Ca2+} values, as is the case with CB. The cited on-rate of 10^8 $M^{-1}s^{-1}$ most probably represents that of the fastest site(s).

diffusible or is bound to structures such as organelles, the plasma membrane, or cytoskeletal structures. The mobility effect may be further complicated if the buffer relocalizes as a result of changes in $[Ca^{2+}]_i$, as in the case for the Ca^{2+} "sensor" visinin-like protein III [13].

Ca^{2+} Buffers as One Component Contributing to Intracellular Ca^{2+} Homeostasis

Following an influx of Ca^{2+} ions into a cell, the role played by Ca^{2+} buffers is apparently a simple one, namely, to bind this cation and thereby lower $[Ca^{2+}]_i$. However, soluble buffers represent but one component of the intricate system implicated in Ca^{2+} homeostasis. A rise in $[Ca^{2+}]_i$ activates also the pumps involved in Ca^{2+} extrusion or Ca^{2+} uptake by organelles, such as the endoplasmic reticulum or mitochondria. These will remain operative until $[Ca^{2+}]_i$ has once again attained its steady-state level and the Ca^{2+} buffers have essentially reverted to their Ca^{2+}-free form, loading at this point being determined by their K_D and by basal $[Ca^{2+}]_i$. It is important to bear in mind that steady-state $[Ca^{2+}]_i$ is determined by the balance obtaining between Ca^{2+}-fluxes across the membranes surrounding the cytosol; it is not influenced by the presence of buffers *per se*. Neither the addition of a Ca^{2+} buffer such as PV or CB28k [7,14] to cells nor its elimination in knockout mice [11,15] affects basal $[Ca^{2+}]_i$, but rather prolongs the time ensuing until the steady-state level has been reattained (Fig.1). In the simplest case, the reduction in amplitude is inversely correlated to the lengthening of the transient, that is the time integral (the product of amplitude and time constant) remains unchanged by the presence of a Ca^{2+} buffer [16].

Intracellular Ca^{2+} transients are often characterized by highly complex patterns in time and space, since several relevant processes, such as Ca^{2+} entry via different pathways, Ca^{2+} binding to buffers (mobile and immobile), and sequestration by pumps, occur on the same temporal scale [7]. Furthermore, saturation of buffers may occur leading to nonlinear (e.g. supralinear) summation of Ca^{2+} signals as demonstrated in cerebellar Purkinje cells [17]. It is evident that the temporal and spatial aspects of Ca^{2+}signals are governed by an intricate interplay of the participating components. In biological systems, nonlinear summation of these signals is rather the rule than the exception, which makes it difficult to analyze the contribution of individual components [16].

Biological Effects of Ca^{2+} Buffers

CR, CB28k, and PV are three major representatives of EF-hand proteins that are classified as "buffers," and each is expressed within a specific subpopulation of neurons. The former two proteins possess, respectively, 5 and 4 Ca^{2+}-specific sites with presumably fast Ca^{2+}-binding kinetics (Table I), whereas PV has two Ca^{2+}/Mg^{2+}-mixed sites with a slow Ca^{2+}-onset rate. In knockout mice for any one of these proteins [11,15,18], the remaining two have been observed to be neither upregulated nor expressed by any types of neurons other than those expressing them in wild-type animals. Hence, neurons are either incapable of inducing the expression of the other two buffers, or the relevant parameters (binding affinities, kinetics or diffusion) are unsuited to these acting as surrogates for the missing one.

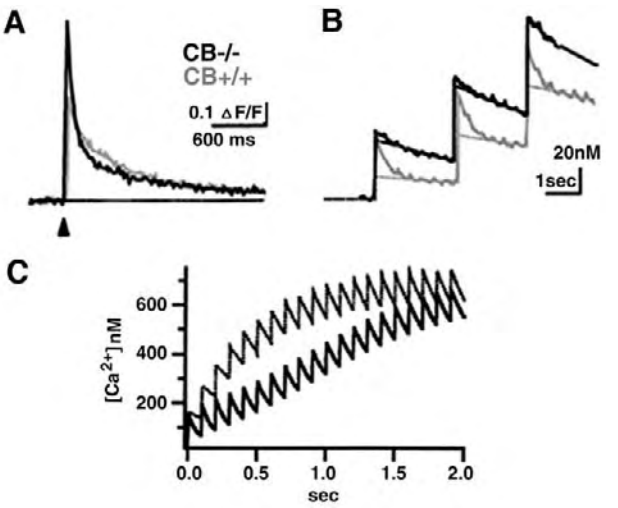


Figure 1 Effect of a fast and a slow buffer on Ca^{2+} transients. (A) Dendritic Ca^{2+} signals in Purkinje cells of CB+/+ (grey trace) and a CB-/- (black trace) mice, elicited by single-shock synaptic stimulation of the climbing fiber (arrowheads; for details, see [15]), CB markedly reduces the amplitude of a Ca^{2+} transient but prolongs the temporal decay of $[Ca^{2+}]_i$. (B) A series of Ca^{2+} transients evoked in a patched chromaffin cell by applying short (20 ms) depolarizing bursts just after break-in (black trace) and after loading with PV via a patch pipette (grey trace, for details, see [7]). PV does not affect the amplitude of the Ca^{2+} transients but increases the initial rate of decay of $[Ca^{2+}]_i$. (C) Simulated Ca^{2+} transients evoked in a neuron by 10 Hz stimulation in the absence (gray trace) or presence (black trace) of 200 µM PV (modified from [7]). Although the build-up of $[Ca^{2+}]_i$ is more rapid in the absence of PV, once the protein is Ca^{2+}-saturated, steady-state $[Ca^{2+}]_i$ is identical under both conditions. Hence, it is the time-course en route to the steady state that is significantly different. From this model, it is likewise evident that it is the metal-binding kinetic parameters that define the frequencies (stimulation intervals) at which a slow-onset buffer is effective in lowering the residual $[Ca^{2+}]_i$ between impulses.

Typical hallmarks of Ca^{2+} transients in excitable cells are their short duration (in the range of 10 to several 100 ms) and often restricted localization, within the axon of neurons, in the subplasmalemmal region of the soma, within parts of the dendrites or even within single spines only. Cytosolic Ca^{2+} buffers have a considerable influence on the spatiotemporal characteristics of such transients. Fast buffers such as CB or CR are able to buffer Ca^{2+} entering via channels from the extracellular space or being released from internal stores with virtually no delay. This reduces the peak amplitude of Ca^{2+} transients but prolongs the decay phase, since proteins such as CB28k or CR act as sources of Ca^{2+} at a later juncture (Fig. 1A). CB28k, on the one hand, is a fast enough buffer to slow down the Ca^{2+}-dependent inactivation of a Ca^{2+} channel and thereby even increases the total Ca^{2+} load [19]. PV, on the other hand, is too slow to affect the peak $[Ca^{2+}]_i$ in most cases, but it can significantly increase the rate of

$[Ca^{2+}]_i$ decay, as revealed in murine fast-twitch muscle fibers [11] or PV-injected chromaffin cells ([7], Fig. 1B).

In the presynaptic terminals or postsynaptic regions (soma, dendrites, and spines) of neurons, repetitive Ca^{2+} transients occurring at short time intervals are a typical physiological signaling event. Whether a particular Ca^{2+} buffer influences the spatiotemporal characteristics of these transients depends upon its concentration, Ca^{2+} affinity, binding kinetics, and diffusion rate. This is exemplified for PV during repetitive stimulations. At short pulse intervals (30 ms), paired-pulse modulation at the synapse between stellate or basket cells and Purkinje cells shifts from depression (Fig. 2A) to facilitation (Fig. 2B), if PV is absent (for example, in PV–/– mice). This phenomenon is attributable to the higher residual $[Ca^{2+}]_i$ obtaining in the absence of PV, which results in the second inhibitory postsynaptic current (IPSC) having a higher amplitude than that of the first (Fig. 2D). Clearly, if the pulses are delivered at longer intervals (300 ms), when residual $[Ca^{2+}]_i$ has decayed to basal levels irrespective of the presence of PV, paired-pulse depression is also observed in PV–/– mice (Fig. 2C). Steady state $[Ca^{2+}]_i$ level during burst-like action potentials (AP) depends upon $\Delta t/\tau$; Δt being the time interval between APs and τ the Ca^{2+} relaxation-time constant of individual Ca^{2+} transients. In the presence of PV, the time course until the equilibrium is reached is delayed, but eventually catches up if all PV molecules are saturated with Ca^{2+} (Fig. 1C; [7]).

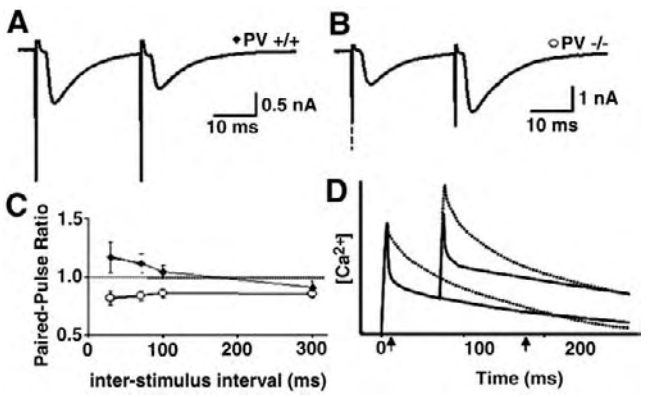


Figure 2 Parvalbumin affects short-term plasticity at the synapse between stellate or basket cells and Purkinje cells in the cerebellum. (A–B) Inhibitory postsynaptic currents (IPSCs) recorded from Purkinje cells during extracellular paired-pulse protocols (at an inter-stimulus interval (ISI) of 30 ms) of GABAergic interneurons. In the presence of PV (i. e. in PV+/+ mice), the second IPSC is depressed (A), whereas in its absence (i. e. in PV–/– mice), facilitation occurs (B). (C) The effect of PV on the paired-pulse ratio ($IPSC_2$/$IPSC_1$) is seen only when the ISI lies between 30 and 100 ms. When the interval is increased to 300 ms, the ratio does not differ between PV–/– and PV+/+ mice. (D) Within a presynaptic terminal, the peak $[Ca^{2+}]_i$ attained during the initial pulse is not affected by the absence or presence of PV, but the decay phase is slower in the former case (broken line). Hence, for a certain period of time (arrows), residual $[Ca^{2+}]_i$ will be elevated. If a second pulse is delivered during this period, then the peak $[Ca^{2+}]_i$ attained will be higher than during the first, a result that leads to enhanced facilitation. At the synapse between PV-containing stellate or basket cells and Purkinje cells, the effect of this Ca^{2+} buffer is maximal at a ISI of 30 ms (A, B, C: modified from [23]; D: modified from [24]).

In *Xenopus* oocytes, the injection or overexpression of PV induces elementary Ca^{2+} release events (Ca^{2+} puffs), which are elicited from discrete clusters of inositol 1,4,5 trisphosphate receptors (IP_3Rs) at low concentrations of IP_3 [20]. Ca^{2+} puff activity has also been detected after the injection of low concentrations of EGTA, but not after that of CB28k, which supports the idea that particular buffers are not simply interchangeable. This circumstance indicates that each buffer has distinct functions, which accord with its specific buffering properties. This is further illustrated by the finding that the changes in spine morphology of Purkinje cell dendrites (increased length and volume) observed in CB-deficient mice are not seen in PV-deficient ones [21]. In the fast-twitch muscles of PV-deficient mice, the volume of mitochondria, organelles also involved in Ca^{2+} sequestration helping to decrease $[Ca^{2+}]_i$ after Ca^{2+} transients (see Chapter 14 by Duchen), is almost twice as large as those in wild-type animals [22]. Ca^{2+} buffers thus constitute an integral part of the finely tuned system involved in Ca^{2+} homeostasis and have a profound effect on many aspects of Ca^{2+} signaling. The removal of such a buffer does not apparently trigger the obvious compensation mechanism (that is, the upregulation of another Ca^{2+} buffer), but leads rather to subtle changes in cell morphology or to discrete modulations in Ca^{2+} uptake or release systems. This may represent the cell's attempt to re-establish a "normal" state of Ca^{2+} homeostasis.

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CHAPTER 135

Mitochondria and Calcium Signaling, Point and Counterpoint

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Introduction

Mitochondria can no longer be considered as static structures whose sole function is the unobtrusive manufacture of ATP. It is now clear that they also represent a storeroom for a number of potentially lethal proteins that are unleashed during programmed cell death and that they are significant participants in the detailed intracellular organization of cellular $[Ca^{2+}]_c$ signaling. While the expression in the mitochondrial membrane of Ca^{2+} transporting mechanisms was established years ago, the physiological significance of these pathways has only recently become apparent. There is now no question that mitochondria will take up and accumulate Ca^{2+} in all cells studied during the routine events of cellular $[Ca^{2+}]_c$ signaling, and that the pathway influences both mitochondrial function itself and the spatiotemporal and quantitative characteristics of the cellular $[Ca^{2+}]_c$ signal. As general issues relating to mitochondrial Ca^{2+} handling have recently been widely reviewed (e.g. [1–3]), I propose in this essay to highlight some of the more controversial and novel developments in the field over recent years and some of the mechanistic, quantitative, and comparative questions that remain.

Fundamentals

When energized mitochondria are exposed to raised $[Ca^{2+}]_c$, Ca^{2+} will move into the matrix. The accumulation of Ca^{2+} by mitochondria depends on the electrochemical gradient for Ca^{2+}, defined by the mitochondrial membrane potential, referred to as $\Delta\psi_m$, and by the intramitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$), which is kept low under resting conditions largely through the activity of a xNa^+/Ca^{2+} exchanger (see below). The mitochondrial potential is established and maintained by respiration and so requires a supply of oxygen and carbon substrate—collapse of $\Delta\psi_m$ due to anoxia, ischemia, damage to the respiratory chain, or the action of biochemical reagents, such as uncouplers, limit mitochondrial Ca^{2+} accumulation. One might ask whether any cell is ever really "at rest" *in situ* in the active organism in contrast to the artificial situation of the cell grown in culture—in which case, what would the normal $[Ca^{2+}]_m$ be in a cell in the living tissue and organism? The closest we get to that information comes from electron probe microanalysis (e.g. see [4]), but multiphoton imaging now holds the promise of being able to study mitochondria within intact tissues.

Machinery of Mitochondrial Ca^{2+} Movement

The Uniporter

Ca^{2+} is taken up through the mitochondrial inner membrane by a uniporter. Remarkably, we do not know the molecular identity or even the precise nature of this pathway. Is it a channel or a carrier? Flux rates are equivalent to those measured for fast gated pores, but rather slower than those seen for channels (see [3] for review). The activity of the uniporter shows little sensitivity to changes in temperature, and it also shows a wide spectrum of cation selectivity, together suggesting that it is a channel rather than a carrier. Ca^{2+} uptake via the uniporter is inhibited by ruthenium red (RuR), a compound that inhibits a variety of cation channels, including L-type plasmalemmal Ca^{2+} channels [5], ryanodine sensitive ER Ca^{2+} release channels [6], and vanilloid receptor operated channels [7], again suggesting that the uniporter may share channel properties.

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One of the most interesting features of the uniporter is an apparent gating by $[Ca^{2+}]_c$, identified primarily through studies of the Ca^{2+} sensitivity of RuR-sensitive mitochondrial Ca^{2+} *efflux* in response to dissipation of $\Delta\psi_m$ [8,9]. Montero *et al*. [9], showed that, while collapse of $\Delta\psi_m$ prevents mitochondrial Ca^{2+} uptake, collapse of $\Delta\psi_m$ *after* the accumulation of mitochondrial Ca^{2+} inhibited mitochondrial efflux, i.e. all mitochondrial efflux pathways were inhibited by depolarization. Addition of Ca^{2+} to the depolarized Ca^{2+} loaded mitochondria then promoted mitochondrial Ca^{2+} release sensitive to RuR, suggesting release through the uniporter. This is consistent with suggestions that the uniporter is allosterically gated by $[Ca^{2+}]_o$ [8], an observation that may also explain why local $[Ca^{2+}]_c$ needs to be higher than one might expect from the behavior of a conducting Ca^{2+} channel in order to see significant increases in $[Ca^{2+}]_m$.

An uptake pathway with properties distinct from those of the uniporter has also been described [10,11] and dubbed the rapid uptake mode (RaM). This pathway has the capacity to transfer Ca^{2+} very rapidly into the mitochondria during the rising phase of a Ca^{2+} pulse. The properties of the pathway differ in different tissues [11], but in heart the pathway saturates quickly and is slow to reset after activation. Again, the functional significance of the pathway remains to be established.

VDAC

The mitochondrial outer membrane has been assumed to be permeant to small ions and so has been largely neglected in considerations of mitochondrial Ca^{2+} handling. However, the outer membrane may play a more significant role in modulating access of Ca^{2+} to the uniporter through the selectivity filter of the voltage-dependent anion channel (VDAC). It appears that VDAC is Ca^{2+} permeant and is regulated both by $[Ca^{2+}]$ and by RuR [12]. This finding raises questions about the extent to which the properties of the uptake pathway are defined by VDAC acting as a first filter. It is also tantalizing that VDAC appears to be part of the mitochondrial permeability transition pore (mPTP; see below), itself regulated by $[Ca^{2+}]_m$, as the mPTP provides a potential efflux pathway for Ca^{2+}, although the physiological relevance of this pathway is debated. Such studies point to the outer membrane as a significant permeability barrier that may itself be regulated.

Mitochondrial xNa^+/Ca^{2+} Exchange

The major route for Ca^{2+} efflux from mitochondria is a xNa^+/Ca^{2+} exchange. Identified about twenty years ago, it has a discrete pharmacology distinct from the plasmalemmal exchanger. The stoichiometry of the exchanger seems still to be controversial. Initially, it was thought to be an electroneutral $2Na^+/Ca^{2+}$ exchanger [13], but this has been questioned, as the exchanger can operate against a $[Ca^{2+}]$ gradient whose energy is over twice that of the Na^+ gradient [14]. Jung *et al*. [14] suggested a stoichiometry of $3Na^+/Ca^{2+}$, in which case the operation of the exchanger will be dependent on $\Delta\psi_m$. The inhibition of mitochondrial Ca^{2+} efflux by mitochondrial depolarization (see above [9], and also [15]) supports this electrogenic stoichiometry. An electrogenic stoichiometry also predicts that Ca^{2+} efflux should be associated with mitochondrial depolarization. To my knowledge, this has not been documented.

The Set Point

Flux studies in isolated mitochondria revealed many years ago that mitochondria will take up Ca^{2+}. With small elevations of $[Ca^{2+}]_o$, the removal of Ca^{2+} from the matrix by the xNa^+/Ca^{2+} exchange may be sufficiently rapid so that net $[Ca^{2+}]_m$ changes little. As $[Ca^{2+}]_o$ rises above ~4–500 nM, the capacity of the exchanger is exceeded and mitochondria show net accumulation of Ca^{2+}. This was termed the "set point" for mitochondrial Ca^{2+} uptake by Nicholls and Crompton [16]. It is worth considering that Ca^{2+} flux into mitochondria is not necessarily synonymous with a net increase in $[Ca^{2+}]_m$, especially given our ignorance of the Ca^{2+} buffering capacity of the matrix. This is not purely semantic, as Ca^{2+} uptake by the uniporter is electrogenic and is therefore associated with small changes in $\Delta\psi_m$. Experimentally, changes in $\Delta\psi_m$ will reflect the rate of Ca^{2+} flux, and may therefore prove a more sensitive measurement of Ca^{2+} movement into mitochondria than measurement of $[Ca^{2+}]_m$. Further, net mito chondrial Ca^{2+} accumulation will be partly set by the activity of the xNa^+/Ca^{2+} exchanger—and we still know little about its regulation.

Many excitable cells respond to depolarization with a rise in $[Ca^{2+}]_c$, which rises rapidly and recovers with an initial rapid phase and a slower second phase that can even form a plateau [17–19]. It has been established in many preparations that the slow recovery phase reflects the redistribution of mitochondrial Ca^{2+} through the activity of the Na^+/Ca^{2+} exchanger, reflecting the set point, typically initiated at a $[Ca^{2+}]_c$ of ~500 nM. The operation of this system has functional consequences at presynaptic terminals, where the $[Ca^{2+}]_c$ plateau that follows repetitive stimulation, maintained by the reequilibration of mitochondrial Ca^{2+}, provides an elevated $[Ca^{2+}]_c$ baseline upon which subsequent stimulation initiates an enhanced synaptic response—the basis for post-tetanic potentiation of synaptic transmission [18,20]. It is also intriguing that the post stimulus plateau phase is not seen in nonexcitable cells following the transmission of $[Ca^{2+}]_c$ signals from ER to mitochondria. Certainly in astrocytes, $[Ca^{2+}]_m$ remains high for a very prolonged period after stimulation [21], suggesting that mitochondrial Ca^{2+} efflux must be very slow and perhaps the activity of the exchanger differs between tissues or cell types.

Quantitative Issues, Microdomains, and the Regulation of $[Ca^{2+}]_c$ Signals

There has been some debate about the quantitative relationships between ambient $[Ca^{2+}]$ and mitochondrial uptake.

In HeLa cells transfected with mitochondrially targetted aequorin and then permeabilized, net mitochondrial Ca^{2+} accumulation was only detectable if the added Ca^{2+} reached concentrations higher than 3 μM, while $[Ca^{2+}]_c$ signals evoked by IP_3 mobilizing agonists were far more effective at raising $[Ca^{2+}]_m$ even though the mean $[Ca^{2+}]_c$ signal might rise to <1 μM [22]. This led to the suggestion that mitochondria must be positioned at privileged sites close to the ER Ca^{2+} release sites where they would be exposed to microdomains of high local $[Ca^{2+}]_c$ sufficient to promote rapid Ca^{2+} uptake.

The proximity of mitochondria to SR or ER Ca^{2+} release sites has been further emphasized through evidence that focal, nonpropagating ER/SR Ca^{2+} release can cause a transient increase in $[Ca^{2+}]_m$ in mitochondria close to the release site. Thus, we found [23] that mitochondria in cardiomyocytes show spontaneous transient mitochondrial depolarizations that were dependent on local SR Ca^{2+} release and were blocked by inhibition of mitochondrial Ca^{2+} uptake. Hajnoczky *et al.* [24] have since shown that local $[Ca^{2+}]_c$ sparks may be associated with the direct transfer of Ca^{2+} to mitochondria visualized as transient increases in $[Ca^{2+}]_m$, which the group termed Ca^{2+} "marks." Further data from cardiomyocytes [25] strongly suggest that, in cardiomyocytes, mitochondria and SR must show very close coupling, as the transfer of Ca^{2+} to mitochondria in response to SR Ca^{2+} release with caffeine in permeabilized cells was sustained despite Ca^{2+} buffering by BAPTA sufficient to suppress the cytosolic signal. The transfer of Ca^{2+} was prevented by disrupting the cytoskeleton, suggesting that the maintained close apposition of mitochondria to SR was central to this signal.

The proximity of mitochondria to Ca^{2+} release sites has functional consequences for $[Ca^{2+}]_c$ signaling. Using $[Ca^{2+}]$ indicators in both mitochondria and ER in permeabilized cells, Csordas et al. [26] showed direct transfer of Ca^{2+} from ER to mitochondria and suggested that the proximity must be ~10–20 nm. This work was extended to show that mitochondrial Ca^{2+} uptake enhances the release of Ca^{2+} from the ER in response to IP_3 by acting as a local buffer [27]. Thus, by removing Ca^{2+} from the microdomain close to the IP_3 Ca^{2+} release channel, mitochondria prevent the Ca^{2+} dependent inactivation of the channel and facilitate ER Ca^{2+} release. This mechanism allows mitochondria to play a significant role in shaping the spatiotemporal patterning of $[Ca^{2+}]_c$ signals. In Xenopus oocytes, energization of mitochondria enhances the propagation and coordination of $[Ca^{2+}]_c$ waves [28], while in astrocytes, which express primarily IP_3 type 3 receptors, energized mitochondria serve as a spatial buffer that limit the rate and extent of propagation of $[Ca^{2+}]_c$ waves [21]. Microdomains of $[Ca^{2+}]_c$ regulated by mitochondria also play a significant role in the regulation of capacitative Ca^{2+} influx [29,30], suggesting that the mitochondria must be positioned close to the plasma membrane. The principle is very much as outlined above for the IP_3 receptor, as the Ca^{2+} influx channel is desensitized by Ca^{2+}. By keeping $[Ca^{2+}]_c$ low in microdomains close to the channels, mitochondria keep the channels open and facilitate Ca^{2+} influx through the channels.

In the blowfly salivary gland and in pituitary gonadotropes, mitochondrial Ca^{2+} uptake may even play a major role in defining the rates of oscillation of the IP_3 generated $[Ca^{2+}]_c$ signal [31,32], suggesting that the interplay between mitochondrial Ca^{2+} uptake and ER Ca^{2+} release contribute significantly to the temporal patterning of the $[Ca^{2+}]_c$ signal.

In pancreatic acinar cells, the mitochondria are concentrated into a band that isolates the secretory pole of these polarized cells, and they seem to act as a "firewall" that limits the spread of $[Ca^{2+}]_c$ signals from their initiation at the apical pole to the basal pole [33]. Furthermore, mitochondria localized close to the basal pole are more sensitive to local Ca^{2+} influx by capacitative entry, and so it seems that the positions of mitochondria within the cell may have a profound influence on their interaction with cellular $[Ca^{2+}]_c$ signals [34]. This issue alone is fascinating but unresolved—what dictates the positions mitochondria occupy within cells? Indeed, imaging mitochondria within cells shows that they move, and that the movement is erratic and unpredictable, and, to my knowledge, we have no idea what might be the functional significance of that movement.

Are $[Ca^{2+}]_c$ microdomains essential for mitochondria to sense changes in $[Ca^{2+}]_c$ associated with $[Ca^{2+}]_c$ signals? In permeabilized adrenal glomerulosa cells, Szabadkai *et al.* [35] found that graded additions of buffered external Ca^{2+} caused a graded but nonlinear increase in $[Ca^{2+}]_m$, showing a response even when the ambient $[Ca^{2+}]$ was only ~200–300 nM. Such data suggest that the close juxtaposition of mitochondria to Ca^{2+} sources is not an absolute requirement if they are to respond to $[Ca^{2+}]_c$ signals. A recent study in HeLa cells [36] also suggests that areas of maximal mitochondrial Ca^{2+} uptake may be divorced from areas of maximal proximity with ER in HELA cells. This study suggested that peripheral mitochondria had larger mitochondrial potentials and that this might provide a mechanism to enhance mitochondrial $[Ca^{2+}]_c$ accumulation into that mitochondrial population. The notion that mitochondria within a single cell may have different potentials remains contentious and the observation is critically dependent on the behavior of the fluorescent indicators used to measure $\Delta\psi_m$. This is probably not the place for further discussion of this issue, but my own view is that the question remains open and has not been satisfactorily resolved either way.

Impact of Ca^{2+} Uptake on Mitochondrial Function

In teleological terms, it seems that the major functional significance of mitochondrial Ca^{2+} uptake is in the regulation of mitochondrial metabolism. In the early 1990s it was shown that the three major rate-limiting enzymes of the citric acid cycle are all upregulated by Ca^{2+} (for review, see [37]). The question that remained was the functional issue—is mitochondrial Ca^{2+} uptake during physiological signaling sufficient for this mechanism to provide a functional regulation of metabolism? First suggestions that such a system operates in intact cells came from measurements of changes

in mitochondrial redox state, reflected as changes in mitochondrial NADH and flavoprotein autofluorescence, in response to changes in $[Ca^{2+}]_c$ [5,38]. These observations showed clearly that (1) mitochondria must be taking up Ca^{2+} during $[Ca^{2+}]_c$ signals, and (2) that this was sufficient to activate the TCA cycle, causing increased net reduction of the coenzymes. More recently, transfection of cells with firefly luciferase allowed a clear and unequivocal demonstration that mitochondrial Ca^{2+} uptake increases mitochondrial ATP production [39]. The relative importance of this mechanism in the regulation of mitochondrial oxidative phosphorylation over the more traditional model, in which the rate of ATP generation is regulated largely by the ATP/ADP ratio, is not clear. It is very attractive to suggest that the transfer of Ca^{2+} from the cytosol to mitochondria during $[Ca^{2+}]_c$ signals represents a major mechanism to couple ATP supply with demand, as in almost all systems, increases in work are associated with increases in $[Ca^{2+}]_c$. Nevertheless, direct evidence for a significant role in intact systems is limited and there are conflicting data (see e.g. [40,41]).

The time course of the changes in $[Ca^{2+}]_m$ and in activation of the enzyme systems becomes crucial. $[Ca^{2+}]_c$ signals are typically brief, transient phenomena. Typically, it seems that the resultant mitochondrial activation is prolonged with respect to the change in $[Ca^{2+}]_c$ [5,42,43], and this in turn will be a function of the rate of mitochondrial Ca^{2+} efflux and the half-life of the activated states of the enzymes.

A further major question that is important in considering the impact of Ca^{2+} on mitochondrial function is, how high does $[Ca^{2+}]_m$ rise during these signals? There is not space here for a detailed discussion, but experiments using low-affinity variants of aequorin suggest that, at least in some mitochondria in some cells, $[Ca^{2+}]_m$ may rise into the millimolar region [44].

Mitochondrial Ca^{2+}, Disease, and Death

Most important, mitochondrial Ca^{2+} uptake may have profound consequences for mitochondrial function under pathological conditions. A combination of mitochondrial Ca^{2+} loading and oxidative stress and/or ATP depletion may promote opening of the mitochondrial permeability transition pore (mPTP). This appears to reflect a pathological conformation of a group of mitochondrial membrane proteins, notably the adenine nucleotide translocase (ANT) and VDAC, with the association of cyclophilin D, a regulatory protein that confers sensitivity of the complex to cyclosporin A, and a possible association of a number of other proteins, including the antiapoptotic Bcl-2, and the benzodiazepine receptor (see [45,46] for reviews). It is not clear whether the mPTP has any physiological function, but its complete opening under pathological conditions will lead inevitably to energetic collapse and cell death, and has been implicated in Ca^{2+} dependent cell death in reperfusion injury in the heart and in glutamate neurotoxicity in the CNS.

CODA

Our perception of the mitochondrion has changed radically over the last few years. Mitochondrial function is critical for the viability of the cell, mitochondria play an integral role in shaping cell signaling, and the dysfunction of the pathways necessary for these functions may trigger cell death. These are not trivial and peripheral functions but are central to cell life and cell death.

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CHAPTER 136

EF-Hand Proteins and Calcium Sensing: The Neuronal Calcium Sensors

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Introduction

EF-hand calcium (Ca^{2+})-binding proteins play many important roles in Ca^{2+}-homeostasis and Ca^{2+}-signaling mechanisms. The EF-hand containing protein calmodulin has been most extensively studied but the EF-hand motif is the most prevalent and widely distributed protein domain in Ca^{2+} signaling. The neuronal Ca^{2+}-sensor (NCS) proteins are a closely-related family of Ca^{2+}-binding proteins whose physiological functions have begun to emerge in recent years.

EF-hand Ca^{2+}-binding proteins fall into two main categories: Ca^{2+} buffers, which act as Ca^{2+} chelators but do not undergo a conformational change, and Ca^{2+} sensors, which upon Ca^{2+} binding undergo a conformational change and transfer the signal to other proteins. This conformational change can alter interactions of EF-hand Ca^{2+} sensing proteins with target proteins or cause a conformation change in proteins already bound to the sensor concurrent with the Ca^{2+} concentration shift. Members of the neuronal Ca^{2+}-sensor (NCS) family (Table I) include proteins expressed only in the retina (e.g. recoverin) and others expressed mainly in neuronal and neuroendocrine cells such as the neurocalcins (*vi*sinin-*li*ke *p*roteins [VILIPs]) and NCS-1 (frequenin). The NCS proteins are ~22 kDa, high-affinity Ca^{2+}-binding proteins, with ~30–70% protein sequence identity to each another, and most members of the family are N-terminally myristoylated. A Ca^{2+}-myristoyl switch mechanism has been proposed for some members of the family in which the proteins bind membranes in a Ca^{2+}-dependent manner via exposure of the myristoyl-group upon Ca^{2+} binding (Table I). The myristoyl group may also be important for protein-protein interactions. Two recent reviews [1,2] cover the NCS proteins in detail, and this chapter will emphasize recent developments in the field.

Class A. Neuronal Calcium Sensor 1 (Frequenin)

Drosophila mutants overexpressing frequenin were found to have frequency-dependent facilitation of evoked neurotransmission at the neuromuscular junction [3], and overexpression of NCS-1 results in enhancement of evoked exocytosis in neuroendocrine cells via an indirect effect of NCS-1 [4]. It is now known that NCS-1 regulates both voltage-gated (VG) Ca^{2+} channels and potassium (K^+) channels. NCS-1 functions in a G-protein-coupled receptor-mediated pathway of VG Ca^{2+} channel inhibition in chromaffin cells that relies on the activity of a Src-like tyrosine kinase [5,6]. NCS-1 has also been shown to increase the current amplitude for Kv4 (A-type) K^+ channels [7]. In a pathway involving glial-derived neurotrophic factor (GDNF), NCS-1 has been implicated in the facilitation of N-type channels in neuromuscular synapses [8]. NCS-1 knockouts and overexpression mutants in *C. elegans* demonstrate that NCS-1 has a role in learning and memory [9].

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Table I General Properties of the NCS Family of Proteins

Protein name	Myristoyl switch mechanism	Structure determined	Ca^{2+} binding affinity	Tissue/cell localization	Subcellular localization	Pathways/ molecular targets	Possible physiological function(s)
NCS-1	No	Yes	0.3 μM	Brain, adrenal gland, COS cells, mouse inner ear, insulin secreting cells, heart, etc.	Golgi, synapses, dendrites, synaptic-like vesicles	PI4 kinase, calcineurin, ion channels	Control of neurotransmitter release , Ca^{2+} and K^+ channel regulation, learning and memory
Recoverin	Yes	Yes	2.1 μM	Photoreceptors	Photoreceptor membranes	Rhodopsin kinase	Retinal phototransduction
VILIP 1	Yes	No	1 μM	Brain-highest expression in cerebellar granule cells, and hippocampus, retina	Unknown	Actin, tubulin, ds RNA, cGMP	Cytoskeletal regulation, cGMP pathways
VILIP 2	Yes	No	Unknown	Brain-except cerebellum	Unknown	Unknown	Unknown
VILIP 3	Yes	No	Unknown	Highest expression in cerebellar purkinje cells	Unknown	Unknown	Unknown
Neurocalcin δ	Yes	Yes	0.6 μM	Highest expression in cerebellar purkinje cells	Plasma membrane, Golgi complex	Actin, tubulins and clathrin	Cytoskeletal regulation, protein trafficking
Hippocalcin	Yes	No	5 μM	Highest expression in hippocampus	Hippocampal pyramidal neurons-soma and dendrites	PLD, NAIP, Cdc42, MLK2	Apoptosis, MAP kinase pathway
GCAP 1	Yes	No	0.26 μM	Photoreceptors	Photoreceptor membranes	Guanylate cyclase	Retinal phototransduction
GCAP 2	Reversed	Yes	0.25 μM	Photoreceptors	Photoreceptor membranes	Guanylate cyclase	Retinal phototransduction
GCAP 3	Yes	No	0.25 μM	Photoreceptors	Photoreceptor membranes	Guanylate cyclase	Retinal phototransduction
KChIP 1	Unknown	No	Unknown	Brain	Plasma membrane	K^+ channels	K^+ channel regulation
KChIP 2	No myr. group	No	Unknown	Brain, heart	Plasma membrane	K^+ channels	K^+ channel regulation
KChIP 3	No myr. group	No	14 μM	Brain, testes	Plasma membrane	K^+ channels, DNA, Presenilins	K^+ channel regulation, transcriptional repressor for pain modulation
KChIP 4	No myr. group	No	Unknown	Brain	Membranes	K^+ channels	K^+ channel regulation, KchIP4b abolishes fast inactivation of Kv4 A-type currents.

Further evidence for a role of NCS-1 in learning and memory comes from the observation of an increase in brain NCS-1-mRNA levels following induction of long-term potentiation [10]. It has been demonstrated both biochemically and in cells that myristoylated NCS-1 binds membranes in a Ca^{2+}-independent manner [11,12]. It has also been reported that yeast frequenin does not need Ca^{2+} for interaction with its major target PI-4-kinase [13]. This suggests that NCS-1 does not rely on the Ca^{2+}-myristoyl switch mechanism for binding interactions. NCS-1 has multiple binding partners in adrenal chromaffin cell fractions; some of these interactions are Ca^{2+}-independent and others are Ca^{2+}-dependent [11].

Class B. Neurocalcins (VILIPs) and Hippocalcin

Neurocalcins (VILIPs) are expressed in certain classes of neurons (Table I). Both VILIPs and hippocalcin have been shown to have a classic Ca^{2+}-myristoyl switch mechanism in cells [12]. VILIP1 and neurocalcin δ have been shown to bind actin, tubulins, and, in the case of neurocalcin δ, clathrin as well [14,15]. These interactions appear to link neurocalcins to cytoskeletal regulation and possibly vesicle trafficking mechanisms. VILIP1 has also been shown to interact with double stranded RNA in a Ca^{2+}-dependent manner [16] and also increases cGMP levels in PC12 cells and cerebellar granule neurons [17]. VILIP1 expression promoted cell death, tau phosphorylation, and appeared to have a role in Ca^{2+}-mediated cytotoxicity in PC12 cells [18]. Hippocalcin is predominantly expressed in mammalian hippocampus and has been implicated in interactions with neuronal apotosis inhibitory protein (NAIP), phospholipase D (PLD)/Cdc42, and mixed lineage kinase 2 (MLK2) [19–21]. These studies link hippocalcin to endocytosis, MAP kinase, and apoptosis pathways.

Class C. Recoverins

Recoverins are the best-studied members of the NCS protein family. The structure of recoverin has been resolved both in the Ca^{2+}-free and Ca^{2+}-bound states. Analysis of its biochemical properties and structure has revealed that recoverin uses the Ca^{2+}-myristoyl switch mechanism. Recoverins are expressed in rod photoreceptors and have an important role in the Ca^{2+}-signaling of retinal phototransduction [22]. Recoverin regulates cGMP levels indirectly via a Ca^{2+}-dependent interaction with rhodopsin kinase inhibiting the kinase in the dark when Ca^{2+} levels are high [22].

Class D. Guanylate Cyclase Activating Proteins

Like recoverins, guanylate cyclase activating proteins (GCAPs) are Ca^{2+} sensors expressed in photoreceptors that play an important role in the signal transduction pathways of the retina by regulating guanylate cyclase (GC) activity. In the retina Ca^{2+} levels are low in the light. In such low Ca^{2+} conditions the GCAPs activate GC. This allows cGMP levels to be directly increased to allow activation of the cyclic-nucleotide gated channels. GCAP2 and GCAP3 directly activate GC in their Ca^{2+}-free state and at increased levels inhibit GC. GCAP2 is bound to membranes at low Ca^{2+} levels and as Ca^{2+} is elevated it dissociates from membranes [22]. EF-hand 1 in all the NCS family members is unable to bind Ca^{2+}. Recently it was determined that GCAP2 interacts with GC via this EF1 domain [22]. This may be an evolutionarily conserved mechanism in the NCS proteins, whereby the ability of EF1 to bind Ca^{2+} was lost in order to gain a protein interaction domain.

Class E. K^+ Channel Interacting Proteins

K^+ channel interacting proteins (KChIPs) [24,25] regulate A-type K^+ channel currents. KChIP1 is the only member of the KChIP subfamily that is myristoylated. Modulation of Kv4.2 K^+ channels in CHO cells and Kv4.2 and Kv4.3 K^+ channels in *Xenopus* oocytes via arachidonic acid has been shown to be dependent on KChIP1 [26]. KChIP2 also regulates KV4.2 and Kv4.3 K^+ channels, and KChIP2 knockout mice are highly susceptible to ventricular tachycardia due to the loss of a transient outward K^+ channel current in the heart [27]. KChIP3 is the same protein as calsenilin, which interacts with presenilin-1 and 2. Presenilin-1 mutations are the most common cause of familial Alzheimer's disease, and calsenilin interacts with the endogenous 20 kDa C-terminal fragment of presenilin 2 that is a product of regulated proteolytic cleavage [28]. KChIP3/calsenilin is also the same protein as downstream regulatory element antagonist modulator (DREAM), which is a Ca^{2+}-regulated transcriptional repressor involved in pain modulation [29,30]. An alternative splice variant of KChIP4 (KChIP4a) encodes a protein with a novel N-terminus called the KIS (K-channel inactivation suppressor) domain. The KIS domain appears to be important for the abolishment of fast inactivation of Kv4.3 channels [31].

Future Perspectives for the NCS Protein Family

The NCS proteins are already known to have multiple binding protein partners. More studies are needed to characterize these interactions so that we can place NCS proteins in known pathways. Further *in vivo* knockout and overexpression studies will reveal more about the roles of the NCS proteins and clarify their range of physiological functions in the regulation of neuronal activity.

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CHAPTER 137

Calmodulin-Mediated Signaling

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Calmodulin (CaM) is a ubiquitous, essential protein that serves as the primary sensor of changes in intracellular Ca^{2+} in all eukaryotic cells [1]. In this way CaM is the Ca^{2+} receptor that orchestrates Ca^{2+}-initiated signal transduction cascades leading to changes in cell function. Some of these cascades are initiated by Ca^{2+} and proceed in a linear fashion that culminate in regulation of the vital cellular response. However, Ca^{2+}/CaM can also influence physiologically important processes indirectly via cross-talk with pathways initiated by other second messengers [2]. These pathways include those regulated by cyclic nucleotides, nitric oxide, and MAP kinases (Fig. 1). Thus, it is with good reason that Ca^{2+} is recognized as the most versatile of the second messengers and CaM as its pivotal receptor.

The exquisite design of CaM allows it to assume an almost limitless number of conformations, each one dictated by the amino acid sequence of the CaM-binding domain of the individual target proteins. Illustrative of the versatility of CaM, over 50 CaM-binding proteins have been described to date [1]. Whereas CaM is generally considered to be a Ca^{2+}-binding protein and binds to many of its targets only in the presence of Ca^{2+}, this is not the only mode of action of CaM in cells. Some physiologically relevant interacting proteins also bind CaM in a Ca^{2+}-independent way. For example, CaM is an integral subunit of phosphorylase kinase (PK). Whereas the subunit association of PK is Ca^{2+}-independent, CaM still serves to sense changes in the concentration of Ca^{2+} as Ca^{2+} can regulate the activity of the enzyme. Alternatively, CaM can bind to a partner in the absence of Ca^{2+} but be released from its binding partner upon Ca^{2+}-binding. CaM-binding proteins of this kind include the unconventional myosins, neurogranin and neuromodulin. However, this review will focus on the central role of CaM as a transducer of Ca^{2+} signals to Ca^{2+}/CaM-dependent effector proteins. In addition, because of space limitations, references are primarily restricted to other reviews that focus on different aspects of CaM structure and function.

Calmodulin is a 148 amino acid protein in which globular domains, each containing a pair of EF-hand Ca^{2+}-binding motifs, are separated by an 8-turn α-helix. Ca^{2+} binding causes a conformational change in CaM that exposes hydrophobic residues and generates a considerable amount of biochemical energy, which is then used to modify the activity of its targets. It is this remarkable ability to serve as a selective allosteric regulator of so many enzymes that allows CaM to react to both global and transient changes in Ca^{2+} concentration within the dynamic and physiologically relevant range experienced by a cell. A rise in Ca^{2+} markedly increases the affinity of CaM for its target proteins and promotes conformational changes in both CaM and the effector [1].

Although generally considered a soluble protein, almost 95% of the CaM in mammalian cells, such as those in smooth muscle, is immobile [3]. However, in neurons and pancreatic acinar cells CaM has been reported to specifically translocate from plasma membrane to nucleus in response to brief stimuli that result in a localized rise in Ca^{2+} [4,5]. Thus, although CaM may relocate in response to Ca^{2+} signals, it seems to primarily move around in cells by diffusion. Calmodulin is found in all subcellular compartments where CaM-binding proteins characteristic to a given organelle serve to tether the Ca^{2+} receptor in a manner appropriate to facilitate Ca^{2+}/CaM-dependent function [6].

In the absence of Ca^{2+}/CaM, the target enzymes for this protein are inactive. In most cases this is due to intrasteric

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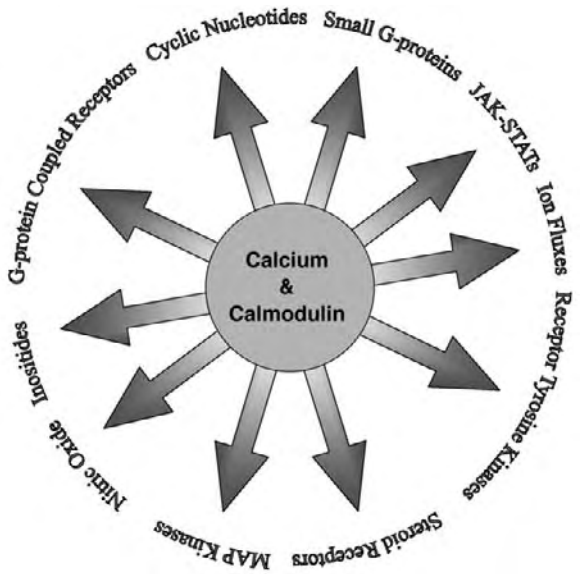


Figure 1 Signaling pathways influenced by Ca^{2+}/CAM.

autoinhibiton in which a portion of the protein is folded in a manner that prevents substrate access to its active site [7]. Ca^{2+}/CaM-binding to a region contiguous to the inhibitory segment relieves autoinhibition and thus promotes enzyme activity. Many of the autoinhibitory segments possess sequence similarity to the protein substrate, which led to the idea that autoinhibition was of the "pseudosubstrate" type. This concept has also been confirmed by both biochemical and structural approaches [7]. Ca^{2+}/CaM binding to a target enzyme can have one of three consequences. First, binding can activate the enzyme and inactivation follows the termination of the Ca^{2+} signal [1]. Enzymes that behave in this way include myosin light chain kinase, protein phosphatase 2B (calcineurin), CaMKI, and phosphodiesterase. Second, Ca^{2+}/CaM can activate the enzyme by promoting intersubunit phosphorylation that results in generation of constitutive activity. CaMKII is the prototypic example of this mode of regulation. As a CaMKII holoenzyme is composed of 10 to 12 subunits, this protein kinase can sense and record incremental increases in the intracellular concentration of Ca^{2+} [8]. Third, Ca^{2+}/CaM binding can enable phosphorylation of the target enzyme by an upstream enzyme in a signaling cascade of reactions. CaMKI and CaMKIV are examples of enzymes subject to this type of control. In response to Ca^{2+}/CaM binding these enzymes are phosphorylated by a CaMKK in a manner similar to the cascade of reactions that characterize MAPK pathways. This analogy has led to the concept of a CaMK cascade [9]. Like CaMKII, CaMKIV activity is maintained even after termination of the Ca^{2+} signal. The second and third control mechanisms provide means of continuing a Ca^{2+}-initiated reaction even after the Ca^{2+} signal has dissipated. Since in these cases the kinases are inactivated by dephosphorylation, kinase activity can also be terminated in the presence of a high level of intracellular Ca^{2+}, thus uncoupling the maintenance and inactivation of the response from the changes in Ca^{2+} required for its initiation.

One biological system that illustrates the multifaceted modes by which CaM can transduce a Ca^{2+} signal is the T lymphocyte. Stimulation of this cell through the T cell receptor results in a rapid and robust increase in intracellular Ca^{2+} that peaks about 2 min after receptor occupancy. The rise in Ca^{2+} is required for activation of two CaM-dependent pathways involved in expression of the interleukin 2 gene (IL-2) that encodes a mitogen necessary to expand this cohort of T cells. On the one hand, Ca^{2+}/CaM activates calcineurin in the cytoplasm. Calcineurin dephosphorylates a subunit of the NF-AT transcription factor, which allows the NF-AT/calcineurin complex to translocate into the nucleus and bind to the IL-2 gene promoter [10]. On the other hand, Ca^{2+}/CaM also activates CaMKIV, which resides in the nucleus and is involved in the phosphorylation of CREB [11], which is required for activation of immediate early genes such as those of the Fos and Jun families of transcription factors. Fos and Jun heterodimerize to form the AP1 transcription factor, which collaborates with NF-AT to activate the IL-2 gene promoter [10]. By 5 min after T cell receptor activation, the Ca^{2+} levels have largely decreased but remain elevated in the nucleus at a level about twice that present in resting T cells. This higher sustained Ca^{2+} concentration, which has to be maintained for approximately 2 hours, is required to maintain the activity of calcineurin and keep the NF-AT/calcineurin complexes in the nucleus [10]. However, because once fully activated CaMKIV becomes independent of Ca^{2+}/CaM, this enzyme is inactivated by dephosphorylation, which is catalyzed by PP2A that is in a complex with CaMKIV [11]. Hence, although a very similar Ca^{2+}/CaM-dependent mechanism is used to activate calcineurin and CaMKIV, the characteristics distinct to each enzyme permit maintenance of calcineurin activity and thus continued IL-2 gene transcription but inactivation of CaMKIV and the immediate early genes it regulates in the face of a sustained elevation of intranuclear Ca^{2+}.

Calcium is mandatory for cell proliferation and plays an important role in all cell cycle transitions [12]. In model organisms such as fungi, nematodes, and flies, the single CaM gene is essential for cell growth. It is interesting that the CaM effector proteins shown to be essential in *S. cerevisiae* are unique, as all bind CaM in the absence of Ca^{2+}. However, in the filamentous fungus *Aspergillus nidulans*, several essential CaM targets are Ca^{2+}-dependent and seem to be very similar to those required in mammalian cells. Thus, for the entry of quiescent cells into the cell cycle, both calcineurin and a CaMK are required and function in mid-G1 upstream of the activation of the cyclin/cdk complex that is a prelude to entry into DNA synthesis. In mammalian cells calcineurin is required to maintain stability of cyclin D, whereas the CaMK is required for activation of the cyclin D/cdk4 complex in the nucleus. Progression of *A. nidulans* and mammalian cells from G2 into mitosis requires a second CaMK. Again the CaMK is required prior to the activation of cyclin B/cdc2 and at least one target protein has been suggested to be the cdc25 phosphatase that activates the cdc2 complex. Finally, Ca^{2+}/CaM is important for mitosis. Presently available

data suggest that Ca^{2+}/CaM is likely to be required for breakdown of the nuclear envelope, the transition from metaphase to anaphase and for cytokinesis. However, the Ca^{2+}/CaM-dependent target proteins appear to be different at all three points in mitotic progression, although the details are unclear and under intense investigation [12].

Based on studies in a variety of cell types except *S. cerevisiae*, it seems clear that all roles for CaM in the cell cycle are heralded by transient changes in the intracellular Ca^{2+} concentration, which emphasizes the ubiquitous role of CaM as a Ca^{2+} receptor [13,14]. In addition, at least six different Ca^{2+}/CaM-dependent effector proteins have been shown to be essential for cell proliferation. These observations illustrate the pleiotypic ability of CaM to relay Ca^{2+} signals to very different effectors. Thus, Ca^{2+}, CaM, and CaM-dependent target proteins are essential components of signaling cascades that monitor progression through the cell cycle.

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CHAPTER 138

The Family of S100 Cell Signaling Proteins

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Introduction

S100 proteins attracted great interest in recent years due to their association with various human pathologies and their use in the diagnosis of these diseases. Twenty members have been discovered so far, and altogether S100 proteins represent the largest subgroup within the EF-hand Ca^{2+}-protein family. S100 proteins show a very divergent pattern of cell- and tissue-specific expression and of affinities for Ca^{2+}, Zn^{2+}, and Cu^{2+}, consistent with their pleiotropic intra- and extracellular functions. Several genetically engineered animal models have now been generated to study the roles of S100 proteins under normal and pathological conditions.

Many cellular events are regulated by oscillations of intracellular Ca^{2+} concentrations wherein the signal specificity is obtained through differences in location, duration, and frequency [1]. Participants in most Ca^{2+}-signaling pathways are members of the large family of Ca^{2+}-binding proteins characterized by the EF-hand structural motif [2]. Certain members, notably calbindin D28k and parvalbumin, serve as cytosolic Ca^{2+} buffers/shuttles whereas others such as calmodulin, troponin C, and the S100 proteins are Ca^{2+}-dependent regulatory proteins.

Unlike the ubiquitous calmodulin, most of the 20 members of the S100 protein family show cell- and tissue-specific expression, which is often deregulated in a number of human diseases including cancer, neurodegenerative disorders, inflammations and cardiomyopathy [3]. S100 proteins have a size of 10 to 12 kDa and form homo- and heterodimers. The monomer is composed of two helix-loop-helix (EF-hand) motifs connected by a central hinge region. The C-terminal EF-hand contains the canonical Ca^{2+}-binding loop, common to all EF-hand proteins. The N-terminal EF-hand consists of 14 amino acids and is characteristic for S100 proteins. Upon Ca^{2+}-binding S100 proteins undergo a conformational change required for target recognition and binding [32]. Generally, the dimeric S100 proteins bind four Ca^{2+} per dimer (K_d = 20–500 μM). Besides Ca^{2+} a number of S100 proteins bind Zn^{2+} with a wide range of affinities (K_d = 0.1–2000 μM). For S100B and S100A5 even Cu^{2+}-binding was reported (K_d = 0.4–5 μM). This suggests that S100 protein target interactions and cellular functions may also be triggered by Zn^{2+} and Cu^{2+}.

Another unique feature is that individual members of S100 proteins are localized within specific cellular compartments from which some of them are able to relocate upon Ca^{2+} or Zn^{2+} activation [4], transducing the signal in a temporal and spatial manner by interacting with different targets specific for each S100 protein. Furthermore, some S100 proteins are even secreted from cells acting in a cytokine-like manner. The individual members seem to utilize distinct pathways (ER-Golgi route, tubulin- or actin-dependent) for their translocation/secretion into the extracellular space [5]. S100B and S100A12 specifically bind to the surface receptor RAGE (receptor for advanced glycation endproduct)—a multiligand member of the immunoglobulin superfamily [6]. The extracellular levels of S100B thereby play a crucial role in that nanomolar concentrations of S100B have trophic effects on cells whereas pathological levels (as found in Alzheimer's patients) induce apoptosis [7].

Unique to the S100 protein family is that most S100 genes are located in a gene cluster on human chromosome 1q21. Within this chromosomal region, several rearrangements and deletions have been reported during tumor development,

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probably linked to the deregulated S100 gene expression observed in various tumor types. Genetically manipulated mice (knockout and transgenics for S100 proteins and RAGE) and microarray technologies are now becoming available that will further advance our understanding of the diverse cell signaling activities of S100 proteins.

Protein Structures and Metal-Dependent Interactions with Target Proteins

The amount of detailed structural data for S100 proteins is growing rapidly. With the exception of calbindin D_{9K} all structures of S100 proteins revealed a tight homodimer whereby the dimerization plane is composed of strictly conserved hydrophobic residues, which are missing in the case of calbindin D_{9K} [2,3]. Each S100 monomer consists of two helix-loop-helix Ca^{2+}-binding domains termed EF-hands (Fig. 1). The N-terminal domain consisting of helices H_I and H_{II} connected by loop L_1 is different from the canonical EF-hand motif and is therefore called S100-specific or pseudo EF-hand, whereas the C-terminal domain (H_{III}-L_3-H_{IV}) contains the canonical EF-hand motif. Upon Ca^{2+}-binding almost all S100 proteins undergo a conformational change exposing a previously covered hydrophobic patch. The Ca^{2+}-dependent conformational change of S100 proteins was characterized by NMR and high-resolution X-ray studies. This conformational change of Ca^{2+}-bound S100 proteins is distinct from Ca^{2+}-dependent changes observed in other EF-hand proteins such as calmodulin or troponin C. In the C-terminal EF-hand (canonical EF-hand) there is a large change in the position of helix H_{III} upon Ca^{2+}-binding. The interhelical angle between helices H_{III} and H_{IV} changes by 90° in S100B compared to the Ca^{2+}-free structure, opening the structure and exposing the residues required for target recognition and binding. A similar change in conformation is observed for Ca^{2+}-bound S100A6 [30,31]. The crystal structures of Ca^{2+}-bound S100A7 [8], S100A8 [9], S100A11 [10], and S100A12 [11] confirmed the observations made for Ca^{2+}-bound S100B. All four structures revealed an open conformation suitable for target binding. A further interesting phenomenon was observed for S100A10, which is not able to bind Ca^{2+}. The crystal structure of S100A10 showed that the Ca^{2+}-free protein is already in a Ca^{2+}-bound like open conformation that enables S100A10 to interact Ca^{2+}-independently with its target molecule [12]. Recently a hexameric form of S100A12 was described [11]. Three S100A12 dimers assemble as a hexamer that is stabilized by six additional Ca^{2+} ions bound to the interface of two adjacent dimers.

S100A3 is a unique member of the S100 protein family; it has a low affinity for Ca^{2+} but a high affinity for Zn^{2+} ions. The crystal structure of S100A3 (Fig. 1A) [13] allowed the prediction of one putative Zn^{2+}-binding site (distinct from the EF-hand) in the C-terminus of each monomer, thus disturbing the hydrophobic interface of the homodimer.

Target Binding

So far three different S100-target complexes have been characterized: S100B complexed with a peptide of the regulatory domain of p53 [14], S100A10 with a peptide of annexin II, and S100A11 complexed with a peptide of annexin I [10]. All three peptides were located in a cavity formed by helices H_{III} and H_{IV} in the open conformation of the C-terminal canonical EF-hand. The binding of the target peptides with the protein matrix is accomplished by hydrophobic and ionic interactions. Furthermore the stoichiometry of the complex is two target peptides per S100 homodimer. However, the binding

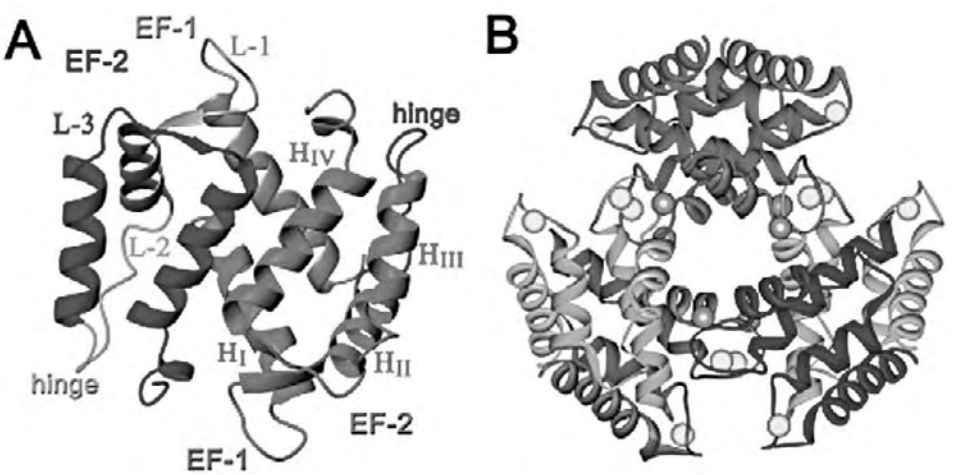


Figure 1 Structure of S100 proteins. (A) Dimeric structure of S100A3. The monomers are depicted in red and blue, respectively. Each monomer consists of two EF-hands connected by a hinge region. (B) Hexameric structure of S100A12 (pdb code 1GQM [11]). Three S100A12 dimers form a hexamer. The Ca^{2+} ions bound to the EF-hands are shown as bright yellow spheres. At the hexamer-forming interface six additional Ca^{2+} ions are located, which are shown as dark yellow spheres. A color representation of this figure is available on the CD version of the *Handbook of Cell Signaling*.

mode of the annexin peptides to S100A10 and S100A11 is strikingly different from that of the p53 peptide to S100B. In contrast to the p53 peptide the annexin peptides interact with both monomers whereby the required residues are located on the helices H_{III} and H_{IV} of one monomer and on helix H_I of the second monomer.

Based on these observations one can suppose that there are further modes of target binding to other S100 proteins. For example, it was proposed [11] that the hexameric form of S100 A12 (Fig. 1B) might interact with three extracellular domains of the RAGE receptor, bringing them together into large trimeric assemblies.

Zn^{2+} Binding

Although a number of S100 proteins bind Zn^{2+} with high affinity, only the structure of Zn^{2+} bound-S100A7 is known so far [8]. The Zn^{2+} binding sites in other S100 proteins, such as S100B, S100A2, S100A3, S100A4, or S100A6, are only preliminarily characterized [15]. The Zn^{2+}-binding residues were identified by spectroscopic and mutational analysis mainly as histidine and cysteine residues; however, a common Zn^{2+} binding motif has not yet been recognized in the sequence of these proteins.

Genomic Organization, Chromosomal Localization, and Nomenclature

The structural organization of S100 genes is highly conserved both within an organism and in different species [3]. A typical S100 gene consists of three exons whereby the first exon carries exclusively 5′ untranslated sequences. The second exon contains the ATG and codes for the N-terminal EF-hand, and the third exon encodes the carboxy-terminal canonical EF-hand. Only a few genes, such as S100A4, S100A5, and the newly identified S100A14 [16], are composed of four exons. In these genes, the first two exons can either be alternatively spliced (S100A4) or noncoding (S100A5), leaving the two exon-splitting of the coding region intact. It is interesting that for both S100A11 and S100A14 this region encoding the corresponding proteins is split into three exons. Whether this finding reflects a functional or evolutionarily close relationship between these two members of the S100 family remains to be seen.

Presently 14 bona fide S100 genes are found in a gene cluster on human chromosome 1q21 (Fig. 2), a finding that led to the introduction of the now widely accepted S100 nomenclature (Table I). Four additional S100 genes are found on other human chromosomes, including the newly discovered S100Z [17] likely to be localized on chromosome 5 (the 3-terminal sequence of the S100Z cDNA is part of a human BAC clone on region 5q12-q13). Hence, one can recognize at least four different subgroups of S100 genes located closely together (S100A1–S100A13–S100A14; S100A2 to S100A6; S100A8–S100A9–S100A12; S100A10–S100A11). This finding raises the question of whether each gene is regulated by its own promoter elements or by as yet uncharacterized locus control elements, as has been suggested for the epidermal differentiation genes. The evidence available today would rather suggest an individual regulatory mechanism for most S100 genes. It is striking, however, that for some of these subgroups of genes, similarities in the function of their encoded proteins have been recognized. Furthermore,

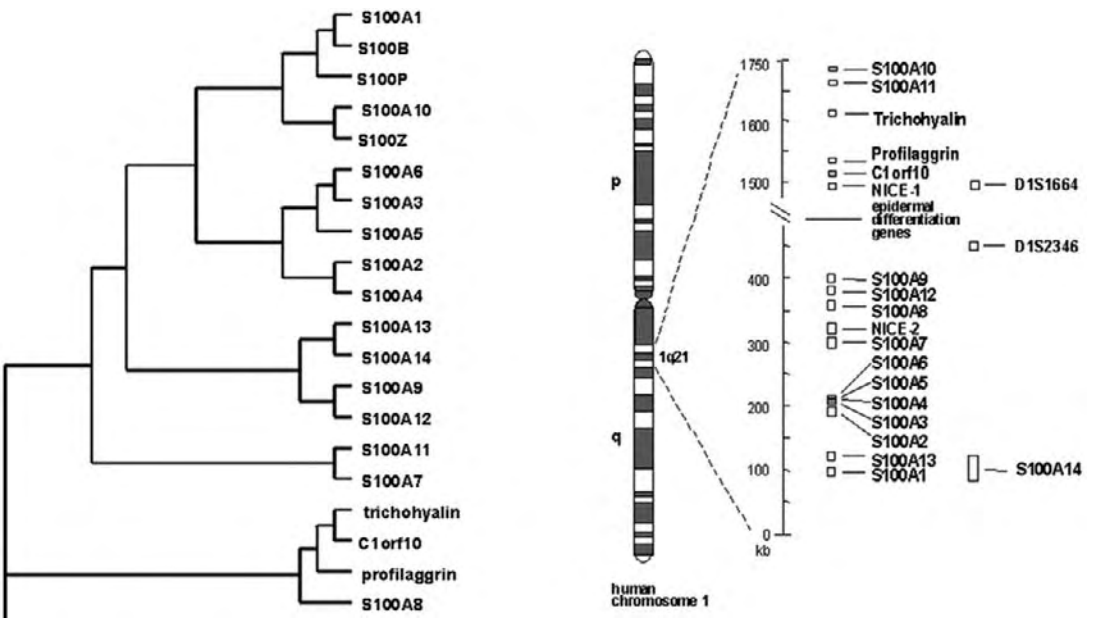


Figure 2 Phylogenetic tree of human S100 proteins in comparison with physical map of the S100 gene cluster on human chromosome 1q21. In the phylogenetic tree each node represents a putative gene duplication. The left-most nodes indicate the earliest gene duplications. Genes lying in the cluster region are indicated as well as two commonly used genomic markers (D1S1664 and D1S2346); p and q represent the long and the short arm of the chromosome, respectively.

Table I Nomenclature for S100 Genes Clustered on Human Chromosome 1q21

New name	Previous symbols/synonyms
S100A1	S100α
S100A2	S100L, CaN19
S100A3	S100E
S100A4	CAPL, p9ka, pEL98, mts 1, metastasin, calvasculin, murine placental calcium protein, Fsp1, 18A2
S100A5	S100D
S100A6	Calcyclin, CACY, 2A9, PRA, CaBP, 5B10
S100A7	Psoriasin, PSOR1, BDA11, CAAF2
S100A8	Calgranulin A, CAGA, CFAg, MRP8, p8, MAC387, 60B8Ag, L1Ag, CP-10, MIF, NIF, calprotectin
S100A9	Calgranulin B, CAGB, CFAg, MRP-14, p14, MAC 387, 60B8Ag, L1Ag, MIF, NIF, (S100A8/A9 dimer)
S100A10	Calpactin light chain, CAL12, CLP11, p11, p10, 42C
S100A11	Calgizzarin, S100C
S100A12	Calgranulin C, p6, CAAF1, CGRP, corned-associated antigen
S100A13	CAAF2
S100A14	—

Nomenclature for S100 Genes Located on Different Chromosomes

Name	Previous symbols/synonyms	Chromosomal location
Calbindin-D9K	9K CALB3, CaBP9k, I CaBP, Cholecalcin	Xp22
S100B	S100β, NEF	21q22
S100P	S100P	4p16
S100Z	—	5

a number of chromosomal abnormalities such as deletions, rearrangements or translocations in this region have been associated with neoplasias, suggesting that the expression of S100 genes might be altered in human cancer. It is also interesting that the clustered organization of the human genes seems to be evolutionarily conserved, at least in the mouse. In other species, S100 genes are less well characterized.

Comparison of a phylogenetic tree constructed on the basis of sequence alignments between the different human S100 proteins revealed a clustering of S100 proteins, which is mirrored in the physical map of the S100 genes on chromosome 1q21 (Fig. 2). For example, S100A2 through to S100A6 cluster tightly together both on the phylogenetic tree and on the chromosome. The small distances on the chromosome and the phylogenetic tree indicate that these S100 proteins most likely originate from late gene duplication events. Similarly, clusters are seen for S100A10 and S100A11, as well as for S100A13 and S100A14. Furthermore, there are three proteins encoded in 1q21 that carry in the N-terminus an S100-like domain, namely Trichohyalin, Profilaggrin, and C1or f10.

Translocation, Secretion, and Biological Functions

S100 proteins are generally involved in a large number of cellular activities such as signal transduction, cell differentiation, regulation of cell motility, transcription, and cell cycle progression (Table II) [3]. S100 proteins are thought to modulate the activity of target proteins in a Ca^{2+}- (and possibly also in a Zn^{2+}- and Cu^{2+}-) dependent manner. During the last decade, a large number of such possible interactions have been described involving enzymes, cytoskeletal elements, and transcription factors.

Apart from these intracellular functions, some S100 proteins, such as S100A8/A9, S100B, S100A4, and probably others, are secreted from cells and exhibit cytokine-like extracellular functions [3]. These include chemotactic activities related to inflammation (S100A8; S100A9 and S100A12), neurotrophic activities (S100B), and a recently described angiogenic affect (S100A4). In all cases, the mechanisms of secretion as well as the nature of high affinity surface receptors remain largely unknown. One candidate receptor to mediate at least some of the described extracellular functions is RAGE, which was shown to be activated upon binding of S100A12 and S100B [6]. It is currently not known whether RAGE is a universal S100 receptor.

Generation of some animal models has been initiated to study the physiological impact of S100 proteins [3]. Ectopic overexpression in the mouse has been described for S100B and S100A4. In the case of S100B, enhanced expression in the brain led to hyperactivity associated with an impairment of hippocampal function. Brains from S100B transgenic mice show a higher density of dendrites in the hippocampus postnatally compared to controls and a loss of dendrites by one year of age. In contrast to this mild phenotype, expression of S100A4 in oncogene-bearing transgenic mice is capable of inducing metastasis of mammary tumors, suggesting that S100A4 has an important role in the acquisition of the metastatic phenotype during tumor progression. While stimulation of angiogenesis might play a role, the exact mechanisms of this function are still under investigation.

Inactivation through homologous recombination in mouse embryonic stem cells has been achieved for S100B and S100A8. While inactivation of S100B has no obvious consequences for life, S100A8 null mice die via early resorption of the mouse embryo, a result that suggests a role for this protein in prevention of maternal rejection of the implanting embryo.

Since S100 proteins can form homo- and also heterodimers and usually more than one S100 protein is found to be expressed in a given cell type, functional redundancy or compensatory mechanisms might explain the lack of phenotype observed in some animal models. Clearly, more animal models inactivating single S100 proteins as well as combinations thereof are needed before their physiological roles can be clarified.

Table II S100 Proteins: Functions and Association with Human Diseases

Protein	Postulated functions	Disease association
S100A1	Regulation of cell motility, muscle contraction, phosphorylation, Ca^{2+} release channel, transcription	Cardiomyopathies
S100A2	Tumor suppression, nuclear functions, chemotaxis	Cancer, tumor suppression
S100A3	Hair shaft formation, tumor suppression, secretion, and extracellular functions	Hair damage, cancer
S100A4	Regulation of cell motility, secretion and extracellular functions, angiogenesis	Cancer (metastasis)
S100A5	Ca^{2+}-, Zn^{2+}-, and Cu^{2+}-binding protein in the CNS and other tissues; unknown function	Not known
S100A6	Regulation of insulin release, prolaction secretion, Ca^{2+} homeostasis, tumor progression	Amyotrophical lateral sclerosis
S100A7	S100A7-fatty acid binding protein complex regulates differentiation of keratinocytes	Psoriasis, cancer
S100A8/A9	Chemotactic activities, adhesion of neutrophils, myeloid cell differentiation, apoptosis, fatty acid metabolism	Inflammation, wound healing, cystic fibrosis
S100A10	Inhibition of phospholipase A2, neurotransmitter release, in connection with annexin II regulates membrane traffic, ion currents	Inflammation
S100A11	Organization of early endosomes inhibition of annexin I function, regulation of phosphorylation, physiological role in keratinocyte cornified envelope	Skin diseases, ocular melanoma
S100A12	Host-parasite interaction, differentiation of squamous epithelial cells and extracellular functions	Mooren's ulcer (autoimmune disease), inflammation
S100A13	Regulation of FGF-1 and synaptotagmin-1 release	—
S100A14	—	malignant transformation
S100B	Cell motility, proliferation, inhibition of phosphorylation, inhibition of microtubule assembly transcription, regulation of nuclear kinase, extracellular functions, e.g. neurite extension	Alzheimer's disease, Down's syndrome, melanoma, amyotrophic lateral sclerosis, epilepsy
S100P	Function in the placenta	Cancer
S100Z	Function in spleen and leukocytes	Aberrant in some tumors
Calbindin D_{9K}	Ca^{2+} buffer and Ca^{2+} transport	Vitamin D deficiency, abnormal mineralization

Associations with Human Diseases

As listed in Table II, various S100 proteins are closely associated with several human diseases. We will briefly discuss a few examples.

S100 A1 is mainly expressed in the human myocardium. Reduced levels measured in the left ventricles of patients with end stage heart failure may contribute to a reduced contractility [18]. This notion is in agreement with the reported interactions of S100A1 with SR proteins, regulating Ca^{2+}-induced Ca^{2+} release [19], and with SERCA2a, phospholamban (own results), and titin [20], modulating Ca^{2+} homeostasis and contractile performance [21]. Therefore, an S100A1 gene transfer to the heart *in vivo* might provide a new therapeutic approach to correct the altered Ca^{2+} signaling pathways that cause abnormal myocardial contractility.

S100A4 has been implicated in invasion and metastasis [22]. The prognostic significance of its selective expression in various cancers has been exploited. Identification of predictive markers of cancer is of major importance to the improvement of clinical management, therapeutic outcome, and survival of patients. In gastric cancer the inverse expression of S100A4 in relation to E-cadherin (a tumor supressor) was found to be a powerful aid in histological typing and in evaluating the metastatic potential/prognosis of patients with this type of cancer [23].

Extracellular functions of S100 proteins such as S100A4 have been described. Recently it was demonstrated [24] that S100A4 could act as an angiogenic factor and might induce tumor progression via an extracellular route stimulating angiogenesis. Inhibiting the process of tumor angiogenesis might be possible by either blocking S100A4 secretion or its extracellular function.

A prognostic significance of S100A2 in laryngeal squamous-cell carcinoma has also been found [25], thus allowing discrimination of high- and low-risk patients in the lymph-node negative subgroup to provide better therapy.

Human S100A8 and S100A9 are associated with chronic inflammatory diseases. Both proteins are also involved in wound repair by reorganizing the keratin cytoskeleton in the injured epidermis [26].

S100B and its interacting tau protein are individually affected in Alzheimer's disease (AD). S100B is overexpressed in AD, and hyperphosphorylated tau constitutes the primary component of neurofibrillary tangles. In addition, S100B interacts with other AD-associated proteins such as presenilin (PS1 and PS2) and with the amyloid precursor protein (APP). These interactions are altered by the phosphorylation state

of tau and the overexpresssion of S100B, a finding that strongly indicates that S100B plays an important role in pathways associated with neurodegeneration [27–29].

Conclusion and Perspectives

S100 proteins have been implicated in pleiotropic Ca^{2+}-dependent cellular events, with specific functions for each of the family members. However, some S100 proteins have also physiologically relevant Zn^{2+} affinities, suggesting that Zn^{2+} rather than Ca^{2+} controls their biological activities. In order to understand how the biological functions of S100 proteins are regulated by Zn^{2+} and Ca^{2+} it will be necessary to determine the three-dimensional structures of the Zn^{2+} loaded S100 proteins and their interactions with target proteins.

S100 proteins are localized in specific cellular compartments from which some of them relocate upon cellular stimulation and even secrete exerting extracellular, cytokine-like activities. This finding suggests that translocation might be a temporal and spatial determinant of their interactions with different partner proteins. Our recent experiments suggest that different S100 proteins utilize distinct translocation pathways, which might lead them to certain subcellular compartments in order to perform their physiological tasks in the same cellular environment.

Some S100 proteins can play a crucial role in physiological responses through their paracrine effects on neighboring cells. The discovery of the surface receptor RAGE for two S100 proteins (S100B and S10012) has shed more light on the extracellular functions of these two proteins. Just how they are secreted and how they interact with RAGE still remains to be investigated. This could be done using animal models with inactivated single S100 proteins or deletion mutants of RAGE. Future research activities will also focus on the deregulated expression of S100 genes, which is a hallmark of a wide range of human diseases.

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CHAPTER 139

C_2-Domains in Ca^{2+}-Signaling

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Structures of C_2-Domains

C_2-domains are widespread protein modules of approximately 130 residues that are defined by a consensus sequence and a common three-dimensional fold [1]. Most C_2-domains bind Ca^{2+} and mediate interactions of their proteins with phospholipids. In the initial analysis of the human genome [2], C_2-domains were the second most abundant Ca^{2+}-regulatory domain (123 genes) after EF-hands (242 genes). C_2-domains primarily occur in membrane trafficking proteins such as synaptotagmin 1 and in signal transduction proteins such as protein kinase C (PKC). Although most C_2-domains bind Ca^{2+}, a significant number of C_2-domains do not bind Ca^{2+}.

As initially revealed in the structure of the synaptotagmin 1 C_2A-domain [3] and confirmed in more than ten C_2-domain structures (see Table I), C_2-domains are composed of a compact β-sandwich containing two β-sheets with four β-strands each. This is illustrated exemplarily for the two C_2-domains for synaptotagmin 1 in Fig. 1. Flexible loops at the top and bottom connect the eight β-strands. The β-strands are conserved between C_2-domains, whereas the loops vary and frequently contain additional secondary structure elements. The eight β-strands in the C_2-domain β-sandwich can be arranged in two distinct topologies. These topologies are circular permutations of each other, and are referred to as type 1 and type 2 (Fig. 2; reviewed in [1]). As a result, the N- and C-termini of the C_2-domains are either on top or at the bottom of the domains. However, C_2-domains with distinct topologies can nevertheless have similar three-dimensional structures. For example, the type 1 C_2A-domain from synaptotagmin 1 [3] and the type 2 C_2-domain from phospholipase Cδ1 [4] exhibit a root mean square deviation of 1.4 Å for 109 equivalent α-carbons. One reason for the different C_2-domain topologies may be that the topology influences the relative orientation of a C_2-domain and the neighboring domains.

The conserved core β-sandwich of C_2-domains serves as a scaffold for the emergence of variable loops at the top and bottom of the domain. In C_2-domains that bind Ca^{2+} and/or phospholipids, these exclusively bind to sites formed by the top loops. In contrast, the function of the bottom loops is unclear. The distinct Ca^{2+}- and phospholipid-binding properties of C_2-domains—as well as other activities—are probably encoded in the variable top and bottom loops which differ in size and sequence. C_2-domains thus are "janus-faced" modules in which a stable β-scaffold supports two variable surfaces: a generally Ca^{2+}-dependent top surface and a Ca^{2+}-independent bottom surface [5].

C_2-domains can be classified based on structural or functional properties. Structurally, in addition to the assignment of C_2-domains to the two principal types of β-strand topology, C_2-domains can be subdivided into classes that share common sequences. For example, the two C_2-domains of synaptotagmins (referred to as the C_2A- and C_2B-domains) exhibit conserved sequence differences. The fourth bottom loop of all C_2B-domains from synaptotagmins and related proteins forms a α-helix that is absent from all C_2A-domains and other C_2-domains [5]. In addition, the C_2B-domains from synaptotagmins 1, 2, and 8 but not other synaptotagmins include an extra C-terminal α-helix [6]. In contrast to this structural classification, C_2-domains can also be divided into functional classes. Again taking synaptotagmins as an example, synaptotagmins 1 and 2 bind Ca^{2+} but synaptotagmin 8

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Table I Atomic Structures of C_2-Domains

Protein/C_2-domain	Method[a]	Ca^{2+}-binding state of structure[b]	Ca^{2+}-binding sites of C_2-domain[c]		References
			Number	Intrinsic affinity (in μM Ca^2)	
1. Trafficking proteins					
Synaptotagmin 1 C_2A-domain	X-ray & NMR	Ca^{2+}-free & Ca^{2+}-bound	3	Ca1 ≈ 50; Ca2 ≈ 500; Ca3 >10,000	3, 8–11, 18
Synaptotagmin 1 C_2B-domain	NMR	Ca^{2+}-bound	2	Ca1 ≈ 350; Ca2 ≈ 550	6
Rabphilin C_2B-domain	NMR	Ca^{2+}-bound	2	Ca1 ≈ 7; Ca2 ≈ 11	5
Synaptotagmin 3 C_2A/B-domain	X-ray	Ca^{2+}-free	n.d.	n.d.	41
2. Signal transduction proteins					
Protein kinase Cα C_2-domain	X-ray	Ca^{2+}-bound ± phospholipids	2	n.d.	17
Protein kinase Cβ C_2-domain	X-ray	Ca^{2+}-bound	3	n.d.	16
Protein kinase Cδ C_2-domain	X-ray	Ca^{2+}-independent		n.a.	23
Protein kinase Cε C_2-domain	X-ray	Ca^{2+}-independent		n.a.	24
Phospholipase Cδ1 C_2-domain	X-ray	Ca^{2+}-free & Ca^{2+}-bound	3	n.d.	4, 12
Phospholipase A_2 C_2-domain	X-ray & NMR	Ca^{2+}-bound	2	Ca1 ≈ 10; Ca2 ≈ 60	13, 14, 42
PTEN	X-ray	Ca^{2+}-independent		n.a.	21

[a]X-ray = X-ray crystallography; NMR = NMR spectroscopy.
[b]Number of Ca^{2+}-ions in structure.
[c]n.d. = not determined; n.a. = not applicable.

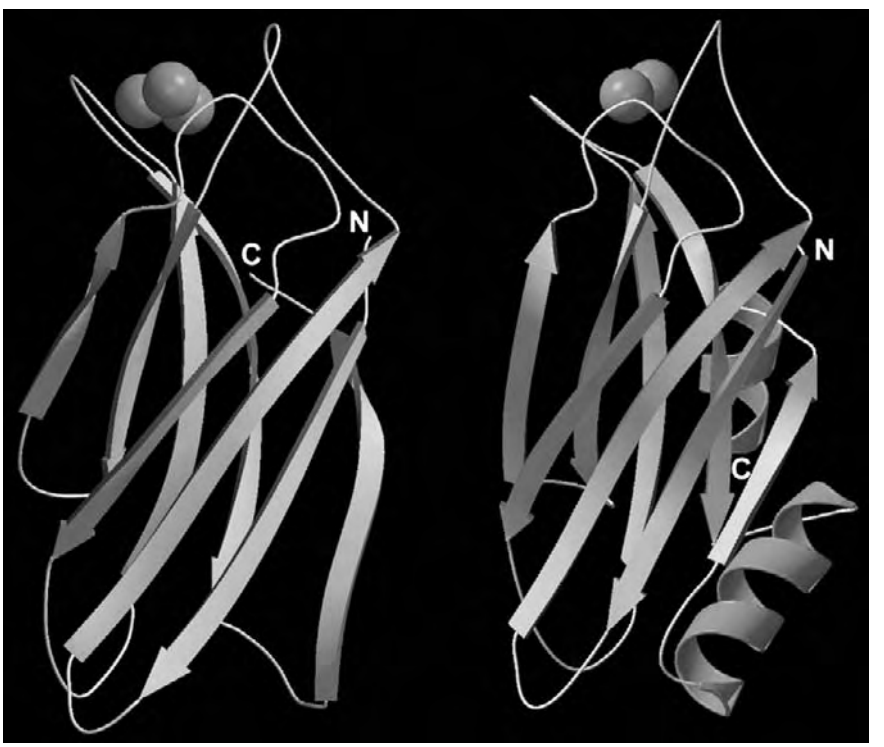


Figure 1 Structures of the synaptotagmin 1 C_2A- and C_2B-domains. Pictures show ribbon diagrams of the synaptotagmin C_2-domains (wide ribbons = β-strands; helices = α-helices; thin light strands = no secondary structure) in the Ca^{2+}-bound state. Three Ca^{2+}-ions are shown bound to the top loops for the C_2A-domain (left), and two Ca^{2+}-ions for the C_2B-domain (right). Note that only the C_2B-domain contains significant α-helices. Positions of N- and C-termini are indicated by N and C.

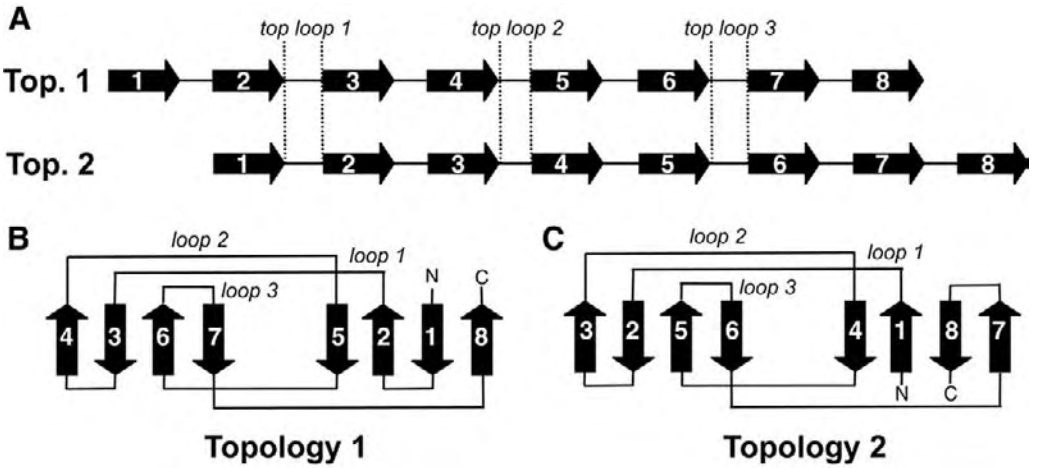


Figure 2 Diagram of the topography of β-strands in various types of C_2-domains. Two types of C_2-domain structures have been described that differ in the arrangement of β-strands; they are circular permutations of each other. In type 1 C_2-domains exemplified by the synaptotagmin C_2-domains (diagrams A and B; see also Fig. 1), N- and C-termini are on top of the C_2-domains, whereas in type 2 C_2-domains exemplified by the phospholipase Cδ1 structure (diagrams A and C; see ref. 4), the N- and C-termini are on the bottom. Note that the position of the top loops changes, but the actual localization of the loops within the three-dimensional structure does not.

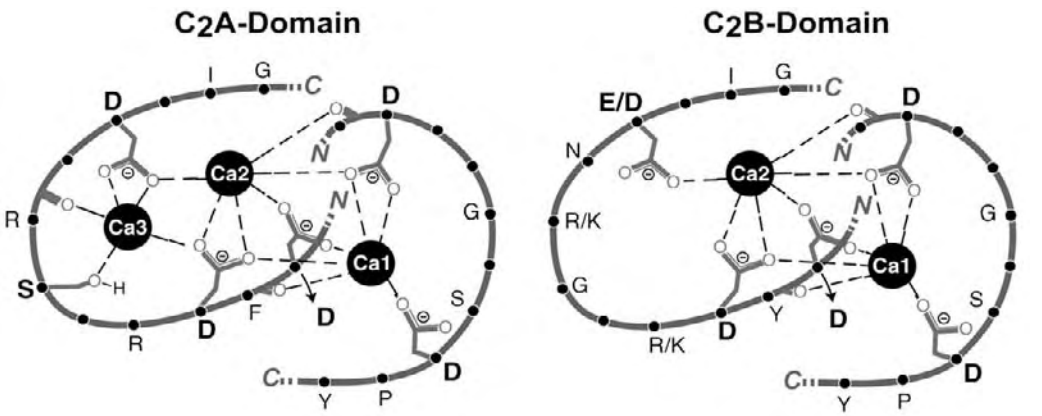


Figure 3 Model of the Ca^{2+}-binding sites of synaptotagmin C_2-domains. In both the C_2A- and C_2B-domains, the Ca^{2+}-binding sites are primarily formed by negatively charged residues located on top loops 1 and 3 (loop 2 is not shown). Most of the Ca^{2+}-coordinating negatively charged residues are multidentate ligands for the Ca^{2+}-ions. The sequences shown for the top loops are consensus sequences present in most but not all synaptotagmins (modified from [40]).

does not, in spite of the fact that the C_2B-domains from these three synaptotagmins share the same extra C-terminal α-helix, which is absent from other C_2B-domains [6,7]. Thus structural and functional classifications do not necessarily overlap.

Ca^{2+}-Binding Mode of C_2-Domains

In all Ca^{2+}-binding C_2-domains, Ca^{2+} binds exclusively to the top loops [3–6,8–17]. C_2-domains usually bind either two or three Ca^{2+}-ions at closely spaced sites that are primarily formed by aspartate residues, as illustrated in Fig. 3 for the two C_2-domains of synaptotagmin 1. The residues that form the Ca^{2+}-binding sites are widely separated in the primary sequences. The Ca^{2+}-binding residues often serve as bidentate ligands for multiple Ca^{2+}-ions [8,11,12]. The coordination spheres of the bound Ca^{2+} ions in the C_2-domain are incomplete in many C2-domains, resulting in low intrinsic Ca^{2+}-affinities (e.g. the synaptotagmin 1 C_2A-domain exhibits an affinity of >1.0 mM for complete Ca^{2+}-binding; [18]). As a result of this design, multiple Ca^{2+}-ions are concentrated in a small region on top of the C_2-domains and contain unsatisfied coordination sites that remain available for interaction with target molecules (see model in Fig. 3). Other C_2-domains, however, exhibit a much higher intrinsic Ca^{2+}-affinity (e.g. in the rabphilin C_2B-domain the two Ca^{2+}-ions bind with intrinsic affinities of 7 and 11 μM; [5]), and may have complete Ca^{2+}-coordination spheres.

The detailed characterization of the Ca^{2+}-binding sites for several C_2-domains, especially the C_2A-domain of

synaptotagmin 1 [8–11,18] and the C_2-domain of phospholipase Cδ [4,12], revealed that the Ca^{2+}-binding sites are very similar, allowing a reasonably reliable prediction of whether a given C_2-domain is likely to bind Ca^{2+}. The aspartate residues involved in Ca^{2+} binding in the synaptotagmin I C_2A-domain are conserved in many C_2-domains, and the conserved Ca^{2+}-binding sequence that they form is referred to as the C_2-motif [8]. However, it has been difficult to predict the precise Ca^{2+}-binding properties of C_2-domains—for example their phospholipid specificities and apparent Ca^{2+}-affinities—presumably because they are determined by the variable sequences of their top loops that do not have defined conformations.

In all C_2-domains studied so far except for the piccolo C_2A-domain [19], Ca^{2+}-binding does not induce a substantial conformational change. For example, comparison of the Ca^{2+}-free and Ca^{2+}-bound forms of the synaptotagmin I C_2A-domain demonstrated that Ca^{2+} binding involves rotations of some side chains but causes no substantial backbone rearrangements [10]. The Ca^{2+}-binding region appears to be flexible in the absence of Ca^{2+}, and is stabilized after Ca^{2+} binding. Similar findings have been obtained for the C_2B-domain of synaptotagmin 1 [6] and the C_2-domain of phospholipase Cδ [4,12]. These results suggest that in most C_2-domains, Ca^{2+}-binding to a small patch on the top surface causes only a local effect that transduces the Ca^{2+}-binding signal. The nature of this effect probably depends on an electrostatic switch, since Ca^{2+}-binding causes a major change in the electrostatic potential of the top surface of the synaptotagmin 1 C_2A-domain [9].

The only C_2-domain that has been shown to undergo a major conformational change in response to Ca^{2+}-binding is the C_2A-domain of piccolo/aczonin [19]. Although this C_2-domain probably has "standard" C_2-domain Ca^{2+}-binding sites, Ca^{2+}-binding appears to induce a rearrangement of β-strands. The fact that a C_2-domain can undergo such a conformational change in response to Ca^{2+} indicates that C_2-domains are more versatile than suggested by the characterization of the initial C_2-domain structures.

Phospholipid Binding Mechanism of C_2-Domains

As first described for the Ca^{2+}-dependent binding of the synaptotagmin 1 C_2A-domain to phospholipids [20], the most common property of C_2-domains is phospholipid binding. This is true even for C_2-domains that do not bind Ca^{2+}; in fact, the function of most Ca^{2+}-independent C_2-domains appears to be to attach their resident proteins to phospholipid membranes [21–24]. Ca^{2+}-independent phospholipid binding is possibly best illustrated by the C_2-domain of PTEN, a tumor suppressor gene that is a phosphatase for the lipid phosphatidylinositol 3,4,5-trisphosphate [21,22]. The C_2-domain of PTEN positions its catalytic domain on top of the substrate. The C_2-domain not only recruits PTEN to the membrane, it also orients the catalytic domain with respect to the membrane substrate. Similar functions have been ascribed to the N-terminal C_2-domains of novel PKCs whose structures have been solved [23,24]. However, although significant evidence exists that phospholipid binding may be an even more general property of C_2-domains than Ca^{2+}-binding, it seems likely that not all C_2-domains bind phospholipids.

In all phospholipid-binding C_2-domains, phospholipids bind exclusively to the top loops similar to Ca^{2+}-binding. In spite of this similarity, however, the mechanism of phospholipid binding and the phospholipid specificity vary greatly among C_2-domains. Some C_2-domains (such as both C_2-domains of synaptotagmin 1) bind promiscuously negatively charged residues [6,20], whereas other (such as the C_2-domain of cytoplasmic phospholipase A2) bind neutral lipids [25]. Both hydrophobic and electrostatic interactions contribute to phospholipid binding but to different degrees in the various C_2-domains (see e.g. [25,26]). The contribution of different types of interactions has been described in detail for the double C_2-domain fragment of synaptotagmin 1 in which both C_2-domains contribute to the overall interaction [27,28]. Here, each C_2-domain separately participates in three types of interactions with the phospholipid bilayer, Ca^{2+}-mediated binding, hydrophobic attachment, and electrostatic interactions via positively charged residues.

Ca^{2+}-ions serve as a bridge that connect the C_2-domains to the phospholipid headgroups. The bound Ca^{2+}-ions are incompletely coordinated by the top loops of the C_2-domains. When phospholipids bind, they probably fill unsatisfied coordination sites on the bound Ca^{2+} ions. This results in a 100 to 1,000-fold increase in the apparent Ca^{2+}-affinity of the C_2-domains, and converts noncooperative intrinsic Ca^{2+}-binding into highly cooperative Ca^{2+}-binding by the C_2-domain/phospholipid complex [17,18,29]. In fact, at least for synaptotagmin 1, intrinsic Ca^{2+}-binding has an unphysiologically low affinity and probably never occurs in the absence of phospholipids, suggesting that the true signaling structure for synaptotagmins is the C_2-domain/phospholipid complex [18].

In addition to Ca^{2+}-ions, the synaptotagmin 1 C_2-domains are connected to the phospholipids by hydrophobic residues that insert into the bilayer [30,31] and by positively charged residues that form electrostatic interactions with negatively charged phospholipid headgroups [18,29]. All three forces contribute; in fact, the hydrophobic interactions, although constitutive, are essential for Ca^{2+}-triggered phospholipid binding [31]. Because the two C_2-domains are so closely spaced, the C_2-domains cooperate, resulting in a higher apparent Ca^{2+}-affinity of the double C_2-domain fragment than for the individual C_2-domains [28,32]. Furthermore, mutations that induce dramatic changes in the properties of isolated C_2-domains have unpredictable effects on the double C_2-domain fragment: Whereas some mutations induce the same change in the double C_2-domain fragment [18], others cause no change at all [32].

It seems likely that other C_2-domains, such as that of PKCα [17], bind phospholipids by a similar mechanism,

although the contribution of the various types of interactions vary dramatically among C_2-domains [25]. In C_2-domains that bind phospholipids Ca^{2+} independently, the two Ca^{2+}-independent types of interactions are presumably sufficient to mediate constitutive binding. Such interactions could easily be modulated by other signaling pathways; for example, phosphorylation as shown for a C_2-domain from *Aplysia* PKC [33]. It would not be surprising if in Ca^{2+}-dependent and Ca^{2+}-independent C_2-domains membrane binding was further modulated by additional mechanisms.

Other Ligands of C_2-Domains

In addition to phospholipids, various C_2-domains have been reported to bind to many other molecules, primarily proteins (reviewed in [1]). Like all protein-protein interactions, the *in vivo* importance of these *in vitro* interactions is difficult to assess, and all of these interactions remain to be validated. Nevertheless, indirect evidence indicates that at least some of these interactions are important. First, some C_2-domains apparently do not bind to either phospholipids or Ca^{2+}. Although this finding does not exclude the possibility that the right lipids have not yet been tested, some of these C_2-domains strongly bind to other ligands that may mediate their functions. For example, the C_2B-domain of the active zone protein RIM does not bind Ca^{2+} or phospholipids but strongly interacts with proteins called α-liprins [34], suggesting that some C_2-domains might function as standard protein-protein interaction domains. Second, as janus-faced modules, C_2-domains have conserved sequence elements on their bottom surfaces that have no role in either Ca^{2+} or phospholipid binding [5]. It stands to reason that these sequences perform a function, although the nature of this activity remains obscure.

Function of C_2-domains

As is evident from their properties, the function of most C_2-domains is to attach their resident proteins to phospholipid membranes, although C_2-domains probably also connect their resident proteins to other ligands. The membrane-attachment function of C_2-domains may differ between trafficking and signal transduction proteins, the two classes that contain most of the C_2-domains in the genome. Signal transduction proteins usually contain a single C_2-domain that serves to position the catalytic domain of these proteins close to their substrates in the membrane. This is most obvious for enzymes that act on lipids such as phospholipase A2 and PTEN, where the C_2-domain is essential for placing the catalytic domain close to the phospholipid substrate either in a Ca^{2+}-dependent or constitutive manner [21,22,35,36]. However, this is also true for enzymes such as ras-GAP and PKC, where the C_2-domain brings the enzyme into proximity with membrane-bound ras (for ras-GAP) or diacylglycerol (for PKC) [37,38].

In contrast to signal transduction proteins, membrane trafficking proteins usually contain tandem C_2-domains (e.g. synaptotagmins), but some proteins include as many as six C_2-domains (e.g. ferlins; [39]). The properties of the tandem C_2-domain architecture has only been worked out for synaptotagmin 1 (reviewed in [40]). Here both C_2-domains bind Ca^{2+} and phospholipids, and both are essential for the function of the protein. Since synaptotagmins are intrinsic membrane proteins, their C_2-domains do not function to recruit these proteins to the membrane. The precise need for two C_2-domains is unknown, but a possible function of this configuration is to effect a physicochemical change in the target membranes to which they bind. For example, the role of synaptotagmin 1 in fast Ca^{2+}-triggered exocytosis may be mediated by a rapid Ca^{2+}-induced rearrangement of phospholipids during the fusion reaction, thereby opening the fusion pore that lets the transmitters escape. Although this is a plausible hypothesis for the need for two C_2-domains in synaptotagmin 1, it does not explain why so many other membrane trafficking proteins also contain two C_2-domains, even membrane proteins such as RIMs in which the C_2-domains do not appear to bind Ca^{2+} and/or phospholipids.

In the emerging universe of protein modules that are used to construct many of the eukaryotic signaling pathways, C_2-domains are remarkable for several reasons. A rigid core composed of a relatively invariant β-sandwich is used as a scaffold to form variable binding surfaces on the top and bottom of the module. The Ca^{2+}-binding sites formed in most C_2-domains are unusual because these sites are built from residues that are widely separated in the primary sequence and because Ca^{2+}-binding does not generally cause a conformational change but induces an electrostatic switch.

Although the progress in understanding C_2-domains has been significant over the last ten years, many questions remain to be addressed. For example, the mechanism and validity of the protein-protein interactions mediated by C_2-domains needs to be examined, the function of the bottom surface of C_2-domains needs to be elucidated, and the molecular basis for the Ca^{2+}-affinity and phospholipid-binding specificity of C_2-domains needs to be clarified. Before these important goals are realized, it will be difficult to postulate general conclusions about the functions of these domains.

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CHAPTER 140

Annexins and Calcium Signaling

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Introduction

The vertebrate family of annexins comprises 12 calcium-binding proteins encoded by distinct genes. Although the functions of annexins are not yet fully elucidated, there is growing evidence that certain members of this family are involved in the homeostatic regulation of intracellular calcium ion concentration [1]. Annexins have a lower affinity for Ca^{2+} than E-F hand proteins, but this affinity is increased in the presence of negatively charged phospholipids. This defining biochemical property forms the basis of a generalized paradigm for annexin function in which elevation of intracellular Ca^{2+} concentration during cell stimulation is accompanied by translocation of annexins from the cytosol to the inner face of the plasma membrane. Ca^{2+}-dependent spatiotemporal regulation of subcellular localization is therefore likely to be a key aspect of annexin function, enabling annexins to influence the activities of other peripherally bound or integral membrane proteins in response to transient increases in cytosolic Ca^{2+} concentration. The precise question of what annexins do once membrane-bound has not been clearly answered, but it is probable that annexins are involved in membrane-associated events such as phospholipid clustering in lipid rafts, control of membrane fluidity, and structural changes to the membrane-cytoskeleton during endocytosis or phagocytosis (for review see [2]). Another possible role for membrane-bound annexins is in the generation and regulation of intracellular Ca^{2+} fluxes, and it is this topic that forms the focus of this chapter.

Annexins as Ca^{2+} Channels

The notion that annexins could function as Ca^{2+} channels first emerged in 1987, with the observation that purified annexin 7 (synexin) displays the properties of a voltage-gated Ca^{2+} channel when added to synthetic phospholipid bilayers [3]. Subsequent studies on the *in vitro* channel activities of various annexins, often supported by parallel structural analyses, have revealed this to be a general property of most members of the family. Annexin 5, which has been most extensively investigated with regard to the relationship between structure and Ca^{2+} channel activity, is approximately doughnut shaped with a slightly convex upper surface on which the Ca^{2+}-binding loops are located, and a slightly concave lower surface [4]. The proposed ion conductance pathway is lined with acidic residues, some of which have been demonstrated by mutagenesis studies to function as ion selectivity filter and voltage sensor [5,6]. Structural analysis of many other annexins has revealed almost superimposable tertiary architectures, and it is unsurprisingly that most family members exhibit Ca^{2+} channel activity *in vitro*.

The convincing structural basis for the Ca^{2+} channel activities of annexins is supported by electrophysiological and pharmacological correlates between the properties of putative annexin channels and as yet uncharacterized Ca^{2+} channels in mammalian cells [7–9]. For example, annexin 5 exhibits the properties of a classic voltage-gated Ca^{2+} channel, with unitary channel conductance values in the 10–20 pS range at both depolarizing and hyperpolarizing membrane potentials. The annexin 5 Ca^{2+} channel activity is inhibited by La^{3+}, which is known to block Ca^{2+} influx in most nonexcitable cells, whereas blockers of the L-, N-, P-, and T-type Ca^{2+} channels, such as nifedipine and Cd^{2+}, are without effect on the annexin 5 channel. Perhaps the most interesting pharmacological antagonist of annexin 5 is a cardioprotective benzothiazepine named K201, which exerts its effect by inhibiting Ca^{2+} influx into cardiomyocytes following ischemia-reperfusion injury [10]. Co-crystallization studies of K201 in complex with annexin 5 revealed the inhibitor to be tightly bound in a cleft at the proposed exit site of the Ca^{2+} conductance pathway [11].

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Despite the weight of the structural, pharmacological, and electrophysiological evidence, the consensus view that annexins are either cytosolic or peripheral membrane-binding proteins presents a conceptual obstacle to the idea that such proteins could function as ion channels. There are few studies that have directly addressed this point, but investigations using bone-derived matrix vesicles [12] and chick DT40 cells containing a targeted disruption of the annexin 5 gene [13] add credibility to this theory. Mineralizing chondrocytes shed vesicles rich in phosphatidylserine and annexin 5 and take up Ca^{2+}, which forms crystals that embed in the collagen matrix during *de novo* bone deposition. The Ca^{2+} entry pathway in matrix vesicles was shown to be inhibited by Zn^{2+} and GTP, and increased by ATP. These characteristics mirror those observed in phospholipid vesicles containing annexin 5, suggesting that annexin 5 may be directly responsible for Ca^{2+} influx in mineralizing matrix vesicles. In annexin 5 null-mutant DT40 cells, Ca^{2+} signals elicited by both thapsigargin and activation of the B-cell receptor are normal, whereas the Ca^{2+} influx component of the biphasic response to hydrogen peroxide is absent. Cells lacking annexin 2 were normal with regard to peroxide-induxed Ca^{2+} fluxes, showing that although both annexins exhibit Ca^{2+} channel activity *in vitro* [14], only annexin 5 seems to be involved in Ca^{2+} signaling *in vivo*. These data show that annexin 5 functions either as a Ca^{2+} channel, Ca^{2+} channel subunit, or signaling intermediate in the peroxide-activated Ca^{2+} influx pathway. Although the case for annexin 5 having a role as a Ca^{2+} channel is not yet proven, it would be a curious denouement if a protein that possesses so many of the structural and biophysical characteristics of a bona fide Ca^{2+} channel were to be shown to function as a Ca^{2+} channel regulator *in vivo*.

Annexins as Ca^{2+} Channel Regulators

A more readily acceptable modus operandi for annexins is in the regulation of intracellular Ca^{2+} fluxes. Annexins could influence Ca^{2+} signals by direct interaction with the cytoplasmic domains of proteins involved either in Ca^{2+} extrusion or in release of Ca^{2+} from the endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) Ca^{2+} stores. The first evidence for such roles came from studies in which annexin 6 was shown to increase the mean open time and opening probability of SR ryanodine-sensitive Ca^{2+} release channels in isolated membrane preparations [15]. However, annexin 6 was shown to exert this effect only when added to the lumenal side of the vesicles, and annexin 6 is generally considered to be a cytosolic protein. Also, these experiments were performed before it was known that annexin 6 itself has Ca^{2+} channel activity [16], raising the possibility that the changes observed in Ca^{2+} conductance may have been directly due to annexin 6. In A431 squamous epithelial carcinoma cells (which normally lack annexin 6), ectopic expression of annexin 6 was found to attenuate the sustained phase of the Ca^{2+} response to epidermal growth factor [17]. Other responses, such as to thapsigargin, were unaffected by annexin 6. An interesting finding is that only the larger of the two splice forms of annexin 6 exhibited this effect, which correlated with slower proliferative rate and tumor growth in nude mice [18].

Further studies in transgenic mice showed that targeted overexpression of an annexin 6 transgene in the heart led to cardiomyopathy, acute myocarditis, and fibrosis [19]. Experiments on isolated cardiomyocytes from these animals revealed lower resting Ca^{2+} levels, decreased amplitude of electrically evoked Ca^{2+} spikes, and impaired contractility. Similar studies on cardiomyocytes from annexin 6 knockout mice failed to identify any changes in resting cytosolic Ca^{2+} levels, but the contractile properties of these cells were significantly enhanced with regard to rate of contraction, extent of contraction, and rate of relaxation [20]. These mechanical changes correlated with accelerated diastolic clearance of Ca^{2+} from the cytosol, perhaps through enhanced activity of either the SR Ca^{2+} ATPase or the Na^{+}/Ca^{2+} exchanger. It is interesting to note that in humans with end-stage heart failure annexin 6 is downregulated [21]. Based on the studies of transgenic and null mutant mice, this would be predicted to enhance cardiomyocyte contractility, suggesting a negative inotropic role for annexin 6 in cardiomyocyte function.

Similar changes in cardiomyocyte function have been reported in mice lacking annexin 7 [22]. In normal mice, the degree of cell shortening increases with increasing frequency of stimulation, but this was not the case in the annexin 7 KO mice. In a separate study, targeted disruption of the annexin 7 gene in mice was reported to be lethal, and the heterozygous mice exhibited severe defects in insulin secretion that were apparently due to abnormally low expression of the inositol trisphosphate (IP_3) receptor [23]. Although these observations suggest a functional link between annexin 7 and IP_3 receptor expression, it is not clear why loss of a single annexin 7 allele should have such a striking effect on the expression of the IP_3 receptor.

Conclusions

The evidence that certain members of the annexin family have roles in Ca^{2+} signaling continues to grow. Most of the studies in this area have focused on annexins 5 and 6, and a combination of structural, electrophysiological, pharmacological, and genetic evidence tends to support the idea that annexin 5 functions as a Ca^{2+} channel. If this is indeed a physiological role of annexin 5, how does one account for the ability of a resident cytosolic protein to channel calcium ions? One study based on theoretical calculations predicted that membrane binding by annexin 5 would lead to foci of increased ion permeability caused by electrostatic destabilization of the lipid bilayer [24]. An alternative mechanism emerged from spin-labeling studies on annexin B12, which suggested that under certain conditions structural changes could occur that would lead to membrane insertion [25]. The availability of annexin null mutant cells and animals provides the opportunity to test these and other models of annexin function *in vivo*. Studies of this type should refine

our understanding of the increasingly firm link between calcium signaling and annexin function.

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CHAPTER 141

Calpain

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Introduction

Four decades of study have provided much understanding of the calpain family of intracellular cysteine proteases [1]. Due to the limitations of investigative tools, earlier work focused on *in vitro* regulation and activities of these proteases, primarily the two ubiquitous isoforms μ- (calpain I) and m-calpain (calpain II) [2]. Structure-function studies have culminated in elucidating the molecular structure of the two ubiquitous calpains and deciphering how these enzymes might be activated and modulated. More recently, investigators have focused on connecting calpain function to physiology and pathology, particularly in regard to motility during wound repair [3,4], injury-mediated apoptosis in stroke and ischemia [5], protein degradation in muscular dystrophies [6], and susceptibility for non-insulin dependent diabetes mellitus [7,8].

Calpain Family

Thirteen distinct mammalian calpain gene products comprise the calpain gene family. The general structure is of a large subunit complexed to a single 30 kDa small subunit [6,9]. These isoforms differ in the length of N-terminal sequences, regulatory domain structures, and presence of calcium-binding domains. Ten calpain isoforms have been studied; most of these appear to be relatively selective for or enriched in cell and tissue types. The two ubiquitous calpains, μ- and m-calpain, are the best understood due to their high level of expression and primacy of discovery. These two isoforms were named according to their relative requirement for calcium *in vitro*, with μ-calpain requiring micromolar concentrations and m-calpain requiring near millimolar levels of calcium to elicit proteolytic activity *in vitro*.

Two other calpains, p94 calpain III and calpain X, also have a high level of interest due to their potential involvement in pathologies. The muscle-specific calpain III is characterized by two inserts, one within domain II and the other between domains III and IV [6]. It is interesting that p94 calpain appears to be independent of high calcium for activation but demonstrates low-level constitutive activity across a wide range of calcium concentrations. Calpain X has recently gained attention because of its identification in a linkage analysis study for type II or non-insulin-dependent diabetes [7,8]. This calpain is present in the β-cells of islets as well as in muscle and liver. Its structure is similar to that of the ubiquitous calpains.

Structure

The calpain molecule can be divided into five domains, initially described in protein structure-function studies and now by the crystal structure [10,11] (Fig. 1). Domain I contains a short 19 amino acid N-terminal sequence that is cleaved during autoproteolysis. The catalytic domain is divided into two parts, with the active site forming in the cleft between them. Domain III is a regulatory domain that has been shown to contain sites for attenuative phosphorylation [12] and a potential phospholipid-binding domain [13]. The fourth domain contains four calcium-binding EF-hand domains.

The crystal structure of calpain provides for the mechanism of activation. Unlike papains, the N-terminal domain is not a prodomain residing in the active site, a finding that confirms that autolysis of the N-terminal is not required for activation [14,15]. The most intriguing aspect is the active site itself.

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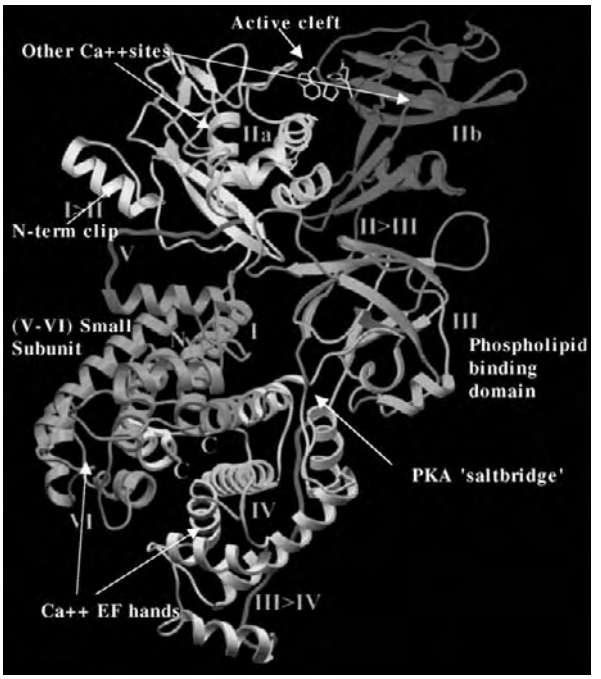


Figure 1 Structural domains of human m-calpain. Ribbon diagram of the crystal structure in the absence of calcium (domain V of the small subunit is absent) [10]. Denoted are sites of calcium binding in domains V and VI and in the active sites. Also noted are potential regulatory sites of phospholipid binding (the C2-like domain III), autoproteolysis of the N-terminal leader, and negative attenuation by PKA phosphorylation at serine/threonine 369/370. Adapted from [3,10].

In the inactive state, the catalytic residues (Cys105, His262, Asn286) are misaligned and too far apart to form a catalytic center [11,16]. Therefore, activating processes such as calcium binding, phospholipid binding, intramolecular cleavage, or phosphorylation must effect a realignment of these domains. Recent crystallography demonstrates that calcium loading at supraphysiological levels can accomplish such a shift. However, how these activating reorganization are effected *in vivo* remains a major challenge.

Modes of Regulation

An intricate strategy for the temporal and spatial regulation of calpain activity is necessary because calpain, which is abundant in the cytoplasm, cleaves many intracellular signaling and structural proteins. However, controversy still exists about how calpain activity is regulated *in vivo*. The lack of progress in this arena stems from many of the earlier studies having focused on calpain behavior *in vitro*. Whatever mechanisms are used to form an active site, the upstream signals triggering this activation and the downstream targets are critical in understanding the physiological roles of calpain. Furthermore, the ways that calpains are activated might vary not only by isoform, but also by subcellular localization as the ubiquitous isoforms are found throughout the cell, including in the nucleus. Therefore, multiple, potentially alternative or complementary mechanisms of activation have been proposed (Table I).

Table I Possible Mechanisms of Modulation

Activation	Inhibition
Calcium fluxes	Endogenous inhibitor Calpastatin
Phosphorylation	Phosphorylation
Proteolysis–limited	Proteolysis–extended
Phospholipids	
Protein cofactors	
DNA	

Calcium

Based on *in vitro* findings, calpains were proposed to be activated by intracellular calcium fluxes. That calcium can activate calpains is well supported *in vitro* [10,16]. *In vivo*, calcium chelation blocks activation of μ-calpain in response to chemokines in keratinocytes [17]. However, the need for seemingly supraphysiological levels of calcium has instigated searches for other modes of activation. Recent advances in calcium imaging suggest that levels high enough for μ-calpain activation could be achieved in highly localized calcium puffs (up to ~600 nM in nonexcitable cells) or sparks (excitable cells)[18]. During traumatic or ischemic compromise of the plasma membrane, calcium influx may reach levels that activate both μ- and m-calpain; but this level of calcium is not compatible with cell survival. Thus, the *in vitro* calcium levels required for m-calpain cannot be attained for other physiological responses. Therefore, a number of mechanisms have been suggested to lower the calcium requirement, even down to ambient cytosolic levels.

Phosphorylation

Most recently, an old standby of signal transduction cascades has been shown to regulate m-calpain. Early studies reported calpains not to be phosphorylated *in vivo* as determined by autoradiography due to the long half-life of calpains in unstimulated cells [19]. However, both m- and μ-calpain have been shown to be phosphorylated *in vivo* [20]. Under unstimulated conditions, there are three sites each of phosphotyrosine, phospho-serine, and phospho-threonine phosphorylation, with the isolated calpains demonstrating varied sub-stoichiometric phosphorylation. Growth factors activate m-calpain downstream of ERK MAP kinase [21]; this is likely to occur by direct phosphorylation at amino acid S50 [22]. This is an intriguing finding, as p94 calpain III, which is considered constitutively active [23], presents a glutamic acid at this site.

Accessory Molecules

Mechanisms to reduce the requirement for calcium to the physiological range have been proposed. These include phospholipid binding, release of calpain from its inhibitor calpastatin, and binding of activator proteins. Phospholipids decrease

the calcium requirement *in vitro* [24,25]. Calpain translocates to the plasma membrane in the presence of calcium, where it associates with phosphatidylinositol bis-phosphate [26]. Of particular interest, there is a putative phospholipid-binding activity in the regulatory domain III [13]. What is especially intriguing, DNA has been reported to lessen the requirement of m-calpain for select nucleoproteins [27], which may provide a mode of activation for the nuclear-localized pool of this protease.

A ubiquitous, endogenous inhibitor of calpains, calpastatin, provided hope that dissociation/re-association would be the mainstay of calpain regulation. Calpastatin binds and inactivates calpains through each of its four repetitive inhibitory domains. However, release of calpain from calpastatin, although it correlates with activity, is insufficient for activation. Furthermore, calpastatin is neither always present in excess molar levels, nor always co-localized with calpain. Calcium fluxes actually enhance calpastatin inhibition of calpains, suggesting that calpastatin might attenuate activated calpains rather than prevent activation [28,29]. Despite the conflicting evidence of physiological relevance, overexpression of this molecule can be employed to prevent calpain activation.

Other protein-protein interactions have been proposed to activate calpain. Select proteins co-purify with active calpains from many cell types. In rat skeletal muscle, bovine brain, and rat brain, activator proteins were found that increased autolysis and lowered the calcium requirement of μ-calpain [30–32]. Acyl-CoA-binding protein has been proposed as an activator for m-calpain [33]. Unfortunately, the association and activation of calpain *in vivo* by these proteins has not been demonstrated, and the mechanism by which these proteins would activate calpains remains unclear.

Inactivation

Key to all enzymes, especially those that cause irreversible signaling such as proteolytic cleavage, is an efficient system to prevent unintended activity. Calpain activity appears to be kept at minimal levels until signaled. How this occurs is still unknown, in part because the activation mechanisms are similarly unclear.

Calpain autoproteolysis and degradation rapidly remove active enzyme. The half-life of active calpain I or calpain II is shortened from almost a week [34] to just hours [21]. This autolysis was thought to activate calpain [35,36], since the N-terminal clipped intermediaries display increased activity. However, as intact calpain can be equally active [15,37,38], these are now considered just steps on the way to degradative removal.

Calpastatin can inhibit calpain activity by acting through each of four repeated domains. Expression of exogenously encoded calpastatin has been used successfully to block calpain activation [39,40]. Still, this does not address whether this endogenous protein acts as such *in vivo*. In fact, the reported discrepancies in subcellular localization [41] argue against this being the only inhibitory mechanism for preventing calpain activity. However, it is possible that calpastatin serves to attenuate activated calpain [42], whereas low calpain activity levels are maintained through lack of positive signals.

Phosphorylation of calpains may serve to either prevent activation or attenuate triggered enzyme. PKA phosphorylation of at least m-calpain limits the ability of growth factors to activate this isoform [12,43]. Whether this mechanism is operative in other isoforms is still an open question. The target serine at amino acid 369 is present in some of the other isoforms, but the recipient residues of the putative ensuing salt-bridge is lacking in μ-calpain.

Calpain as a Signaling Intermediate: Potential Targets

Evidence supports a critical role for calpain as a signaling intermediate downstream of both integrin and growth factor signaling pathways. The role for calcium, phospholipids, and phosphorylation in calpain regulation supports a central role for calpain in basic signal transduction mechanisms. However, the key question for understanding calpain function remains frustratingly unsolved—what are the operative targets of calpains? Cell behaviors dependent on calpain have provided hints as to what these targets might be, and many substrates have been identified both *in vitro* and *in vivo* (Table II). However, establishing whether proteolysis of these targets is either sufficient or required for the cellular responses has remained challenging due to the difficulties in generating calpain-resistant functional target molecules. The structure, primary or tertiary, of the proteolytic sites remains unknown, a fact that has confounded attempts to identify key target molecules or negative calpain cleavage of putative targets to allow assessment of functional role. Originally, calpain was proposed to cleave downstream of PEST sequences [9]; although further identification of targets demonstrated that the presence of a PEST sequence was not required [10].

The limited proteolysis of calpain suggests that it functions as an irreversible step in signaling cascades, generating constitutively active or dominant-negative versions of signaling proteins, rather than serving a degradative function. A number of structural and signaling molecules have been

Table II Potential Targets of m- and μ-Calpain

Signaling	Adhesion	Proliferation/ survival	Cytoskeletal components
EGF receptor	β-integrins	cyclin D1	spectrin
Protein kinase C	ezrin	caspases	MAP2
Src	talin	p53	filamin
Rho A	paxillin	p35	fodrin
Myosin light chain kinase	vinculin		tau
Focal adhesion kinase	α-actinin		

identified *in vitro* and in cells as targets of calpain. An early identified target of calpain was the EGF receptor, wherein calpain removes most of the carboxy-terminal domain that serves both as an autoinhibitory and a docking domain. Thus, it is not obvious whether calpain cleavage would increase or decrease EGF signaling or generate a signaling-restricted EGFR. Many of the other targets of the ubiquitous calpains are involved in cell adhesion and motility, being linked to the cytoskeletal machinery. These include FAK, ezrin, talin, paxillin, src, MLCK, RhoA, and the cytosolic tails of some of the β-integrins [3]. Recent studies with calpain-deficient embryonic fibroblasts adherent to fibronectin substrata demonstrate *in vivo* cleavage of talin but not FAK, paxillin, α-actinin, or vinculin, suggesting that talin may be a critical calpain substrate *in vivo* [44]. In accordance with previously published reports [45], Capn4-/-embryonic fibroblasts have reduced stress fibers, thus implicating a role for calpain in the formation of Rho-mediated stress fibers [44]. It must be mentioned that various investigators report different spectra of cellular targets in very similar systems, thereby suggesting that calpain targeting is likely to be plastic and redundant and possibly dependent on the mode of activation and analysis.

Functional Roles

Selective inhibitors for calpain have provided functional indications of calpain's roles in a wide range of physiological processes, including cell motility, cell proliferation, and apoptosis. However, confusion and controversy have existed regarding calpain's functional role, to a large extent, because of a lack of specificity of many of the cell-permeable inhibitors. More recent studies using calpain-deficient embryonic fibroblasts or ectopic expression of the endogenous calpain inhibitor calpastatin have helped clarify calpain's physiological role; however, the current efforts are also not isoform specific but target both m- and μ-calpain. These studies support a critical role for calpain in regulating the actin cytoskeleton and cell migration [44] but have called into question its role in other processes such as cell proliferation [24]. However, part of calpain's widespread functional profile is likely to be due to the various calpain isoforms and their cell-specific functions. In many cases these various functions of calpain can be seen in the same cell system, thus suggesting that these different functions may also be subserved by the different localized pools of calpain that exist throughout a cell.

The critical importance of ubiquitous m- and μ-calpain for normal development has been demonstrated by transgenic mice deficient in the regulatory subunit that eliminates detectable calpain activity [24]. These mice die during embryonic development with vascular defects, thus supporting a role for calpain in blood vessel formation.

Platelet Activation

Initial studies of the role of calpain were conducted in platelets, an interesting system in which calpain clearly plays a role in secretion, adhesion, and aggregation. Inhibition of calpain via overexpression of calpastatin prevents α-granule secretion, platelet aggregation, and spreading on glass surfaces [46]. Platelets uniquely express predominantly μ-calpain and have negligible levels of M-calpain. Therefore, molecular inhibition of μ-calpain is sufficient to down-regulate all detectable calpain activity, and antibodies to the autolyzed form of μ-calpain can yield meaningful results.

In this context, calpain was shown to be part of the signal transduction apparatus. Calpain is activated following signaling by the platelet integrin αIIbβ3 [47,48]. Calpain associates with focal adhesion proteins in platelets, regulates the attachment of αIIbβ3 to the cytoskeleton, and relaxes the retraction of fibrin clots. Activation of calpain by ionophore A23187 increased the proteolysis of pp60c-src and PTP-1B, which then dissociated from the cytoskeleton, thereby inactivating these proteins. This correlated with the inhibition of fibrin clot retraction observed in aggregated platelets in the presence of calcium. Calpain inhibition also blocked the cleavage of the actin-binding protein talin, whereas calpain activation caused the movement of both cleaved talin and integrin αIIbβ3 from the Triton X-100 insoluble fraction (cytoskeleton) to the Triton X-100 soluble fraction [49]. Calpain therefore functions as a signaling molecule in platelets by coordinating the cellular response of aggregation and clot formation.

Adhesion Modulation—Spreading and Motility

Calpains regulate cell adhesion to the substratum and thereby affect spreading and motility of many cell types. Cell spreading requires active remodeling and turnover of adhesion sites to enable cells to extend processes subsequent to attachment. It is also considered to be similar to forward protrusion during active cell locomotion. In bovine aortic endothelial cells, calpain enables spreading by allowing formation of Rac-induced adhesions under the extended lamellae [45,50]. Inhibition of calpain caused a marked reduction in cell spreading and adhesion formation, without affecting initial attachment. Calpain acting to enable new supramolecular assembly is also noted in T cells, in which integrin ligation activates calpain to promote integrin diffusion to form focal complexes and ultimately cell spreading [51,52]. Calpain inhibition may have very different effects on cell spreading in different cellular contexts. For example, enhanced membrane protrusion and filopodia formation is observed in calpain deficient embryonic fibroblasts ([44]; A. Huttenlocher, unpublished).

Calpain-mediated regulation of cell/substratum adhesion is critical not only during spreading and forward protrusion but also in rear release during productive motility [40,53]. Haptokinetic motility, signaled by adhesion receptors, primarily integrins, is calpain dependent. β1 and β3 integrin-mediated CHO cell migration is sensitive to calpain inhibition [40]. Calpain inhibition stabilized peripheral focal adhesions and decreased the detachment rate. If the effect of

calpain was to alter adhesion to the substratum, one would predict a varied effect dependent on substrate density, with calpain being required most for migration over highly adhesive surfaces but only minimally involved for low adhesive regimens [40,54]. That calpain modulated cell motility dependent on adhesive strength identically to alterations in integrin affinity for fibronectin [54] indicates that calpain is acting effectively as a physiologic rheostat for adhesion.

Although the calpain isoform that functions downstream of integrin-mediated adhesion and migration has not been clearly identified, some evidence supports a role for μ-calpain in this regulation, most specifically during endothelial cell and platelet spreading. A growing body of evidence suggests that m-calpain may be involved in growth factor motility [53]. Growth factor–induced chemokinesis also requires de-adhesion [55], dependent on calpain [21]. It is interesting that this de-adhesion and motility occurs via ERK MAP kinase phosphorylating and enabling activation of m- but not μ-calpain in the absence of a calcium flux ([21]; A. Glading, unpublished). The site of phosphorylation appears to be S50, which is absent in μ-calpain. This finding provides an imposed rationale for the evolutionary duplication of the ubiquitous isoforms.

Calpain in Muscular Dystrophy

Calpain 3 is the skeletal muscle–specific calpain isoform. Defects in the human calpain 3 gene are responsible for a form of muscular dystrophy, limb girdle muscular dystrophy type 2A [56]. A calpain 3–deficient mouse model also shows a progressive muscular dystrophy with perturbations in membrane architecture and apoptosis-associated regulation of the IkappaB pathway [57]. It is interesting that in the mouse model of Duchenne's muscular dystrophy there is an increase in the expression and activity of the ubiquitous calpain isoforms, suggesting that perturbation of the muscle calpains, and not just enhanced proteolysis, may contribute to the pathogenesis of muscular dystrophy.

Apoptosis

Calpain has been implicated in necrotic and apoptotic cell death [58]. Previous reports have shown that calpain inhibitors have protective effects in *in vivo* models of CNS [58] and cardiac ischemia [59]. The combined treatment of neurons with both calpain and caspase inhibitors may have an additive protective effect against neuronal apoptosis [58]. These studies support the intriguing potential of calpain inhibitors as a therapeutic target to treat cerebral or cardiac ischemia. How calpain inhibitors exert anti-necrotic and anti-apoptotic effects remain unclear. During ischemic compromise, calcium influx may reach levels that activate both μ- and m-calpain. Under these conditions, calpain may cleave multiple substrates, including signaling, cytoskeletal proteins, and transcription factors. It is likely that calpain-mediated cleavage of focal adhesion and cytoskeletal proteins contributes to cell rounding and loss of focal adhesions during apoptosis and necrotic cell death. However, a direct modulation of apoptotic signaling pathways, i.e. by the cleavage and regulation of caspase activity, for example, may also contribute to calpain's role during apoptosis [60].

Proliferation

Substantial controversy exists about calpain's role during cell proliferation and cell cycle progression. Capn4-/- embryonic fibroblasts exhibit normal proliferation rates. However, ectopic expression of calpastatin reduces CHO cell proliferation [61] and Src-mediated transformation [62]. The calpastatin-induced inhibition of cell cycle progression in Src-transformed cells is associated with a decrease in pRb phosphorylation and reduced levels of cyclin A and D. However, although calpain may cleave cell cycle proteins such as cyclin D1 [40], a substrate for calpain's effects on cell cycle progression has not been identified. Defining calpain's role during cell cycle progression will be an important challenge for future investigation.

Future Considerations

Much is known about this ubiquitous family of limited intracellular proteases. Many investigators have defined the extended family and begun to establish structural bases of calpain activation and regulation. In addition, a number of functional roles have been established by calpain family–-selective inhibitors, which in turn have provided potential target proteins. However, much remains to be learned about this complex family of enzymes.

A glaring gap in our knowledge is what precise roles the various members serve in cells, and how the different calpain localizations contribute to these cellular responses. For instance, if membrane-associated μ- and m-calpain contribute to rear detachment during motility [3,12,40], what do the majority of cytosolic and nuclear μ- and m-calpains do? Only by linking the function of isoform pools of calpain to specific cellular behaviors will we be able to understand the key targets of calpains and whether calpain clipping results in an active or dead molecule. To achieve this level of understanding will require significant advances in our tool sets. First, isoform-specific inhibitors have been attempted without widespread adoption. Second, calpain activity or activation needs to be imaged within subcellular compartments; the ubiquitous nature of calpain distribution throughout the cell renders simple localization and colocalization data of limited utility. That these advances will occur is ever more likely due to the increased realization that calpains function in motility during wound repair and tumor progression, in ischemia-induced apoptosis that aggravates stroke and myocardial infarction, and in myosin degradation of muscle-wasting syndromes. That calpain may prove a target for intervention in these major medical conditions ensures a burgeoning body of work on these fascinating molecules.

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CHAPTER 142

Regulation of Intracellular Calcium through Hydrogen Peroxide

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Introduction

H_2O_2 production, as a result of normal metabolism, environmental factors, and ligand-receptor interactions, is generally associated with an increase in cytoplasmic Ca^{2+} concentration. This Ca^{2+} increase can be attributed partly to the fact that H_2O_2 causes selective oxidization of certain reactive cysteine residues of the ryanodine receptor and the inositol(1,4,5)P_3 receptor, leading to enhanced Ca^{2+} channel activity of these two receptors. The Ca^{2+} elevation may also arise indirectly from inactivation of protein tyrosine phosphatase and PTEN, both of which contain an essential cysteine residue that is especially sensitive to H_2O_2-dependent oxidation.

Sources and Chemical Properties of ROS

Incomplete reduction of O_2 during respiration produces superoxide anion ($O_2^{\bullet -}$), which is spontaneously or enzymatically dismutated to H_2O_2. H_2O_2 can be reduced further to hydroxyl radicals ($HO^{\bullet}$) in the presence of catalytic amounts of iron and electron donor molecules such as thiols and ascorbic acid (reviewed in [1,2]). These reactive oxygen species (ROS), $O_2^{\bullet -}$, H_2O_2, and $HO^{\bullet}$, are also produced in response to environmental factors such as inflammation and UV radiation. Furthermore, substantial evidence suggests that $O_2^{\bullet -}$ and H_2O_2 are generated transiently upon interaction of various ligand-cell surface receptor pairs and function as intracellular messengers (reviewed in [3]). Therefore, $O_2^{\bullet -}$ and H_2O_2 are not merely damage-causing agents but are also mediators of physiological functions.

Calcium homeostasis is controlled by (1) Ca^{2+} channels such as the ryanodine receptor (RyR), inositol(1,4,5)P_3 receptor (IP_3R), dihydropyridine receptor (DHPR), and L-type voltage-sensitive channels, (2) Ca^{2+} pumps such as the sarcoplasmic reticulum Ca^{2+}ATPase (SERCA pump) and sarcolemmal Ca^{2+}ATPase, and (3) Na^{+}/ Ca^{2+} exchangers [4]. A shift in the cellular redox status to a more oxidized state generally causes a rapid increase in the concentration of intracellular calcium ($[Ca^{2+}]_i$) (reviewed in [5–9]). The effect of ROS on $[Ca^{2+}]_i$, however, is variable, depending on the cell type, the type of ROS, the level of ROS production, and the duration of exposure to ROS. The effects of ROS on Ca^{2+} homeostasis have been studied extensively in vascular endothelial cells, smooth muscle cells, cardiomyocytes, and neuronal cells because of the pathophysiologic role of oxidative injury in myocardial ischemia-reperfusion, atherosclerotic lesion formation, and trauma. Despite abundant studies, the target molecules on which ROS act and the chemical nature of ROS-induced modification are largely unknown. Considerable differences in the chemical reactivity of $O_2^{\bullet -}$, H_2O_2, and $HO^{\bullet}$ also add complexity to such studies.

Hydroxyl radicals are extremely reactive, with a lifetime of several nanoseconds in the cellular milieu, and inflict indiscriminate damage on proteins, DNA, and lipids. Oxidation of membrane lipids by $HO^{\bullet}$ alters the physical properties of membranes and membrane-associated proteins, leading to nonspecific ion leakage. It is therefore unlikely that $HO^{\bullet}$ functions as a specific mediator of redox regulation.

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$O_2^{\bullet-}$ and H_2O_2 are less reactive species that are known to display selective oxidation of particular target molecules. H_2O_2 is a mild oxidant that can oxidize the sulfur atom of methinone and cysteine residues in proteins. Cysteine is oxidized to cysteine sulfenic acid or disulfide, both of which are readily reduced back to cysteine by various cellular reductants. The pK_a (where K_a is the acid constant) of the sulfhydryl group (Cys–SH) of most cysteine residues is ~8.5. Because Cys–SH is less readily oxidized by H_2O_2 than is the cysteine thiolate anion (Cys–S^-), few proteins might be expected to possess a cysteine residue that is vulnerable to oxidation by H_2O_2 in cells [10]. However, certain protein cysteine residues have low pK_a values and exist as thiolate anions at neutral pH because of nearby positively charged amino acid residues that are available for interaction with the negatively charged thiolate. Proteins with low-pK_a cysteine residues can be the targets of specific oxidation by H_2O_2, and such oxidation can be reversed by thiol donors such as glutathione and thioredoxin. Methionine (Met) is more susceptible to oxidation by H_2O_2 and is converted to Met sulfoxide, which is reduced back to Met by specific enzymes called Met sulfoxide reductases (reviewed in [11]). Furthermore, reversible oxidation of Met residues can serve as a control switch for the regulation of protein function, as exemplified by calmodulin, which loses the ability to activate plasma membrane Ca^{2+}ATPases when its COOH-terminal Met residues are oxidized [12]. However, it is not known if H_2O_2 can effect selective oxidation of specific Met residues among many solvent-exposed Met residues.

Although $O_2^{\bullet-}$ is a poorer oxidant than H_2O_2, it specifically oxidizes certain metal ions bound to proteins and consequently modifies the function of these proteins (e.g. inactivation of calcineurin due to oxidation of its Fe-Zn center [13]). It seems that $O_2^{\bullet-}$ is also able to react selectively with certain proteins as the result of electrostatic attraction between the negatively charged $O_2^{\bullet-}$ molecules and positively charged amino acid residues of the targeted proteins. One such example is the vascular smooth muscle SR Ca^{2+}ATPase, which is inactivated by $O_2^{\bullet-}$ but not by H_2O_2([14]. However, the amino acid residues affected by $O_2^{\bullet-}$ have not been identified in this case. Furthermore, the cardiac muscle SR isoform, which shares 90% homology with the smooth muscle isoform, is insensitive to $O_2^{\bullet-}$.

Activation of Ryanodine and IP_3 Receptor Ca^{2+} Release Channels by H_2O_2

At the present time, H_2O_2-mediated oxidation of Cys residues residing within special microenvironments appears to provide the most well defined mechanism underlying the reversible and specific effects of ROS [3]. Good examples of this phenonmenon are RyR and IP_3R, both of which are activated when specific Cys residues are oxidized. RyRs, which are involved in Ca^{2+} release from the SR in skeletal and cardiac muscles, are composed of four subunits and form a complex with triadin. RyR contains about 21 cysteine residues per subunit. Some of the 21 cysteine residues have higher reactivity than others toward H_2O_2 and various sulfhydryl reagents, but these have not been mapped precisely (reviewed in [7,15]). Oxidation by H_2O_2 or modification by sulfhydryl reagents of the reactive Cys–SH residues decreases the K_d for ryanodine as well as the EC_{50} for Ca^{2+} activation [16]. Single-channel reconstitution experiments indicate that H_2O_2, at submicromolar concentrations, enhances the Ca^{2+} release that follows fusion of SR vesicles to planar lipid membranes [16,17]. Cysteine oxidation also contributes to the stabilization of a RyR/triadin complex during channel activation, probably through intermolecular disulfide bonding. The stimulatory effects of peroxide are reversed by thiol-reducing agents such as dithiothreitol and glutathione (GSH). Increased oxidative stress produced by ROS and nitric oxide is generally reflected by an increased ratio of GSSG to GSH in cells. GSSG is capable of forming a mixed disulfide with a reactive cysteine or converting two neighboring cysteines to a disulfide. In accordance with this capacity, GSSG, like H_2O_2, has been demonstrated to enhance the binding affinity of RyR to ryanodine and enhance its reconstituted single channel Ca^{2+} release [18].

Sensitivity to sulfhydryl oxidation also appears to be a property of the endoplasmic reticulum (ER) Ca^{2+} channel IP_3R. H_2O_2 and GSSG were shown to cause spontaneous release and oscillation of Ca^{2+} by sensitizing IP_3R to endogenous IP_3 [19–22]. Recent reports suggest that H_2O_2 generated intracellularly as the result of ligation of cell surface receptors also contributes to Ca^{2+} mobilization. For example, histamine produces H_2O_2 through activation of NADPH oxidase in endothelial cells, and the NADPH oxidase-derived H_2O_2 is critical for the generation of Ca^{2+} oscillations during histamine stimulation [23]. Many other agonists induce Ca^{2+} oscillations as well as H_2O_2 production. Therefore, receptor-mediated H_2O_2 production is likely to be a key process affecting Ca^{2+} signaling. However, care should be taken not to attribute the H_2O_2 effect entirely to the oxidation of IP_3R, as H_2O_2 is also known to cause the production of IP_3 (see below).

Many studies on other Ca^{2+} channels (dihydropyridine receptors, L-type voltage-sensitive channels), Ca^{2+}ATPases, and Na^+/Ca^{2+} exchangers also suggest that H_2O_2 affects their activity through cysteine oxidation (reviewed in [4]). However, the results remain inconclusive and, at times, controversial.

Enhancement of $[Ca^{2+}]_i$ through H_2O_2-mediated Inactivation of Protein Tyrosine Phosphatase and PTEN

The changes in Ca^{2+} homeostasis need not be entirely due to the modification of Ca^{2+} transporters (channels, pumps, and exchangers) but may also arise indirectly from the modification of other proteins. Candidates for such modification include protein tyrosine phosphatases (PTPs) and PTEN. All PTPs contain an essential cysteine residue (pK_a, 4.7 to 5.4) in the signature active site motif HCXXGXXRS/T (where X is any amino acid residue) that exists as a thiolate anion at

neutral pH [24]. This active site cysteine is the target of specific oxidation by H_2O_2, and the ability of intracellularly produced H_2O_2 to inhibit PTP activity has been demonstrated in cells stimulated with EGF, PDGF, and insulin [25–27]. Furthermore, EGF- and PDGF-induced protein tyrosine phosphorylation of cellular proteins, including their respective receptor protein tyrosine kinases (RPTKs) and PLC-gamma, requires H_2O_2 production [28,29]. These results indicate that the activation of an RPTK *per se* by binding of the corresponding growth factor may not be sufficient to increase the steady state level of protein tyrosine phosphorylation in cells. Rather, the concurrent inhibition of PTPs by H_2O_2 may also be required. As such, H_2O_2 plays a major messenger role in the activation (tyrosine phosphorylation) of PLC-gamma and subsequent production of IP_3 in cells stimulated with PDGF and EGF. Exogenous H_2O_2 alone, in the absence of a growth factor, induces tyrosine phosphorylation of various cellular proteins including PLC-gamma and elicits IP_3 production [30]. This probably reflects the background activity of various protein tyrosine kinases, which is apparently sufficient to enhance the level of protein tyrosine phosphorylation when the activity of most PTPs is suppressed by H_2O_2.

PTEN is a member of the PTP family and reverses the action of phosphoinositide (PI) 3-kinase by catalyzing the removal of the 3′-phosphate of $PI(3,4,5)P_3$. H_2O_2 induces reversible inactivation of PTEN through specific oxidation of the catalytic site cysteine [31]. As with protein tyrosine phosphorylation, it is likely that the activation of PI 3-kinase in receptor-stimulated cells may not be sufficient to achieve the accumulation of $PI(3,4,5)P_3$ because of the opposing activity of PTEN; the concomitant inactivation of PTEN by H_2O_2 might thus be necessary to increase the abundance of $PI(3,4,5)P_3$ sufficiently to trigger downstream signaling events. However, production of $PI(3,4,5)P_3$ was shown to be necessary for PDGF-induced H_2O_2 production [32]. This is probably because $PI(3,4,5)P_3$ activates Rac, an essential component of the activated NADPH oxidase complex, by binding to the pleckstrin homology domains of the Rac guanine nucleotide exchange factors [33]. Thus, through its effect on the concentration of $PI(3,4,5)P_3$, the oxidation of PTEN by H_2O_2 constitutes a positive feedback loop that increases the production of H_2O_2. This positive feedback loop is expected to result in a rapid increase in Ca^{2+} concentration. Because many inositol polyphosphate phosphatases also contain a cysteine at their active site [34], degradation of IP_3 might be inhibited by H_2O_2. There are observations that support this possibility.

In all likelihood, PTPs and PTEN represent the first examples among many more proteins that connect H_2O_2 and Ca^{2+} signaling. Hence, we are merely taking our first steps in understanding how oxidants modulate Ca^{2+} signaling.

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SECTION D

Lipid-Derived Second Messengers

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CHAPTER 143

Historical Overview: Protein Kinase C, Phorbol Ester, and Lipid Mediators

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Retrospectives of Phospholipid Research

Nearly 200 years ago, a French chemist, L. N. Vauquelin, found phosphorus in the brain material extracted with hot alcohol. This material was probably a mixture of crude phospholipids. Thirty years later, choline-containing phospholipid was obtained from the brain by F. Fremy (oleophosphoric acid) and from the egg yolk by M. Gobley (lecithin). Since then, during a period of more than 100 years, several phospholipids were isolated and structurally identified. The existence of inositol in plants was known in the nineteenth century but was unknown in animal tissues until 1941, when D. W. Woolley found it in the mammalian brain. In the next year, J. Folch and Woolley at Rockefeller Institute in New York fractionated several phospholipids and identified the chemical structure of inositol phospholipid. In the late 1940s Folch, then at Harvard University, noticed that additional phosphate was attached to the inositol moiety.

In the subsequent years many efforts were made to clarify the metabolic and synthetic pathways of various lipids, including inositol phospholipids. In parallel with these investigations, in the decade of 1960s, phospholipids were shown to be cofactors essential to the catalytic activity of enzymes such as β-hydroxybutyrate dehydrogenase (D. E. Green), Na^+/K^+ ATPase (T. Tanaka), NADH-cytochrome C reductase (S. J. Wakil), and many others. Nevertheless, with some exceptions such as the production of platelet-activating factor (D. J. Hanahan) and eicosanoid (S. K. Bergström and B. I. Samuelsson), membrane phospholipids were generally viewed as a biologically inert entity that provide a semipermeable barrier between exterior and interior compartments within and between cells.

In the early 1950s, with radioactive orthophosphate, Hokin and Hokin [1] observed that acetylcholine induced rapid labeling of acid-precipitable materials of some exocrine tissues such as pancreas. It became evident soon that the materials were inositol phospholipid and phosphatidic acid. Namely, the rapid labeling of these lipids resulted from the enhanced breakdown and resynthesis of inositol phospholipid, but its biological significance remained to be clarified for many years. In 1975, Michell postulated that this phospholipid hydrolysis may open the Ca^{2+} gate [2].

Protein Kinase C and Diacylglycerol

In 1977, when protein kinase C (PKC) was first found as an undefined protein kinase present in many mammalian tissues, the enzyme was activated by limited proteolysis with Ca^{2+}-dependent protease, and no obvious evidence was available for its role in signal transduction. Before long it became clear that without proteolysis the enzyme could be activated by a membrane factor in the presence of Ca^{2+}. The membrane factor was identified as anionic phospholipids, particularly phosphatidylserine. Curiously, crude phospholipids extracted from brain membranes could activate the enzyme in the absence of added Ca^{2+}, whereas pure phospholipids obtained from erythrocyte membranes could not

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produce any enzyme activation unless a higher concentration of Ca^{2+} was added to the reaction mixture. Analysis of the lipid impurities on a silicic acid column led us to conclude that diacylglycerol is an essential activator.

To explore the link of PKC activation to inositol phospholipid hydrolysis, we developed a procedure to activate this enzyme in intact cells by applying membrane-permeant diacylglycerols. Diacylglycerols having two long fatty acyl moieties could not be readily intercalated into the cell membrane. If, however, one of the fatty acids is replaced with a short chain, the resulting diacylglycerols, such as 1-oleoyl-2-acetyl-glycerol (OAG), obtain detergent-like properties and could be dispersed into the phospholipid bilayer and activate PKC directly. In the initial studies, human platelets were employed. Thrombin and collagen induce release of serotonin with the concomitant hydrolysis of inositol phospholipid and phosphorylation of two endogenous proteins with 20 and 47 kDa molecular size. It was already known that the 20 kDa protein is myosin light chain, and a specific calmodulin-dependent kinase is responsible for its phosphorylation. Before long we knew that the 47 kDa protein, pleckstrin we call it today, was a substrate specific to PKC. Thus, the phosphorylation of these two proteins served as excellent markers for the increase of Ca^{2+} and diacylglycerol-dependent activation of PKC, respectively. In 1980, we were able to show that both Ca^{2+} increase and PKC activation were essential and acted synergistically to elicit full activation of platelets and release of serotonin. Similarly, it was possible to show unequivocally that PKC activation is indispensable for neutrophil release reaction and T-cell activation, thus establishing the biological role of PKC in cellular responses. In 1983, Berridge and his colleges announced at Cambridge the important inositol 1,4,5-trisphosphate story [3].

Phorbol Ester and Cell Signaling

In the summer of 1981, M. Castagna visited our laboratory from Villejuif, France. We discussed a possible role of tumor-promoting phorbol ester in the PKC signaling pathway. It was already known that phorbol ester shows pleiotropic activities by mimicking hormone actions. When platelets were stimulated by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), the 47 kDa protein was remarkably phosphorylated, but against our expectation diacylglycerol was not produced. It was extremely disappointing to us because this meant that our idea that diacylglycerol is the mediator for PKC activation was not correct. A few days later, however, an idea flashed: What would happen if TPA could activate PKC directly because the phorbol ester contains a diacylglycerol-like structure very similar to the membrane-permeant lipid molecule OAG that we had used? This insight occurred near the end of August. The following year several groups of investigators showed that PKC is the major target of phorbol ester. It was also shown that phorbol ester could cause translocation of PKC from the cytosol to the membrane. As a result, the traditional concept of tumor promotion originally proposed by I. Berenblum at Oxford in 1941 was replaced by an explicit biochemical explanation providing for an understanding the role of PKC. Along this line of study, phorbol esters and membrane-permeant diacylglycerols have since then been used as crucial tools for the manipulation of PKC in intact cells, and have allowed the determination of the wide range of cellular processes regulated by this enzyme [4]. It was realized much later, however, that phorbol ester can bind to other cellular proteins, such as chimaerin and RasGRP [5], and potentially affect cell functions through additional targets.

Structural Heterogeneity and Mode of Activation

Although PKC was once considered as a single entity, molecular cloning and enzymological studies in the mid 1980s revealed the existence of multiple isoforms of PKC. The mammalian PKC family consists of at least ten isoforms encoded by nine genes. These isoforms are divided into three subgroups based on their primary structures and biochemical properties: classical PKC isoforms (cPKC), novel PKC isoforms (nPKC), and atypical PKC isoforms (aPKC) [6]. The PKC isoforms are conserved in a variety of species, including yeast, nematoda, fly, fish, and frog. On the one hand, the serine-threonine protein kinase region that is located in the C-terminal half does not show much difference and exhibits similar enzymatic properties when tested in *in vitro* systems. On the other hand, the N-terminal half of the enzyme molecule contains multiple characteristic functional domains, such as the C1 domain, which binds diacylglycerol or phorbol ester; the C2 domain, which binds phospholipid in the presence of Ca^{2+}; and the OPR (octicosapeptide repeat) domain, which is involved in protein-protein interaction. The structural feature and multiple functional domains of the PKC isoforms are well investigated, as documented in excellent reviews [7,8]. In addition, several protein kinases that share kinase regions closely related to the PKC family are isolated and characterized [9]. These include protein kinase N (PKN or PRK), protein kinase D (PKD or PKCμ), and protein kinase B (PKB, Akt or rac-PK). The N-terminal regions of these enzymes contain multiple distinct functional domains such as PH and HR1 domains.

Structural analysis also made it clear that the mode of activation of the PKC family is far more complicated than we initially had thought. Newton [8] has shown that the newly synthesized kinase is catalytically inert and is regulated by phosphorylation by itself and also by other kinases, including PDK1 and related enzymes. A unique cross-talk thus emerged between the PKC signal pathway and one branch of the inositol phospholipid 3-kinase pathway that was described first by L. Cantley in the mid-1980s [10]. Another cross-talk with tyrosine kinase pathway for the activation of PKC is becoming clearer. PKC was initially recognized as an enzyme that can be activated by limited proteolysis, but later this proteolysis was recognized as a process of downregulation. More recently, however, the PKC δ-isoform is proposed to be a target of caspase 3 for its activation during apoptosis.

Translocation and Multiple Lipid Mediators

The specific functions of individual PKC isoform have been studied for many years, but whether the isoforms exert functional redundancy or functional specialization remains unclear, although some of them obviously play unique specific roles (see PKC minireview series, isoform-specific functions, *J. Biochem.* 2002–2003).

In addition to phospholipase C, phospholipase A2, phospholipase D, and sphingomyelinase appear to be indispensable players in signal transduction. In fact, it becomes increasingly clear that fatty acids, lysophospholipids, ceramide, and other lipid products may play roles in cell signaling, as described elsewhere [6] and in this chapter. In addition, the products of phosphatidylinositol 3-kinases play key roles in the transmembrane control of cellular processes as proposed by Toker and Cantley [10]. Multiple lipid mediators produced in membranes may recruit various protein kinases and other signal molecules to "lipid rafts" through lipid-lipid, lipid-protein, and protein-protein interactions. The lipid-mediated translocation of protein kinases to selective intracellular compartments such as plasma membrane, Golgi complex, and cell nucleus represents an essential step for stable access to their substrate proteins. It is attractive to surmise, then, that the N-terminal half of the enzyme molecule with multiple membrane-binding domains governs the enzymatic activity as well as the functional specificity of the C-terminal half. It is curious that such lipid-mediated translocation of PKC isoforms to membranes sometimes shows oscillation back and forth from the cytosol to the membrane. The mechanism of this oscillation is not clear, but lipid mediators appear to oscillate after receptor stimulation, presumably due to a repetitive feedback mechanism. The destination and reversibility of such translocation appear to differ with the isoform, lipid mediator, and cell type. The dynamic behavior of the PKC isoforms and related kinases can be visualized with the enzymes fused to green fluorescence protein.

Conclusion

In the last decade, our knowledge of the PKC family and related enzymes as well as the lipid mediators derived from membrane phospholipids has expanded enormously. It appears that the enzymes anchor to specific protein complexes through interaction with some adapter proteins, as directed by multiple lipid mediators. Such interactions may be essential for the function of each protein kinase at selective intracellular compartments. Further exploration of the dynamic aspects of such lipid mediators and identification of interacting proteins may unveil more of the transmembrane control of physiological and pathological cellular processes.

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CHAPTER 144

Type I Phosphatidylinositol 4-phosphate 5-kinases (PI4P 5-kinases)

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Introduction

Type I phosphatidylinositol 4-phosphate 5-kinases (PI4P5Ks) phosphorylate phosphatidylinositol 4-phosphate (PI4P) in the 5-position to form phosphatidylinositol 4,5-bisphosphate ($PI45P_2$). Because metabolic evidence suggests that *in vivo* the major route of synthesis of $PI45P_2$ in animal cells is by the 5-phosphorylation of PI4P, both in the plasma membrane [1,2] and the nucleus [3] , type I PI4P5Ks are obviously the enzymes primarily responsible for regulating levels of this multifunctional lipid. In the test tube type I PI4P5Ks have been reported to catalyze other reactions. For example, both Iα and Iβ isoforms can convert PI into PI5P [4], and PI3P into $PI34P_2$ [4, 5] or $PI35P_2$ [4], or even eventually $PI345P_3$ [4,5]. A PI4P5K from *Arabadopsis* shows a similar flexibility when expressed in insect cells [6]. However, the 5-phosphorylation of PI4P is the major activity of the Type I enzymes, and the physiological significance (or even natural occurrence) of these other reactions remains unclear. The exception is the demonstration that an endogenous type I PI4P5K (isoform unknown) makes a physiologically significant contribution to the synthesis of $PI345P_3$ from $PI34P_2$ in response to cell stress [7].

Several fuller reviews have discussed these enzymes directly or indirectly (e.g. [8–10]).

Basic Properties

Cloning

Our current understanding of type I PI4P5Ks is that there are three distinct mammalian isoforms, and no other obvious candidate emerges from a scan of the current human genome database. Nomenclature is rather confusing, as the type Iβ cloned from mouse [11] and the human isoform called type Iα cloned shortly afterwards by Loijens and Anderson [12] are exact orthologues (and similarly, mouse type Iα and human type Iβ). As the type Iγ [13] has come from the same species (mouse) and lab as the original cloning of the Iα and Iβ, we have in Fig. 1 used the mouse nomenclature. The isoform that Carvajal *et al*. [14] identified as the STM7 gene, mapping close to the Friedrich's ataxia gene (X25), is the human type Iβ isoform.

Loijens and Anderson [12] reported two splice variants of the human type Iα and one of the type Iβ, and the mouse type Iγ also has at least two splice variants [13].

Structure

The lineup in Fig. 1 tells a superficially simple story of a highly conserved central core, which consists of the catalytic

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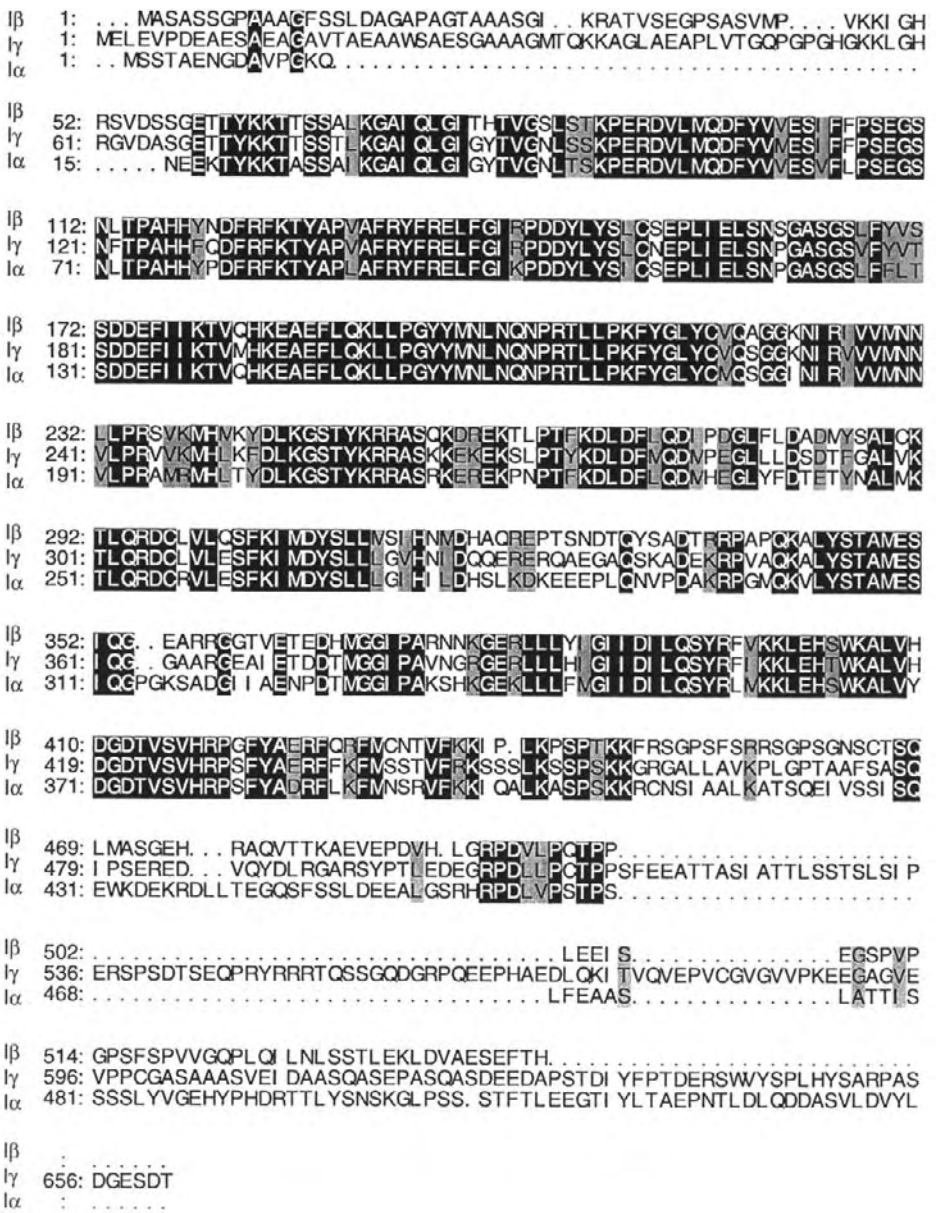


Figure 1 The sequences of the mouse type I PI4P5Ks are shown (Genebank accession numbers: Iβ, NM_008847; Iγ, NM_008844; Iα, NM_008846).

site interspersed with some loops of significant variation between isoforms, and then virtually no sequence similarity whatsoever between the isoforms at the C- and N-termini. The latter, in turn, implies diverse isoform-specific regulation, which has implications in the discussions about regulation, below.

There is also a close similarity in the catalytic "core" with the yeast gene Mss4 [11,15], but again no similarity in other parts of the sequence between the yeast and the mammalian enzymes. There is a limited amount of similarity with the members of the Fab1 family, both yeast and mammalian; these are PI3P 5-kinases, now given the name type III PIP kinases. Also, there is similarity in the catalytic core with the type II PI5P4Ks, and from this, and from the x-ray structure of the type IIβ PI5P4K [16], some deductions can been made about probable crucial residues for catalytic activity in the type I enzymes. Ishihara *et al*. showed that lysine 138 of the type Iα PI4P 5K, which they identified as being in the putative ATP-binding site, is essential for catalytic activity [13].

Substrate Specificity

Kunz *et al*. [17] have shown that the substrate specificity of the type I and II PIP kinases is dictated largely by their "activation loop," that is, transferring the activation loop from type IIβ PIPK into type Iβ (human) converted the type I enzyme into a PI5P4K activity (the activation loop of the orthologous mouse type Iα is residues 347–387 in Fig. 1). The converse (converting a type II PIPK into a PI4P5K activity by inserting a type I loop) was also observed. These observations have recently been taken a stage further by some elegant site-directed changes in this loop [18].

A remarkable finding is that changing a single residue, glutamate 362 in human type Iβ (equivalent to E362 in murine Iα, see Fig. 1), to an alanine transformed its substrate specificity to that resembling a type II activity in that it would use PI5P as a substrate (though its activity against PI4P was diminished rather than lost). Kunz *et al*. have suggested that the activation loop might fold into an α-helix *in vivo* [18]. The structure of type IIβ PI5P4K [16] suggests how the activation loop might lie adjacent to the presumed active site where the PI5P substrate head-group binds. Although the activation loop did not crystallize [16], it seems likely that it will influence the orientation of two loops that link contiguous β strands; both of these loops contribute residues that interact with the inositol 1,5 bisphosphate moiety of the substrate [16].

Localization

These latter studies on substrate specificity [18] have implications also for the localization of the type I enzymes. So far, localization studies have suggested that they are all primarily in the plasma membrane (though see below for some possible regulation of this). The combined data of the two papers on the influence of the activation loop [17,18] demonstrated that changing the substrate specificity from favoring PI4P to PI5P also changes the localization of the type Iβ enzyme from plasma membrane to cytosol. This in turn implies that the plasma membrane localization is governed primarily by interaction with the PI4P substrate. This conclusion is subject to the caveat that these studies use transfection, which might saturate endogenous (protein) binding sites in locations other than the plasma membrane, and it is then the "excess" that is being visualized, bound to its substrate.

Chatah and Abrams have reported a different localization of human type Iβ PI4P5K, that is, perinuclear, with a translocation to the plasma membrane after prolonged activation of the cells [19]. Our own experience is that there is some variation in subcellular localization of transfected type I PI4P5Ks between cell types, and also some dependence on culture conditions and length of transfection time. There is still a lot to learn about the localization *in vivo* of endogenous type I PI4P5Ks and how it is controlled.

Regulation

Given their self-evident role in cell regulation, it is not surprising that type I PI4P5Ks have been found to be subject to a variety of regulatory influences. Only a brief summary of the literature to the end of 2001 is possible here.

Phosphatidic Acid

This lipid has long been known to be a potent stimulator of type I (but not type II) PIPKs [20]. Under some circumstances it can be essential—for example, Honda *et al*. could only see the effects of Arf-6 (below) if PA was supplied [21]. Jones *et al*. [22] have produced evidence that endogenous PA may be a significant regulator of type I PI4P5Ks *in vivo*. PA is of course the product of PLD, itself an enzyme frequently tied in with $PI45P_2$ and with type I PI4P5Ks (e.g. [23]), and it may be that the two enzymes have a complex interregulatory relationship.

Monomeric G Proteins

There is abundant evidence that members of the Rho and Arf family can regulate type I PI4P5Ks, though to a significant degree we do not know the physiological veracity of these events, nor the isoform involved. The clear difference between the three isoforms (Fig. 1) raises the possibility that *in vivo* there may be significant specificity in the G-protein-PI4P5K interaction.

Arguably the strongest evidence supports regulation by members of the Arf family. For example, Honda *et al*. [21] purified from brain cytosol the major GTPγS-dependent activator of murine type Iα PI4P5K, and found it to be Arf-1. They went on to show that its localization in HeLa cells was not consistent with its being a natural regulator of type Iα PI4P5K (Arf-1 being predominantly in the Golgi in these cells), but rather that Arf-6 fitted the bill under all the criteria they addressed. Martin *et al*. [24] also thought that Arf and not Rho (see below) was the endogenous regulator of a type I PI4P5K (isoform unknown). Brown *et al*. [25] have implicated Arf-6 in endosome formation, and showed that human Type Iα PI4P5K can mimic the effects of a constitutively active Arf-6 (though again the endogenous Type I PI4P5K is unknown). Arf-1 may regulate $PI45P_2$ synthesis in the Golgi, though in these experiments it most likely recruited the type I PI4P5K from the cytosol [26,27].

There is also a reasonable case for type I PI4P5K activation by Rho family members, though it is sometimes confusing. Thus using the Rho-specific C3 *Botulinum* toxin, Chong *et al*. [28] implied that Rho regulates a type I PI4P5K activity in fibroblasts, whereas others have failed to see a Rho-type I PI4P5K interaction in experiments where it did interact directly with Rac [29]. Rac interaction with type I PI4P5Ks has been suggested in other experiments [30,31], and there are convincing data placing type Iα or Iβ PI4P5Ks in the signaling pathway from the thrombin receptor, *via* Rac, to actin polymerization [32]. For the most part the evidence for Rho involvement still remains indirect [33]. Some of these simplistic contradictions may be due to differences in isoforms, though Honda *et al*. [21] stated that this was unlikely to be the reason they could not see an effect of Rho in their experiments. An interaction with RhoGDI has also been reported [31], and we think that a fair summary of the state of play is that the involvement of monomeric G-proteins in regulation of type I PI4P5Ks is real, and important, but incompletely understood.

Phosphorylation

Several protein kinases have been reported to associate with or regulate type I PI4P5Ks; for example, casein kinase I in *S. pombe* [34], Rho-kinase [35] (which might explain some of the contradictions about Rho, though see ref [33]),

and PKCμ (a.k.a. PKD) [36]. Also, Park *et al*. [37] showed that all three type I PI4P5Ks can be negatively regulated by PKA, and also suggested that receptor activation led to a dephosphorylation (and thus activation) by an uncharacterized mechanism that may involve PKC. Wenk *et al*. [38] have shown a stimulation-dependent dephosphorylation of type Iγ PI4P5K in synapses (where it is the major type I isoform). Another intriguing possible regulatory mechanism has been suggested by Itoh *et al*. [39]. All three isoforms of type I PI4P5K are capable of autophosphorylation, an activity that is stimulated by PI and that leads to an inhibition of the enzyme's activity against PI4P. The physiological relevance of this awaits further study, as does the even more intriguing (and as yet untested) possibility that, like some of the type I PI3Ks [40], type I PI4P5Ks might phosphorylate other proteins.

Other Regulation Mechanisms

Mejillano *et al*. [41] have suggested that human type Iα PI4P5K is cleaved by caspase during apoptosis, an event that, because they also suggest $PI45P_2$ to be anti-apoptotic, serves as part of the amplification of the apoptotic process once it has started. Recently, Barbieri *et al*. have shown an isomeric specificity for the involvement of type I PI4P5Ks in EGF receptor-mediated endocytosis [42], in that the mouse type Iβ PI4P5K was required but the type Iα was not. How the type Iβ is regulated in this process is an intriguing question for further exploration.

Function

The physiological role of type I PI4P 5Ks is self-evidently well established (in contrast with the more enigmatic type II PI5P 4-kinases; see the next chapter by Rameh), because their primary function is to synthesize $PI45P_2$. Thus the question, what is the function of type I PI4P 5Ks, is essentially the same as the question, what is the function of $PI45P_2$. This is now a huge topic, with upwards of 20 suggested physiological functions (e.g. see [8,10] for reviews) and therefore is outside the scope of this short review.

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CHAPTER 145

Type 2 PIP4-Kinases

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Introduction

The type 2 PIP4-kinase family of enzymes appeared relatively late in the evolution of eukaryotes. Homologous to the type 1 PIP5-kinases, they also catalyze the synthesis of phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2). However, the type 2 PIP4-kinases are 4-kinases that use phosphatidylinositol-5-phosphate (PtdIns-5-P) as substrate, while the type 1 are 5-kinases that use phosphatidylinositol-4-phosphate (PtdIns-4-P) as substrate. Unlike type 1 PIP5-kinases, type 2 PIP4-kinases are not found in yeast (*S. cerevisiae* and *S. pombe*), but they are present in lower multicellular eukaryotes (such as *C. elegans*). Thus, the type 2 PIP4-kinases probably diverged from the type 1 PIP5-kinase to fulfill a specialized but essential function in multicellular organisms. Despite the fact that the type 2 PIP4-kinases were the first phosphoinositide kinases to be isolated, cloned, and crystallized, their purpose in cells still remains elusive. Type 2 PIP4-kinases appear be regulated by extracellular factors, which suggests a role for these enzymes in cell-cell signaling. Here I review the history of the type 2 PIP4-kinases along with their structure and regulation. I present their potential roles in phosphoinositide metabolism and in the transduction of extracellular signals.

History

The kinases capable of synthesizing PtdIns-4,5-P_2 were first purified from erythrocytes in the late 1980s [1]. Two distinct activities, type 1 and type 2, were separated and initially distinguished from each other based on biochemical and immunogenic characteristics [2]. In the literature prior to 1997, type 1 and type 2 PIP-kinases were assumed to carry out the same reaction-conversion of PtdIns-4-P to PtdIns-4,5-P_2. In fact, they were first named PtdIns-4-P 5-kinases. In 1997, a surprising observation led to the realization that the type 2 PIP-kinases actually produce PtdIns-4,5-P_2 by phosphorylating the 4 position of PtdIns-5-P (a contaminate in commercial PtdIns-4-P) [3]. This observation demonstrated that PtdIns-4,5-P_2 can be synthesized through two independent pathways. The pathway catalyzed by the type 1 PIP5-kinase uses PtdIns-4-P as intermediate and is referred to as the canonical pathway for PtdIns-4,5-P_2 synthesis. The pathway catalyzed by the type 2 PIP4-kinase uses PtdIns-5-P as intermediate and is referred to as the alternative pathway for PtdIns-4,5-P_2 synthesis, because it accounts for only a fraction of the total PtdIns-4,5-P_2 in cells. PtdIns-5-P levels in cells are very small when compared to PtdIns-4-P and cannot be easily detected via conventional HPLC separation protocols [3]. For this reason, PtdIns-5-P was not known to exist *in vivo* prior to this discovery. *In vitro*, the type 2 PIP4-kinases can also convert PtdIns-3-P to PtdIns-3,4-P_2, but PtdIns-5-P is the preferred substrate (50-fold better) [3]. In summary, it is now clear that the type 1 and 2 PIP-kinases have different biological and metabolic functions in cells, even though they both synthesize the same lipid product.

Structure

The domain structure of the type 2 PIP4-kinase protein is fairly simple (Fig. 1). Its predicted molecular weight is approximately 47 kDa, but the α and β isoforms migrate with apparent molecular weight of 55 kDa in SDS-polyacrylamide gels. The kinase domain is located in the carboxy-terminal portion of the protein and accounts for most of the protein. The amino-terminal portion of the protein is involved in dimerization. Crystals of the type 2β PIP4-kinase revealed that these enzymes have structures similar to protein kinases. The homodimer forms an elongated disc shape and a large flat surface containing the two catalytic pockets of the subunits [4].

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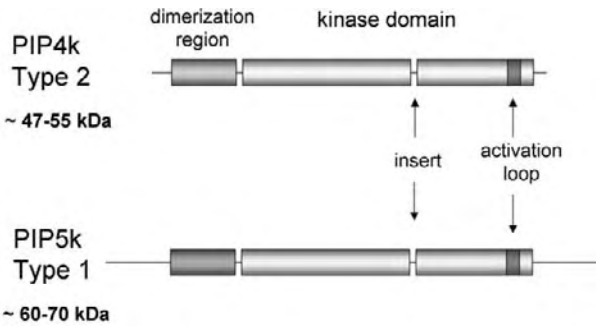


Figure 1 Structure of the type 2 and type 1 PIP-kinases.

A high concentration of positive residues on this flat surface suggests that this region is involved in membrane interaction through electrostatic forces. The substrate pocket is not as deep as protein kinase's substrate pocket, suggesting that the homodimer can float across the surface of membranes and phosphorylate PtdIns-5-P without a necessity for the lipid to significantly protrude from the membrane [4].

The kinase domain of the type 1 and the type 2 PIP-kinases are both interrupted by an insert that does not resolve in the type 2 crystal structure. The type 1 and type 2 are 35 percent identical at the kinase domain. However, the sequence of the type 1 and type 2 PIP-kinases are significantly divergent at a stretch of about 25 amino acids in the region of the kinase domain that corresponds to the activation loop of protein kinases. This region is highly conserved between the different isoforms of a given isotype. Anderson and collaborators showed that this activation loop region is sufficient to determine substrate specificity to the type 1 and type 2 PIP-kinases [5]. When the activation loop of the type 1 PIP5-kinase was swapped with the activation loop of the type 2, the chimeric enzymes lost their original substrate specificity and acquired the catalytic properties of the donor enzyme.

Type 2 PIP4-Kinase Isoforms

There are three isoforms of the type 2 PIP4-kinase in mammalian cells, namely the α, β, and γ isoforms [6–8]. At the protein level, the α and β isoforms are 83 percent identical and the γ isoform is about 60 percent identical to either one of them. All isoforms are ubiquitously expressed, but the α isoform is predominantly found in brain and platelets, the β isoform in brain and muscle, and the γ isoform in brain and kidney. Type 2 PIP4-kinase orthologs are present in the *C. elegans* (F535H12.4) and *Drosophila* (CG17471) genomes. Even though the biochemical properties of the products of the *C. elegans* and *Drosophila* genes have not yet been demonstrated, they are likely to be enzymatically active, based on conservation of critical residues in the active site. Because this gene family has been conserved from worms to humans, it is likely that the type 2 enzymes serve an important function in multicellular organisms. Similarities between the type 1 and type 2 PIP-kinases suggest that they have a common ancestor.

Regulation

The levels of PtdIns-4,5-P_2 in cells can be affected by extracellular signals, thereby suggesting that the activity of PIPkinases may be regulated [9–11]. Although the mechanisms by which the type 2 PIP4-kinases are regulated in cells are not completely clear, the existing data suggest that subcellular localization, interaction with membrane receptors, phosphorylation, and substrate availability are important factors.

Subcellular Localization

The first indication that the type 2 PIP4-kinases respond to extracellular factors came from studies in platelets. Thrombin-stimulated aggregation of platelets induced the redistribution of type 2 PIP4-kinase to the cytoskeleton [12]. This phenomenon correlated with increased cytoskeleton-associated PIP-kinase activity and increased levels of cytoskeleton-associated PtdIns-4,5-P_2. This thrombin-stimulated PIP4-kinase re-localization to the cytoskeleton was mediated by integrins, and the results suggested a role for this enzyme in controlling cell morphology and adhesion.

The subcellular localization of the type 2 PIP4-kinases (α and β) was also examined in fibroblasts by immunofluorescence and by expression of GFP-tagged fusion proteins. A surprising finding was that a fraction of these enzymes, together with the type 1 PIP5-kinases, was present in the nucleus, in structures that appear as nuclear speckles and contain pre-mRNA processing factors [13]. In a different study, the type 2β was found in the nucleus and cytosol, but the α was found exclusively in the cytosol [14,15]. Mutations in the β isoform revealed that α helix-7 of type 2 β is necessary for its nuclear localization. The function of the type 2 PIP4-kinase in the nucleus remains to be determined. Nonetheless, many studies have indicated that phosphoinositide metabolism in the nucleus is an active process.

Interaction with Membrane Receptors

The type 2β isoform was first cloned from a yeast two-hybrid screen by using the p55/tumor necrosis factor (TNF) receptor as bait [7]. Later, it was also shown to associate with the EGF receptor and with ErbB2 [16]. Association with the TNF receptor is specific for the p55 subunit and involves the juxta-membrane region of the receptor. Very little is known about how these interactions affect type 2 PIP4-kinase activity. It is possible that association with receptors may bring PIP4-kinase in close proximity with its substrate or with other regulatory proteins. Association with receptors is independent of ligand stimulation, thus it is not clear whether PIP4-kinase can be regulated by TNFα, EGF, or neuregulin stimulation or whether it participates in signaling by these growth factors.

More recently, the type 2α PIP4-kinase was shown to be present in bovine photoreceptor rod outer segments (ROS), a compartment of retinal photoreceptor cells in which

phosphoinositide metabolism is active and responsive to light stimuli [17]. It is interesting that in tyrosine-phosphorylated ROS, the type 2 enzyme can be precipitated with anti-phosphotyrosine antibodies. The type 2 protein itself does not seem to be phosphorylated in these preparations, indicating rather that it associates with phosphotyrosine-containing proteins. These results suggest that receptor tyrosine kinases may regulate type 2 PIP4-kinase activity, although the phosphotyrosine containing partner of the type 2 PIP4-kinase has not been identified in these studies. Furthermore, these studies indicate that the type 2 enzymes may have a role in the transduction of signals initiated by light. Further analysis will be necessary to confirm these hypotheses.

Phosphorylation

The type 2α PIP4-kinase present in platelets was shown to be phosphorylated on serine and threonine residues [18]. Unlike other lipid kinases, such as the type 1 PIP5-kinase and PI3-kinases, the type 2 PIP4-kinase is not autophosphorylated [19]. The protein kinase CK2 was identified as a PIP4-kinase kinase and shown to phosphorylate Serine 304 (S304), a residue that is not conserved in the β and γ isoforms [20]. The phosphorylation state of S304 in resting versus activated platelets has not been determined. Since CK2 is a constitutively active kinase in cells, it is not clear whether S304 is involved in PIP4-kinase regulation in response to extracellular factors.

The type 2γ isoform was also found to be phosphorylated [8]. In polyacrylamide gels, this isoform migrates as a doublet, and the upper band was shown to be phosphatase-sensitive. *In vivo* labeling of cells with [^{32}P]-phosphate demonstrated that this enzyme is phosphorylated on serine and threonine but not on tyrosine. The phosphorylation state of the type 2γ changes in response to various signals, including EGF and serum. This is strong evidence that the type 2 PIP4-kinases may be regulated by extracellular signals. However, the exact role of phosphorylation on the type 2γ activity in cells remains to be determined.

Substrate Availability

The levels of PtdIns-5-P in cells are comparable to the levels of 3′-phosphorylated phosphoinositides, such as PtdIns-3-P, but are much lower than the levels of PtdIns-4-P [3]. This suggests that the availability of PtdIns-5-P substrate may be the limiting step in the production of PtdIns-4,5-P_2 by the type 2 PIP4-kinases. Although the type 2 PIP4-kinases are subject to posttranslational modifications and protein-protein interactions, no direct effect on kinase activity was reported, as discussed above. In addition, expression of these PIP4-kinases in bacteria results in active enzymes (except for the type 2γ isoform) and indicates that the type 2 α and β may be constitutively active in cells. Therefore, it is possible that the local and temporal activation of the alternative pathway for PtdIns-4,5-P_2 synthesis is dependent upon PtdIns-5-P synthesis and the co-localization of type 2 enzyme with this substrate. PtdIns-5-P levels in cells were shown to be regulated by thrombin, by cell cycle progression, and by serum stimulation ([21,22] and personal unpublished data). However, the pathways for PtdIns-5-P synthesis *in vivo* have not been determined. *In vitro* PtdIns-5-P can be generated through phosphorylation of PtdIns by 5′-kinases, such as the type 1 PIP5kinase [23] and PIKfyve [24], or by dephosphorylation of PtdIns-4,5-P_2 by SHIP [3].

Putative Models for the Function of the Type 2 PIP-Kinases

Despite more than a decade of research on type 2 PIP4-kinases, the biological role of the alternative pathway for PtdIns-4,5-P_2 synthesis is not clear. Nevertheless, it is clear that the type 1 and the type 2 PIP-kinases have nonoverlapping biological functions. For example, overexpression of the type 1 PIP5-kinase, but not the type 2, leads to a dramatic reorganization of actin cytoskeleton [25].

Pulse labeling of phosphoinositides in cultured cells has indicated that the phosphate at the 5′ position of the inositol ring is incorporated last in the majority of PtdIns-4,5-P_2 synthesized *in vivo* [26]. Therefore, the type 2 PIP-kinase is not involved in maintaining the bulk of the PtdIns-4,5-P_2 in cells. At this point we can only speculate on the roles for this enzyme in phosphoinositide metabolism and cell signaling. Here are a few possibilities:

Model 1: to Regulate the Synthesis of Specific Pools of PtdIns-4,5-P_2 in Cells. One possibility is that the type 2 PIP4-kinases may contribute to PtdIns-4,5-P_2 synthesis in specific subcellular compartments where PtdIns-5-P is present (model 1, Fig. 2). This would permit the regulation of local synthesis of PtdIns-4,5-P_2 independent of the bulk of PtdIns-4,5-P_2 synthesis. Even though total PtdIns-4,5-P_2 levels in cells are high, there are reports that demonstrate that a large fraction of cellular PtdIns-4,5-P_2 is unavailable [27]. For instance, the PtdIns-4,5-P_2 synthesized through the alternative pathway could be the main source of substrate for the

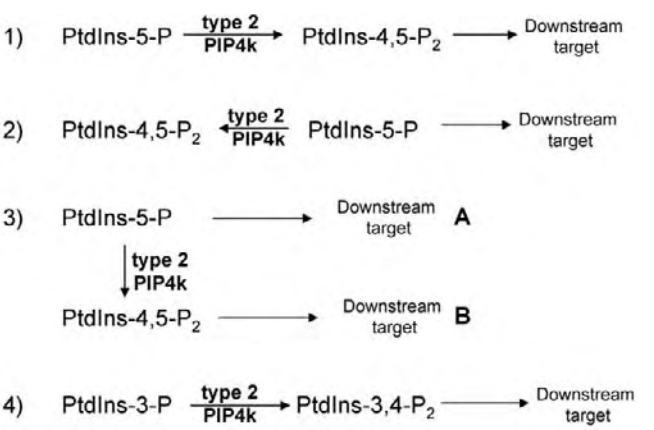


Figure 2 Putative models for the type 2 PIP4-kinase function in cells.

enzyme PLCγ to generate IP_3 and diacylglycerol, despite the availability of PtdIns-4,5-P_2 in the cell.

Model 2: to Regulate the Levels of PtdIns-5-P in Cells. As discussed above, PtdIns-5-P levels in cells are small and comparable to the levels of other signaling phosphoinositides. This observation raises the possibility that the important function of the type 2 PIP4-kinases is to get rid of PtdIns-5-P rather than generate PtdIns-4,5-P_2. This implies that PtdIns-5-P has a specific role in cells that may not be related to its role as an intermediate for PtdIns-4,5-P_2 synthesis. PtdIns-5-P could be a signaling molecule with specific downstream targets or a substrate for other enzymes such as PI 3-kinases. In this case, the function of the type 2 PIP4-kinase could be to assure that the levels of PtdIns-5-P are kept low and tightly regulated (model 2, Fig. 2). New data showing that PtdIns-5-P can be regulated by extracellular factors make this an attractive model.

Model 3: to Coordinate PtdIns-5-P Consumption with PtdIns-4,5-P_2 Synthesis. Models 1 and 2 are not mutually exclusive and it is possible that PtdIns-5-P and PtdIns-4,5-P_2 generated through PtdIns-5-P can trigger opposite cellular responses. In this model, the type 2 PIP4-kinase could serve as a switch between these two modes of signaling, necessary to assure that the termination of the signal generated by PtdIns-5-P is coupled to the initiation of the PtdIns-4,5-P_2 signal (model 3, Fig. 2).

Model 4: to Regulate the Synthesis of PtdIns-3,4-P_2 in Cells. The type 2 PIP-kinase could possibly be responsible for PtdIns-3,4-P_2 synthesis in cells, independent of PtdIns-3,4,5-P_3 (model 4, Fig. 2). In this case, PtdIns-3-P would be the major substrate for type 2 PIP4-kinases. This model is unlikely, based on the strong preference that these enzymes have for PtdIns-5-P.

Conclusion

Despite new biochemical, genetic, and structural information that has been acquired in recent years, the type 2 PIP4-kinase family remains a mystery to cell biologists who are trying to identify the physiologic role for the alternative pathway for PtdIns-4,5-P_2 synthesis, catalyzed by these lipid kinases. Future experiments involving inactivation or suppression of the type 2 activity in cell or animal models are likely to shed a light on this intriguing question.

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CHAPTER 146

Phosphoinositide 3-Kinases

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Introduction

Engagement of a great variety of cell surface receptors triggers the activation of phosphoinositide 3-kinase (PI3K). The lipid products of PI3K serve as second messengers by interacting with phosphoinositide-binding domains in certain cytoplasmic proteins, thereby recruiting these "PI3K effectors" to specific sites in cellular membranes. PI3K and its effectors have been implicated in diverse cellular functions, including vesicle trafficking, cell proliferation, survival, cytoskeletal remodeling, migration, and glucose uptake. The importance of PI3K signaling in cellular and organismal function has been illustrated by the striking phenotypes of animals with genetic perturbations of PI3K signaling.

This chapter is intended to provide a brief summary of PI3K signaling with an emphasis on recent advances. The reader is referred in the text to several excellent reviews that provide more detail on specific topics.

The Enzymes

Phosphoinositide 3-kinase (PI3K) isoforms have been divided into three classes that differ in subunit structure, substrate selectivity, and regulation [1,2]. Class I PI3Ks exist as heterodimers with a tightly bound regulatory subunit (Fig. 1). In addition to the kinase domain, each class I catalytic subunit possesses a Ras-binding domain, a C2 domain for interaction with phospholipid membranes, and a helical "PIK" domain that is also conserved in PtdIns-4-kinases. In a landmark paper describing the crystal structure of the p110γ isoform, Walker and colleagues showed that the helical domain acts as a scaffold or spine on which the other three functional domains are organized [3]. This report also confirmed that the kinase domain is similar in structure to protein kinases, as predicted from primary sequence comparison and limited protein kinase activity of PI3K enzymes. The shape of the substrate binding pocket helped explain the selectivity for phosphoinositide recognition and the likely basis for differential recognition of single and multiply phosphorylated substrates by different PI3K classes. Subsequent structural work from this group has clarified the mode of binding of various PI3K inhibitors to the active site of p110γ and has provided evidence for allosteric activation by Ras [4,5].

Class I PI3Ks are further subdivided by their modes of regulation. The class I_A subgroup (p110α, p110β, and p110δ) associates with regulatory subunits (p85α, p55α, p50α, p85β, or p55γ) that have multiple modular protein-protein interaction domains (Fig. 1). Class I_A PI3Ks function downstream of receptors with intrinsic or associated tyrosine kinase activity. Full activation of class I_A PI3K is thought to require occupancy of both Src-homology 2 (SH2) domains of the regulatory subunit by tyrosine phosphopeptides, along with the binding of catalytic subunit to Ras-GTP [6]. This normally occurs only in proximity with membrane-associated tyrosine kinases that also activate Ras. Other domains of the regulatory subunits may also contribute to activation or localization [1]. The class I_B enzyme (p110γ) interacts with a distinct regulatory subunit, p101, with no significant homology to other known proteins (Fig. 1). The class I_B enzyme is activated by βγ subunits of heterotrimeric G proteins [1,2] following engagement of G-protein-coupled receptors (GPCRs). The presence of a Ras binding domain within p110γ suggests that this isoform also integrates signals from tyrosine kinase pathways.

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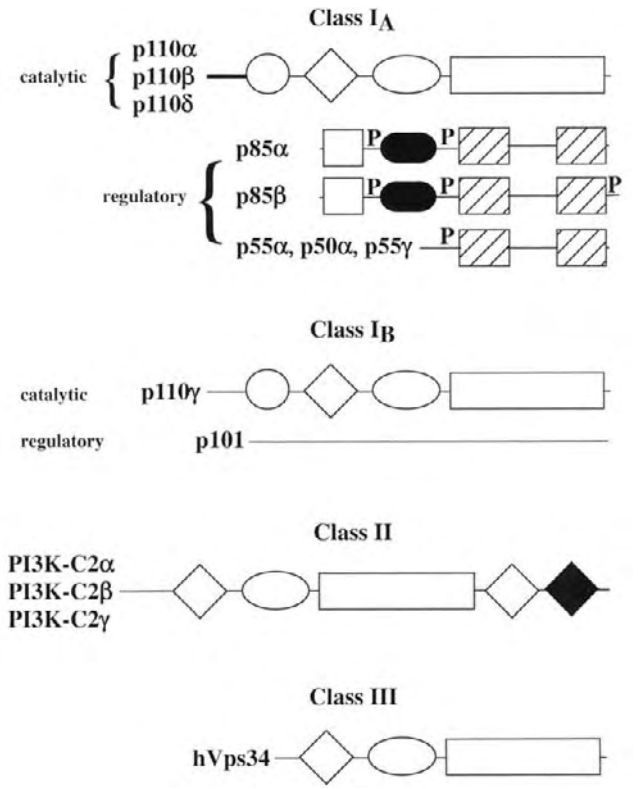


Figure 1 Schematic diagram of the domain structure of mammalian PI3Ks. The common names of the proteins are listed at the left of each structure (hVps34 = human homolog of class III PI3K [Vps34] first cloned from *S. cerevisiae*). The three class I_A catalytic subunits associate with each of the five regulatory subunits without any apparent preference. p85α, p55α, and p50α are alternative transcripts of a single gene. Open boxes, kinase domain; open ovals, PIK domain; open diamond, C2 domain; open circle, Ras-binding domain; open square, SH3 domain; hatched rectangle, SH2 domain; closed oval, RhoGAP-homology domain; closed diamond, PX domain; P, proline-rich motif.

Class II PI3Ks are distinguished by the presence of an additional C-terminal C2 domain and a PX domain (Fig. 1). Although comparatively little is known about class II PI3K regulation, there is growing evidence that these enzymes can be activated by extracellular signals [2]. Genes for class I and class II enzymes have been found in all multicellular animals. Class III PI3Ks are found in all eukaryotes from yeast to humans. These enzymes appear to have a housekeeping function related to vesicular transport and protein sorting [1,2]. They interact with an associated serine kinase in both yeast (vps15p) and humans (p150).

The Products

PI3Ks phosphorylate the 3′-hydroxyl of the D-*myo*-inositol ring of phosphatidylinositol (PtdIns) (Fig. 2A). Four D-3 phosphoinositides exist in mammalian cells: PtdIns(3)P, PtdIns $(3,4)P_2$, PtdIns $(3,5)P_2$, and PtdIns $(3,4,5)P_3$. The pathways of synthesis and degradation of these lipids have been reviewed recently in detail [2,7] and are summarized in Fig. 2B. PtdIns $(3,4,5)P_3$ is produced only by class I PI3Ks with PtdIns $(4,5)P_2$ as a substrate. PtdIns $(3,4)P_2$ can be generated from PtdIns(4)P by class I or class II PI3Ks, or by 5′-phosphatase action on PtdIns $(3,4,5)P_3$, or by a PtdIns(3) P-4-kinase. The signaling functions of PtdIns $(3,4,5)P_3$ and PtdIns $(3,4)P_2$ are well studied and will be discussed further below. Although PtdIns(3)P can be produced by all PI3Ks *in vitro,* the majority of PtdIns (3)P in cells appears to be made by class III PI3Ks and is detected primarily in endosomal vesicles [8]. PtdIns $(3,5)P_2$ is generated *in vivo* probably by a PtdIns(3)P-5-kinase and like PtdIns(3)P may be involved in vesicle trafficking.

Lipid-Binding Domains

Pleckstrin homology (PH) domains are small (~60aa) protein modules that mediate protein-lipid and protein-protein interactions. There is a growing list of PH domains shown to bind selectively to D-3 phosphoinositides [2,9]. It is important to note that PtdIns(4)P and PtdIns $(4,5)P_2$ are much more abundant in cellular membranes compared to D-3 phosphoinositides; thus, for a PH domain to be considered D-3-specific, the binding affinity for D-3 lipids must be considerably higher than the affinity for PtdIns(4)P or PtdIns $(4,5)P_2$. Subgroups have been identified that show greater affinity for either PtdIns $(3,4,5)P_3$ or PtdIns $(3,4)P_2$, with others that bind these lipids comparably [2,9]. A combination of biochemical and structural approaches has helped define features of PH domain primary sequence that determine selectivity for different D-3 phosphoinositides [2,10,11]. D-3-selective PH domains are found in a variety of proteins involved in signal transduction, some of which are discussed below.

PX domains are found in a diverse list of proteins involved in vesicle trafficking, protein sorting, and signal transduction [12,13]. Like PH domains, different PX domains exhibit selectivity for different phosphoinositides. Two components of the oxidative burst complex in phagocytes, p40phox and p47phox, possess PX domains that bind preferentially to PtdIns(3)P and PtdIns $(3,4)P_2$, respectively [14,15]. These interactions are thought to be important for targeting the cytosolic components of the NADPH oxidase complex to the phagolysosome, where they meet with the membrane-bound components p22phox and gp91phox to initiate the oxidative burst. The PX domain of class II PI3K (Fig. 1) binds selectively to PtdIns $(4,5)P_2$ [16]. The PX domain of cytokine-independent survival kinase (CISK) binds both PtdIns $(3,5)P_2$ and PtdIns $(3,4,5)P_3$ [17]. The crystal structure of the PX domain of p40phox bound to PtdIns(3)P shows that the lipid binds in a positively charged pocket and suggests how phosphoinositide binding specificity is determined [18].

The FYVE domain, originally identified in several yeast proteins, is a protein module that binds selectively to PtdIns(3)P. The structure of FYVE domains and their binding to PtdIns(3)P are distinct from the PH domain/D-3 lipid interaction [2,19]. Although some mammalian FYVE domain-containing proteins are involved in signal transduction,

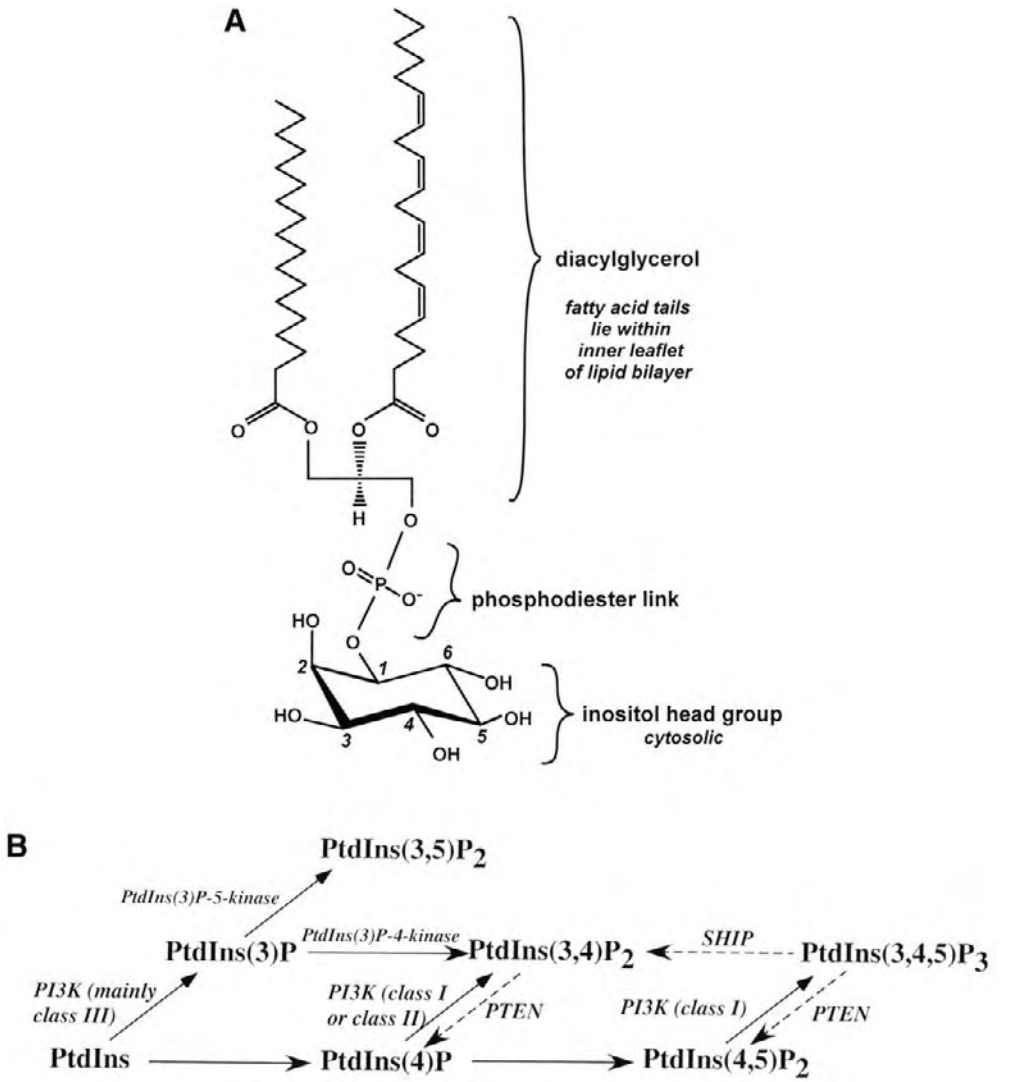


Figure 2 (A) Structure of D-*myo*-phosphatidylinositol (PtdIns). Note that the head group is positioned to interact with cytoplasmic molecules. Although free hydroxyls exist at positions 2–6, phosphorylation *in vivo* has only been detected at positions 3, 4, and 5. Reprinted with permission from the *Annual Review of Biochemistry*, Volume 70 ©2001 by Annual Reviews www.AnnualReviews.org. (B) Pathways of synthesis and degradation of D-3 phosphoinositides. The major enzymes responsible for particular reactions are indicated. For simplicity, many of the enzymes involved in metabolism of other phosphoinositides are omitted.

the primary role of most mammalian and all yeast proteins with this module is in membrane trafficking.

A recent study described the use of phosphoinositide affinity matrices to purify and clone a number of D-3 lipid-binding proteins [20]. Many of these were known proteins with PH or FYVE domains previously shown to bind to PI3K products, helping to validate the method. A novel protein with five PH domains, termed ARAP3, was found to possess distinct domains with GAP activity for Arf and Rho family G proteins. The ARAP family, along with other regulators of Arf and Rho function [2], may thus play an integral role in PI3K-regulated cytoskeletal changes (Fig. 3). This study also identified the Sec14 homology domain, originally identified in the yeast PtdIns transfer protein Sec14p, as a putative phosphoinositide-binding module.

Effectors and Responses

PI3K activation has been linked to distinct cellular responses downstream of different receptors. For example, PI3K is required for proliferation induced by numerous growth factors and cytokines, for glucose uptake triggered by insulin, and for cell migration in response to chemoattractants [1,2]. A major challenge in PI3K research has been to determine how specificity in signaling is achieved. With all the factors that can trigger increases in D-3 phosphoinositides, how is it that different stimuli evoke distinct responses through PI3K?

There are several answers to this puzzle. One level of specificity is conferred by differential expression of PI3K isoforms in distinct tissues and cell types. For example, the p110δ isoform is leukocyte specific, and antibody-blocking

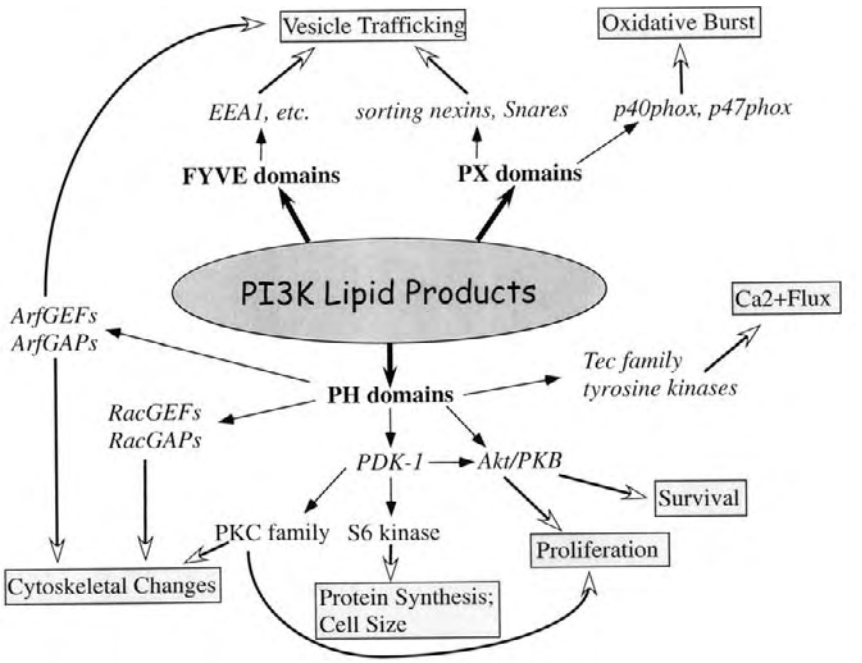


Figure 3 Overview of the effector proteins and signaling pathways regulated by PI3K lipid products. D-3 lipid-binding modules are in bold print. Functional responses are in boxes. The diagram shows selected effector proteins (in italics) whose lipid-binding domains have been well studied and whose activation has been linked to particular responses. GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein.

experiments suggest that in macrophages p110δ is required for migration whereas p110α is required for proliferation triggered by the CSF-1 receptor [21]. The regulatory isoform p50α is expressed at highest levels in the liver, and loss of p50α is associated with hepatocellular necrosis [22]. Similarly, some PI3K effectors are differentially expressed. An example is Btk, a PH domain-containing tyrosine kinase expressed primarily in B lymphocytes and mast cells. Mice lacking either Btk or the predominant class I_A regulatory isoform, p85α, exhibit similar defects in B cell development and function [23, 24]. Another factor in signaling specificity could be the compartmentalization of PI3K activation. In other words, distinct localization of receptors in membrane subdomains affects the pool of PI3K substrates and effectors utilized. D-3 lipids accumulate at the leading edge of cells migrating in response to chemoattractants, thus resulting in localized activation of PI3K effectors [25,26]. Finally, full activation of a given PI3K effector may require synergy with other signals, which may be differentially provided by distinct receptors. For example, full activation of Btk requires phosphorylation by Src family tyrosine kinases that are also activated by B cell antigen receptors [27].

Figure 3 summarizes current knowledge of the linkage of certain PI3K effectors to distinct responses. It is important to note that this diagram is simplified for clarity, and some effectors have been linked to additional functions. A central player in many responses to PI3K activation is phosphoinositide-dependent kinase-1 (PDK-1) [28]. This serine/threonine kinase has a PH domain that binds both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. Current evidence suggests that PDK-1 is constitutively active but only gains access to substrates upon binding D-3 lipids. Phosphorylation by PDK-1 contributes to the activation of many downstream kinases, including Akt/PKB, S6kinase, and some protein kinase C isoforms.

Phosphatases

The membrane-targeting signal provided by D-3 phosphoinositides can be modulated by the action of phosphoinositide phosphatases (PPases). PTEN (*p*hosphatase and *ten*sin homology deleted on chromosome 10) hydrolyzes the 3′-phosphate of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, effectively reversing the action of PI3K (Fig. 2A) [2,29]. Although the importance of PTEN is well established (see next section), it is not yet clear how PTEN is regulated or recruited to sites of PI3K activation. SHIP1 and SHIP2 are related 5′-PPases that contain N-terminal SH2 domains (*SH2*-containing *I*nositol polyphosphate 5-*p*hosphatase). SHIPs can remove the 5′-phosphate from PtdIns(3,4,5)P_3 to produce PtdIns(3,4)P_2 (Fig. 2A) [2,30]. Hence, these enzymes may alter the spectrum of PI3K effectors recruited to the membrane rather than simply turning the signal off. The SH2 domains of SHIP1 and SHIP2 are selective for phosphotyrosines within a particular sequence context known as the immunoreceptor tyrosine-based inhibitory motif (ITIM). ITIMs are found in a number of receptors (for example, FcγRIIB) whose ligation attenuates signaling through antigen receptors [30].

Genetics

Pharmacological inhibitors of PI3K enzyme activity impair proliferation in a variety of cell systems [1,2]. Natural and engineered mutations in PI3Ks and lipid phosphatases have further established the fundamental role of PI3K signaling in promoting growth of normal and transformed cells. The transforming oncogene of an avian sarcoma virus, ASV16, encodes a membrane-targeted variant of p110 whose expression in cells causes accumulation of D-3 phosphoinositides [31]. A truncated variant of p85α (termed p65), first isolated from a T-cell lymphoma, increases basal activity of class I_A catalytic subunits and promotes lymphoproliferation when expressed as a transgene in the T lineage [32,33]. Mice heterozygous for a disrupted PTEN gene exhibit a similar lymphoproliferative disorder [34,35]. Mice with homozygous loss of PTEN specifically in the T lineage develop autoimmune symptoms associated with spontaneously activated T cells that are resistant to apoptosis [36]. Inherited mutations in PTEN are the cause of three autosomal dominant cancer syndromes in humans: Cowden's disease, Lhermitte-Duclos disease, and Bannayan-Zonana syndrome [29]. Moreover, loss of PTEN function is seen in a large fraction of sporadic human cancers, especially glial, prostate, and endometrial tumors [29]. Mice lacking SHIP1 develop a myeloproliferative disorder and have lower activation thresholds for a variety of immune cell stimuli [30]. In addition to these examples of increased PI3K signaling promoting proliferation and tumorigenesis, there are also examples of decreased PI3K signaling causing impaired proliferation. Forced expression of PTEN in PTEN-deficient embryonic fibroblasts and tumor cells impairs growth by inducing cell cycle arrest and/or apoptosis [29]. Loss of the class I_A regulatory isoform p85α in mice abrogates B lymphocyte proliferation in response to antigen receptor engagement, diminishes interleukin-4-mediated B cell survival, and reduces stem cell factor-driven mast cell growth [23,24,37,38]. These mice show impaired immune responses to T-cell-independent antigens, bacteria, and parasitic worms [24,38].

Genetic studies have also implicated PI3K signaling in responses to insulin and insulin-like growth factors. In *C. elegans*, a class I PI3K functions downstream of the insulin receptor homolog in a pathway that regulates both dauer entry and lifespan [39]. This pathway involves the worm orthologs of PDK-1 and Akt and is antagonized by PTEN. In mice, SHIP2 phosphatase is a critical modulator of insulin signaling as SHIP2-deficient mice show increased insulin sensitivity [40]. Based on these findings and a wealth of cell culture experiments, it was expected that mice deficient in class I_A PI3K would show insulin resistance. However, in every case examined, the opposite result has been observed. Mice lacking p85α alone, or all p85α gene products (including p55α and p50α), exhibit hypoglycemia and decreased glucose tolerance [41,42]. Mice lacking p85α alone or p85β also show increased insulin sensitivity [41,43]. Fibroblast experiments suggest that class I_A regulatory isoforms are expressed in excess of catalytic subunits, producing a "buffer" effect that is overcome when regulatory subunit expression is reduced genetically [44]. However, it is not yet known whether this mechanism explains altered insulin sensitivity *in vivo*. Deletion of the mouse class I_A catalytic isoform p110α causes early embryonic lethality, preventing the analysis of insulin signaling in these animals [45].

In *Drosophila*, class I PI3K acts downstream of the insulin receptor ortholog in a pathway that controls cell size [2,46]. Overexpression of class I PI3K in wing imaginal discs increases cell size and yields enlarged wings in the adult fly. Mutation of *Drosophila* PTEN has a similar effect. Conversely, mutation of class I PI3K genes or expression of dominant-negative PI3K reduces cell and wing size. PI3K signaling was also shown to regulate the size of mouse cardiac myocytes [47]. The critical downstream effectors of PI3K in the *Drosophila* system are Akt and S6K, a serine/threonine kinase that regulates protein synthesis [2,46]. These kinases are also regulated by PI3K signaling in mammalian cells (Fig. 3), but their role in controlling the size of cardiac myocytes or other cells has not yet been reported.

p110γ, the class I_B isoform, is expressed primarily in leukocytes. Disruption of the mouse p110γ gene causes defects in inflammatory responses that correlate with defective chemotaxis to GPCR ligands such as f-Met-Leu-Phe and C5a [48–50].

Summary

Signaling through PI3K is an evolutionarily conserved process that enables reversible membrane localization of cytoplasmic proteins. Three modular domains (PH, PX, and FYVE) that interact with D-3 phosphoinositides are broadly distributed among proteins of different function. The recruitment and activation of specific subsets of PI3K effectors in a receptor-specific and cell type–specific manner allows PI3K activation to be linked to different functional responses. Given the pleiotropic effects of pharmacological PI3K inhibitors, therapeutic modulation of PI3K signaling is likely to require targeting of specific effectors that govern particular responses.

Note Added in Proof

Since submission of this chapter, new mouse genetic models have yielded a number of important advances in the PI3K field. Of particular interest are studies demonstrating lymphocyte defects in mice lacking functional p110δ [51-53], analysis of more tissue-specific PTEN knockouts (reviewed in ref. [54], also see [55]), studies showing a role for Akt and S6 kinase in regulation of mammalian cell and organ size [56,57], and a study establishing a role for GPCR signaling through the p110γ isoform in cardiac muscle contractility [58].

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CHAPTER 147

PTEN/MTM Phosphatidylinositol Phosphatases

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PTEN

Introduction

PTEN (*p*hosphatase and *ten*sin homolog deleted on chromosome 10) was first identified as a tumor supressor gene localized on chromosome 10q23. PTEN mutations are found at high frequencies in certain tumors, including endometrial carcinomas, gliomas, and breast and prostate cancers. Furthermore, germline mutations in the *PTEN* gene are found in the related autosomal disorders Cowden disease and Lhermitte-Duclos and Bannayan-Zonana syndromes. Biochemical and genetic analyses of PTEN and its role in these diseases have placed it in a group of gatekeeper genes essential for controlling cell growth and development [1–3].

Activity and Function

PTEN is a member of the protein tyrosine phosphatase (PTP) superfamily of enzymes characterized by the invariant Cys-x_5-Arg (Cx_5R) active site motif (Table I). However, unlike other PTP superfamily enzymes, PTEN utilizes the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP_3) as its substrate [4]. This places PTEN as a negative regulator of phosphatidyl inositol 3-kinase (PI3K) signaling [5]. PTEN has been reported to regulate signaling through Akt/PKB, PDK1, SGK1, and Rho GTPases and therefore as a modulator of a broad range of cellular processes [6,7]. Loss of PTEN function can lead to tumor development through defects in cell cycle regulation, apoptosis, or angiogenesis.

Homozygous $PTEN^{-/-}$; mice die before birth, and embryos display regions of increased proliferation and disturbed developmental patterning. Heterozygous $PTEN^{+/-}$ mice are viable but spontanously develop various types of tumors [3]. Cells from both $PTEN^{-/-}$ -mice and $PTEN^{+/-}$ have constitutively activated Akt and are resistent to apoptotic stimuli. A direct role of PTEN and its lipid phosphatase activity in the regulation of Akt has been demonstrated in several tumor cell lines [2,3]. Furthermore, $PTEN^{+/-}$ mice have a tendency of developing both T-cell lymphomas and autoimmune disorders. A role for PTEN in this postulated link between autoimmune disorders and cancers are further supported by studies of mice where PTEN is conditionally targeted in T cells [8].

Mice in which PTEN is conditionally deleted in neuronal brain cells develop macrocephaly as a result of increased cell numbers, decreased cell death, and enlarged soma size [9,10]. Targeted deletion early in brain development suggests a role for PTEN in controlling the proliferation and potency of stem cells, whereas restricted deletion of PTEN in postmitotic neurons does not result in increased cell proliferation, but rather causes a progressive enlargement of soma size resulting in enlarged cerebellum and seizures. It is of note that the abnormal phenotype of these mice resembles that of Lhermitte-Duclos disease, suggesting that loss of PTEN function is sufficient to cause this disease in humans.

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Table I PTEN and Myotubularin-Related Genes in Human.

Name		Active site	C-terminal domains	Length (aa)	Chromosome	Disease
PTEN	PTEN	IHCKAGKGRT	PDZ-binding	403	10q23.3	Multiple cancers, Cowden and Lhermitte-Duclos
	PTENP1	IHCKAGKGRT	PDZ-binding	>405	9p21	—
	PTENR1	IHCKGGKGRT	—	>408	—	—
	TPIP	IHCKGGKGRT	—	445	13	—
	TPTE	IHCKGGTDRT	—	551	21 and others	—
MTM	MTM1	VHCSDGWDRT	PDZ-binding	603	Xq28	Myotybular myopathy
	MTMR1	VHCSDGWDRT	PDZ-binding	669	Xq28	—
	MTMR2	VHCSDGWDRT	PDZ-binding	643	11q22	Charcot-Marie-Tooth 4B
	MTMR3	VHCSDGWDRT	FYVE	1199	22q12.2	—
	MTMR4	VHCSDGWDRT	FYVE	1195	17q22-23	—
	MTMR6	VHCSDGWDRT	—	>567	13q12	—
	MTMR7	VHCSDGWDRT	—	>574	8p22	—
	MTMR8	VHCSDGWDRT	—	704	Xq11.2-12	—
	MTMR5 (SBF1)	VGLEDGWDIT	PH	1930	22pter	—
	MTMR9 (LIP-STYX)	IHGTEGTDST	—	549	8	—
	MTMR10	LQEEEGRDLS	—	>451	15	—
	MTMR11	LQERGDRDLN	—	710	1	—
	MTMR12 (3-PAP)	LLEENASDLC	—	>637	5	—

Length of predicted protein products (amino acids) and chromosomal localization are listed for each gene. Conserved amino acids within predicted active site sequences are presented, including the catalytic cysteine (yellow) and arginine (light-blue), and non-catalytic basic (blue) and acidic (red) residues. Non-catalytic domains predicted for carboxy-terminal regions as well as related diseases are also listed.

A conserved role for PTEN as a PIP_3 phosphatase and negative regulator of PI3K signaling has also been demonstrated by genetic studies of *D. melanogaster* (dPTEN) and *C. elegans* (Daf-18). By balancing signals from the insulin receptor, dPTEN controls cell size and number in flies, whereas Daf-18 regulates metabolism and longevity in worms [11,12].

The crystal structure of PTEN has revealed several features that contribute to its unique substrate specificity [13]. A 4-residue insertion in one loop of the PTP domain results in the widening and extension of the catalytic pocket and enough space for the bulky PIP_3 headgroup. In addition, the two lysines (Lys125 and Lys128) within the Cx_5R active site sequence, as well as an upstream histidine (His93), coordinate the D1 and D5 phosphate groups of the inositol ring. Hence, the specificity of PTEN toward PIP_3 is generated by a larger active site pocket combined with the conserved residues within the Cx_5R active site. C-terminal to the PTP catalytic domain PTEN contains a Ca^{2+} independent C2 domain, two PEST sequences, and a PDZ-binding motif (Fig. 1). These domains are likely to play important roles in PTEN regulation (see below).

The human genome contains several PTEN-related genes, but so far little is known about their function. Most of these genes exhibit restricted expression pattern and/or subcellular localization different from PTEN and do not appear to regulate Akt phosporylation. In that respect it is interesting to note that these genes have a different active site sequence, which might suggest a different substrate specificity [14,15].

Regulation

The crystal structure of PTEN revealed an extensive interface between its PTP-domain and C2-domain, suggesting that membrane targeting and lipid phosphatase activity are interdependent [13]. This is further supported by the observation that mutations affecting this interface are frequently found in cancers [3]. *In vitro*, the C2-domain of PTEN binds phospholipids independent of Ca^{2+} and its structural characteristics predict a direct membrane association. Mutations in critical lipid binding residues inhibit the ability of PTEN to function as a tumor suppressor and cannot be rescued by artificial membrane targeting. Hence, both structural and functional analysis suggests that the C2-domain play a dual role of both membrane recruitment and positioning of the PTP-domain.

The extreme C-terminus of PTEN contains tandem PEST sequences and a consensus PDZ-binding domain. Whereas the regulatory role of the PEST sequences remain elusive, the PDZ-binding motif has been demonstrated to associate with several PDZ-domain containing proteins [3,16]. The identification of phosphorylation sites in the C-terminal tail of PTEN regulating PDZ-binding and

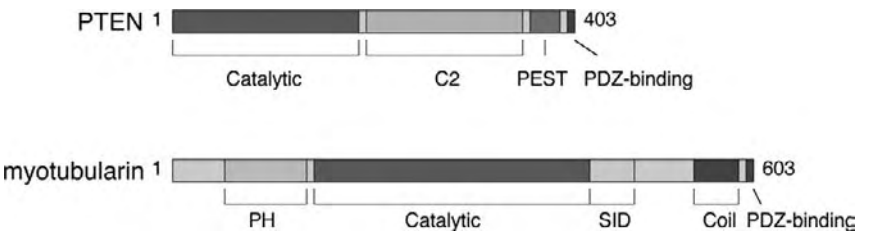


Figure 1 Structural features of PTEN and myotubularin phosphoinositide phosphatases. PTEN and myotubularin contain a catalytic domain that encompasses the CX_5R active site motif of PTP. In addition, both proteins possess several other domains/motifs that are likely to facilitate membrane association and protein-protein interactions. The C2 domain of PTEN is required for binding to lipid vesicles, whereas the carboxy-terminal PDZ-binding motif mediates interaction with PDZ-containing proteins. Phosphorylation in this region inhibits PDZ binding. Myotubularin contains a PH domain that may function to regulate membrane association. Furthermore, myotubularin contains a coiled coil motif as well as a putative PDZ-binding motif.

complex formation (Fig. 1) suggests additional levels of PTEN regulation [3,16].

Finally, several regulatory elements have recently been identified in the PTEN promoter, including binding sites for the tumor suppressors p53, early growth response-1 (Egr-1), and the perioxisome proliferator–activator receptor γ (PPARγ) [17–19]. The inducible transactivation of PTEN by these genes leads to reduced Akt activity and increased cell survival.

Myotubularin: a Novel Family of Phosphatidylinositol Phosphatases

Myotubularin-related proteins constitute one of the largest and most highly conserved protein tyrosine phosphatase (PTP) subfamilies in eukaryotes [14,19]. The MTM family includes at least eight catalytically active proteins as well as five catalytically inactive proteins in human [14,20]. Phylogenetic analysis of MTM family proteins allow a division of myotubularin family onto six subgroups, which include the catalytically active MTM1/MTMR1/MTMR2, MTMR3/MTMR4, and MTMR6/MTMR7/MTMR8 enzymes, as well as MTMR5 (Sbf1), MTMR9 (LIP-STYX), and MTMR10/MTMR11/MTMR12 (3-PAP) inactive forms [14,20]. One gene from *D. melanogaster* and *C. elegans* corresponding to each of these subfamilies has been identified [14].

Phosphatase Activity

Myotubularin (MTM1), the first characterized member of this novel family, utilizes the lipid second messenger phosphatidylinositol 3-phosphate (PI(3)P) as a physiological substrate [21,22]. In addition, recent findings demonstrate that other MTM-related phosphatases MTMR1, MTMR2, MTMR3, MTMR4, and MTMR6 also dephosphorylate PI(3)P, a finding that suggests that activity toward this substrate is common to all active myotubularin family enzymes [23,24].

The consensus CX_5R active site motif of PTP/DSP (dual specificity protein phosphatase) is found in the myotubularin family proteins, and the sequence"CS**D**GW**D**R" is invariant within all members of the active phosphatase subgroups. Unlike PTEN, in which two lysine residues within its active site (C**K**AG**K**GR) contribute to substrate specificity by interacting with the D1 and D5 phosphates of PIP_3, two aspartic acid residues are found in myotubularin family phosphatases. It is possible that interactions between the active site aspartic acid residues and phoshoryl groups at either the D4 or D5 position of the inositol ring may contribute to the high degree of specificity for PI(3)P found in MTM family emzymes.

One of the most notable characteristics of the human MTM family is the existence of at least five catalytically inactive forms, which contain germline substitution in catalytically essential residues within the PTP active site motif (Table I). Myotubularin-related inactive forms may function to regulate PI(3)P levels by opposing the actions of myotubularin phosphatases or directly affect the activity and/or subcellular localization of their active MTM counterparts [25].

Myotubularin Family and Human Diseases

To date, two myotubularin-related proteins have been associated with human disease. The myotubularin gene on chromosome Xq28, *MTM1*, is mutated in X-linked myotubular myopathy (XLMTM), a severe congenital muscular disorder characterized by hypotonia and generalized muscle weakness in newborn males [26]. Myogenesis in affected individuals is arrested at a late stage of differentiation/maturation following myotube formation, and the muscle cells have a characteristically large centrally located nuclei [26].

Mutations in a second MTM family member, *MTMR2* on chromosome 11q22, have recently been shown to cause the neurodegenerative disorder, type 4B Charcot-Marie-Tooth disease (CMT4B) [27]. CMT4B is an autosomal recessive demyelinating neuropathy characterized by abnormally folded myelin sheaths and Schwann cell proliferation in peripheral nerves.

Because these two highly similar genes, *MTM1* and *MTMR2* (64 percent identity, 76 percent similarity) utilize the same physiologic substrate, have a ubiquitous expression pattern, and are mutated in diseases with different target tissues and pathological characteristics, myotubularin and

MTMR2 may be subjected to differential regulatory mechanisms that preclude functional redundancy. Although their specific physiological roles are not known, a recent study has shown that developmental expression and subcellular localization of myotubularin and MTMR2 are differentially regulated, resulting in their utilization of specific cellular pools of PI(3)P [23].

Structural Features

In addition to the phosphatase domain, myotubularin-related proteins possess several motifs known to mediate protein-protein interactions and lipid binding. A PH domain, which was previously defined as a GRAM domain in myotubularin, is present in the N-terminal region of all myotubularin family members, including the catalytically inactive MTMs (Fig. 1). Although the physiologic relevance of this domain is not known, its presence in the myotubularin family lipid phosphatases suggests a role in membrane targeting of these proteins. A coiled coil motif is also present in all family members (Fig. 1) and may play a role in the regulation of MTM proteins through interactions with protein effectors and/or subcellular location. Some myotubularin family members have additional lipid-binding domains. For example, MTMR3 and MTMR4 contain a FYVE domain, and MTMR5 has an additional PH domain in its C-terminal region (Table I).

Although the role of the PH and FYVE domains in MTM function has yet to be determined, it is possible that they serve as targeting motifs to direct the lipid phosphatase domains to specific subcellular environments where PI(3)P is abundant. The physiologic function of myotubularin and related proteins in cell development and signaling processes remains unknown. Studies directed toward clarifying the regulation of myotubularin-related enzymes, as well as identifying downstream effectors, will be of significant value in understanding their roles in cell signaling and development.

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CHAPTER 148

SHIP Inositol Phosphate Phosphatases

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Introduction

The SHIP (*SH*2 domain-containing *i*nositol 5-*p*hosphatase) class of cytoplasmic signaling proteins in higher eucaryotes currently includes a pair of distinct gene products, each encoding an N-terminal SH2 domain, a central amino acid region with inositol 5-phosphatase enzymatic activity, and a C-terminal tail region. The two proteins, named SHIP1 and SHIP2, designating their domain structure and sequence of discovery (gene symbols INPP5D and INPPL1, respectively), are currently the only known members of this family. However, extended family members include many more proteins with inositol phosphatase activity (such as PTEN, type II IP5P, OCRL, and the synaptojanins). A search of the human genome yields only a single orthologue for SHIP2 (Ch. 11q13.3, contig. NT_030106.2) whereas the human genomic sequence for SHIP1 (Ch. 2q37, contig. NT_030597.1) is still incomplete. This review will focus on the principal structural, biochemical, and biological features and familial relationships of the two, so far identified, SHIP proteins. Additional details can be found in recent reviews [1–3].

SHIP1 Structure, Expression, and Function

The 27 exons encoding the SHIP1 protein stretch along an approximately 100 kb region of the murine genome (Ch1, 57.0 CM, C4 to band C5), and are spliced into an approximately 5 kb mRNA as shown in Fig. 1 [4]. The largest SHIP1 protein product, encoded by the co-linear expression of all genomic exons, results in a 1190-amino acid protein termed SHIP1α. This prototypical product contains an N-terminal SH2 domain, an ~450 amino acid inositol 5-phosphatase enzymatic domain, and a C-terminal tail containing multiple motifs for binding potential effector proteins with PTB, SH2, and/or SH3 domains. The SH2 domain has binding specificity for the Y-phosphorylated YxxL motif [3], and the 5′-phosphatase enzymatic activity of the central domain converts PtdIns 3,4,5-P3 to PtdIns 3,4-P2 [5,6]. Either inositol 1,3,4,5-P4 or phosphatidylinositol 3,4,5-P3 can serve as substrate but must contain phosphate at the 3′ position, suggesting that the substrate for SHIP1 is the end product of PI3K activity. Within the C-tail region, notable are the two NPXY motifs, which, when tyrosine phosphorylated, interact with the PTB domain of Shc [6,7]. The NPNY motif also interacts with p85/PI3K, and the Y within this motif plus the three adjacent amino acids comprise the canonical YIGM, which binds the C-terminal SH2 domain of the p85 most avidly [8,9]. The adapter protein, Grb2, contains two SH3 domains, and at least one interacts avidly with SHIP1, probably via one of the "PxxP" motifs in the C-terminal tail region [5,10].

The apparent molecular mass of SHIP1α on SDS acrylamide gel electrophoresis is 145 kDa; however, a large number of additional SHIP-related proteins are detectable (by immunoprecipitation, for example). These additional proteins may be ascribed to spliced isoforms [3], usage of an alternative internal SHIP promoter [11], and C-terminal proteolysis, which affects each of the above protein products [12].

Three SHIP1α isoforms result from three distinct splicing reactions (see Fig. 1), and two complete cDNAs and their

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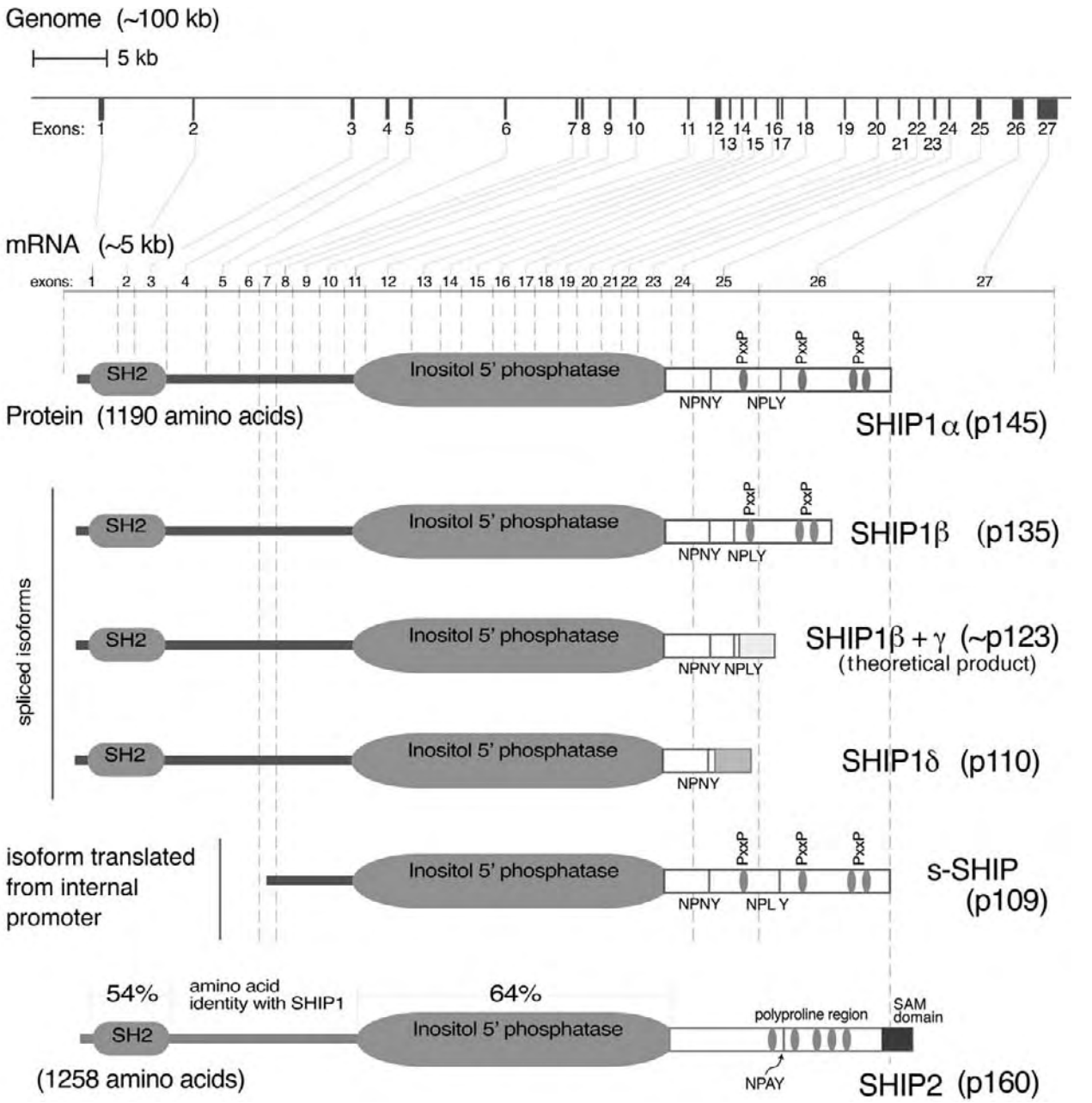


Figure 1 SHIP1 and SHIP2 proteins. The genomic organization of *ship1* is at the top with the mRNA and protein isoforms diagrammed below. At the bottom is the single known protein for SHIP2. See text for details.

protein products have been described for two of the spliced products (the β and δ isoforms) [4,9]. These splicing events, outlined in detail elsewhere [3], in general result in removal of one or several of the C-tail motifs required for binding PTB-, SH2-, or SH3-domain containing proteins. The splice within the β + γ and δ isoforms results in addition of new amino acid sequences at the C-terminal end. The biological function of each shorter isoform is not completely understood.

Recent experiments have established the existence of an SH2-less form of SHIP1 [11]; this isoform is termed s-SHIP (GenBank AF184912) for stem- or short-SHIP. This protein probably results from the usage of a potential promoter region within intron 5 [11]. Transcription of s-SHIP originates at least 44 nucleotides upstream of exon 6 and includes all downstream exons. Translation would probably not begin until exon 7, where the first ATG in the appropriate Kozak motifs is found. The β spliced product has been observed in s-SHIP (GenBank AF184913). In ES cells grown in LIF, s-SHIP is not tyrosine phosphorylated and is not associated with Shc. s-SHIP does, however, form a constitutive complex with Grb2 and is found in the membrane fraction of ES cells. The structure and expression (see below) of s-SHIP suggests it may have a function different from SHIP1 expressed in growth factor stimulated mature cells.

SHIP1 is expressed throughout hematopoietic cell development and tyrosine phosphorylated by a broad range

of cytokines and growth factors of blood cells [3,13,14]. Adult uterus and kidney express SHIP1 detectable by RT-PCR [4], and immunohistochemistry shows strong testis expression [14]. SHIP1 is located within the seminiferous tubules of the testis and exhibits an interesting expressional relationship to both SHIP2, also in these structures of the testis, and the well-defined sequence for spermatozoa development in this organ (discussed in the SHIP2 section). Within the hematopoietic program for blood cell development, the largest SHIP1α product is found in more mature cells [9,13], especially in cell lines, and although numerous spliced isoforms are produced from the SHIP gene, their function and the cellular cues for their production are not understood. Some evidence suggests a developmental role [13]. The s-SHIP product is an exception, as good evidence exists for a function in primitive or stem cells of the blood [11]. s-SHIP is expressed only in very early progenitor or stem cells of the bone marrow and vanishes as cells mature. s-SHIP is also expressed in embryonic stem cell lines. The cDNA for s-SHIP predicts a "stem cell" promoter within intron 5 (see Fig. 1), and the correct size mRNA and protein are expressed in stem or progenitor cells. The exact function of s-SHIP in these cells is not known.

Gene knockout studies in mice and *in vitro* studies have convincingly reconfirmed the negative regulatory role of SHIP1 in myeloid cell development, mast cell activation, and antigen-induces B cell activation [15, 16]. SHIP1$^{-/-}$ mice exhibit a myeloproliferative disorder and inability to regulate mature blood cell functions [3]. Different molecular mechanisms can account for the negative regulatory role of SHIP in each cell type, but a few common themes are apparent (Fig. 2). One mechanism, initiated through receptor tyrosine kinases such as Kit or the M-CSF receptor, may regulate the activation of the survival factor Akt/PKB by eliminating the phosphatidylinositol lipid, PIP3, necessary for Akt/PKB activation (Fig. 2A). How SHIP is recruited into this pathway is not clear; however, one possibility is via the Gab-family of proteins. All three Gab proteins contain the consensus YxxL SHIP SH2 binding motif, and both Gab1 and Gab2 interact with SHIP after growth-factor receptor stimulation [17]. A second general mechanism is shown in Fig. 2B. Here the SHIP SH2 domain is recruited to an Ig-binding receptor (FcγRIIB in B cells and macrophages, FcεRI in mast cells). In B cells the FcγRIIB-SHIP complex terminates a positive signal from the B cell receptor (BCR) [18–20]. In mast cells aggregation of FcεRI is sufficient alone for degranulation, a step regulated by SHIP [21]. In contrast to the above mechanisms, the interaction of SHIP with DOK and RasGAP presents a negative regulatory mechanism altogether different [22,23]. RasGAP in this complex is sufficient to convert active RasGTP to the inactive GDP-bound form and attenuate the MAPK pathway (Fig. 2C). No doubt, additional negative regulatory mechanisms will be uncovered in the future, and it is unlikely that all will be mutually exclusive. Future tasks will be directed at understanding the cellular "when, where, and how" of these different mechanisms.

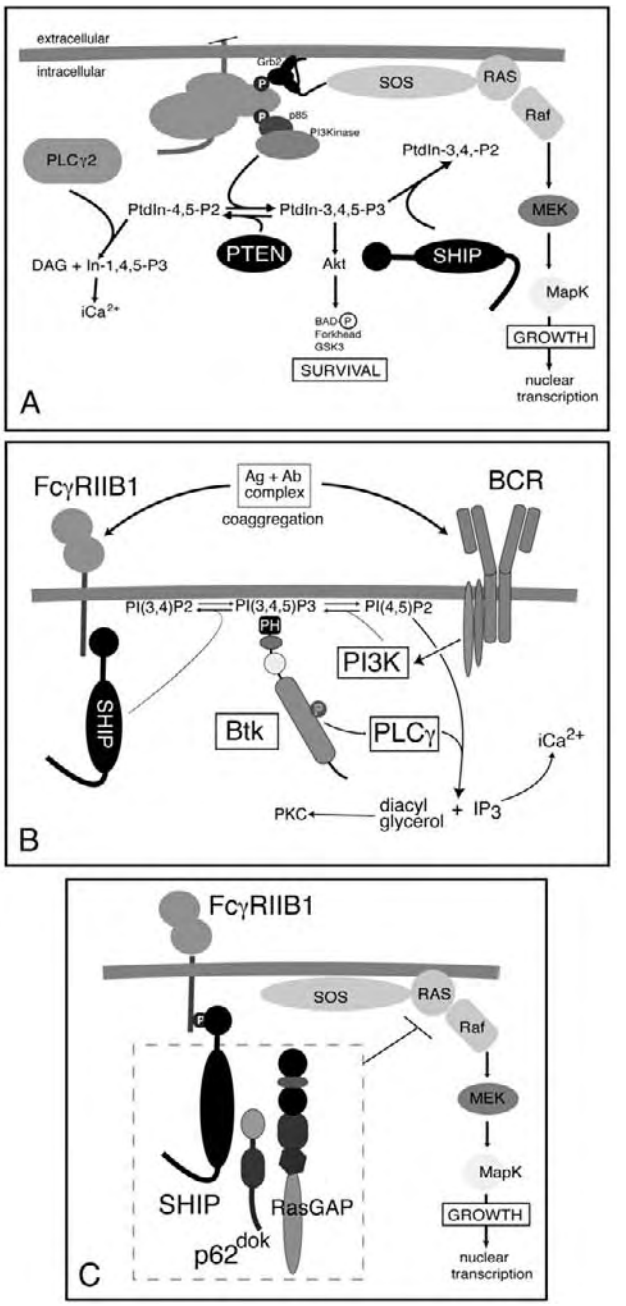


Figure 2 Mechanisms for the negative regulatory function of SHIP in cell signaling. See text for details.

SHIP2 Structure, Expression, and Function

The second member of the SHIP family, the SHIP2 protein, was first isolated by homology to the 51C protein [24]. 51C had been thought, incorrectly, to be a Fanconi anemia protein [25,26]. The 51C protein was also identified simultaneously with SHIP1 as containing an NPXY motif interacting with the PTB domain of Shc [6]. The murine SHIP2 gene is encoded in 29 exons [27]. The full sequence is complete for the mouse genome (AF162781). Translation is predicted to start in the second exon, which would encode part of the SH2 domain, and complete translation would

produce a predicted protein of 142 kDa. Antibodies to the C-tail of the SHIP2 protein recognize a protein with the apparent mass of 160 kDa, a size corresponding roughly to the predicted full-length SH2 domain-containing protein [28].

Spliced isoforms of the SHIP2 protein have not been reported; however, if exons 3–29 alone were transcribed, they might encode an alternative protein product from this gene. The 51C protein might be such a product. The nucleotide sequence for the 51C cDNA (GenBank L36818) comprises exons 3–29 but contains 393 different nucleotides at the 5′ end. These nucleotides appear to be the intron immediately upstream of SHIP2 exon 3. If a protein were translated from this 51C mRNA, it would initiate translation at the methionine homologous with the same site in the s-SHIP protein. Therefore, the 51C cDNA could represent an s-SHIP version of the SHIP2 protein; however, it is also possible that this 51C cDNA is merely derived from incompletely-spliced mRNA.

SHIP2 contains, in general, a structure highly related to SHIP1 but the regions between the SH2 domain and the inositol 5-phosphatase domain and the C-tail region exhibit the least identity [24]. The specificity of the 5′-phosphatase enzymatic activity is similar to that in SHIP, but the inositol polyphosphates may be weaker substrates than the phosphatidylinositol analogues [29,30]. The C-tail region contains a single NPXY motif, several potential SH3-domain binding sites, and a C-terminal SAM (Sterile Alpha Motif) domain of yet unknown function in SHIP2 signaling. The amino acid sequence of the SH2 domain of SHIP2 suggests a binding specificity similar to that of SHIP1, and indeed, both are reported to interact with the phosphorylated immunoreceptor tyrosine-based inhibitory motif (ITIM) of FcγRIIB [31]. In addition, the SH2 domain of SHIP2 was found to bind tyrosine phosphorylated $p130^{Cas}$ and therefore may have some role in the actin-based cytoskeletal reorganization accompanying cell spreading or migration [32].

Unlike SHIP1, SHIP2 is expressed in a broader range of cells and tissues of the mouse [24,26,27] and is present and constitutively tyrosine-phosphorylated in chronic myelogenous leukemia [30]. Brain and thymus exhibit the most prominent expression in both adult and 15.5 day embryos; liver expression was also highest in the embryo, but all other major embryonic or adult organs express some SHIP2. Testes express both SHIP1 and SHIP2 within the seminiferous tubules. Expression of SHIP2 is strongest at the periphery of the tubules where Sertoli cells and spermatogonia precursors reside. Also strongly positive are the mature spermatozoa occupying the inner portions of the seminiferous tubules. In contrast, SHIP1 expression is strongest in membranes of the developing spermatids located between the periphery and central core of the tubules. Therefore, the largely exclusive expression patterns of SHIP1 and SHIP2 in this tissue follow the developmental stages of spermatozoa production from the immature cells at the periphery of the seminiferous tubules to the mature cells in the central core. Expression of SHIP2 appears strongest in the most immature cells and decreases in spermatids while SHIP1 increases; expression levels again reverse in the mature spermatozoa.

Several growth factor receptors stimulate tyrosine phosphorylation of SHIP2 and activation of the Ras/Map kinase and Akt pathways [24,26,28]. The insulin receptor is extremely proficient at stimulating rapid and prolonged SHIP2 tyrosine phosphorylation and Akt/PKB activity. A negative regulatory role for SHIP2 in insulin-induced glucose uptake and glycogen synthesis has been shown by two independent methods. One study utilized wild-type and an inositol phosphatase-inactive mutant of SHIP2, demonstrating the requirement of the phosphatase activity in suppressing insulin-induced metabolic activities [33]. Another study generated SHIP2 knockout mice (lacking exons 18–29) and concluded that SHIP2 is necessary for the negative regulation of insulin signaling and sensitivity to insulin [34]. The homozygous mice lacking functional SHIP2 exhibited perinatal death, and heterozygous mice expressed symptoms of adult-onset diabetes mellitus. Additional abnormalities were not detected in the SHIP2 knockout mice, suggesting that negative regulation of insulin signaling may be a primary function of SHIP2.

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CHAPTER 149

Structural Principles of Lipid Second Messenger Recognition

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Introduction

Structural analyses have shown that domains with a variety of different folds can recognize a single type of lipid second messenger and that a single type of fold can evolve different binding sites and alternative modes of interaction for the same lipid second messenger. Specificity in lipid recognition is achieved by both electrostatic and shape complementarity. A common theme suggested by the structures of the lipid-modifying enzymes and the specific recognition modules is that secondary, nonspecific membrane interactions cooperate with specific lipid recognition to increase membrane avidity. Most binding domains have evolved mechanisms such as partial membrane penetration to bind lipids without removing them from the bilayer.

A wide range of lipid second messengers that are generated in response to external signals has been characterized in terms of their molecular biology. This review will focus on underlying structural principles involved in recognizing these messengers both by the enzymes that produce or consume them and by the downstream effector domains. In most cases, the lipid-modifying enzymes are structurally homologous to enzymes that catalyze an analogous reaction using soluble substrates, suggesting that the constraints imposed by the catalytic chemistry are a stronger determinant of fold than specific lipid binding. For example, the lipid kinases are homologous to protein kinases [1,2]. The phosphoinositide phosphatases are homologous to protein phosphatases and endonucleases [3,4]. The phosphoinositide-specific phospholipases C have a catalytic domain with a TIM-barrel fold similar to many other enzymes and an arrangement of catalytic residues similar to nucleases [5]. A variety of domains present in downstream effector proteins also specifically recognize the lipid second messengers. In contrast to the metabolizing enzymes, these effector domains typically bind lipids with higher affinity and have unique folds.

Phospholipid Second Messenger Recognition by Active Sites of Enzymes

The phosphoinositides are the most diverse family of lipid messengers. All of them share a phosphatidyl D-*myo*-inositol (PtdIns) scaffold that can be phosphorylated at all possible combinations of the 3-, 4-, and 5-hydroxyls to generate lipid messengers with specific roles in intracellular signaling. Several generalizations can be made regarding the recognition of phosphoinositides by proteins. The enzymes that recognize phosphoinositides as substrates tend to envelope the headgroup and make Van der Waals contacts with both faces of the inositol ring (Fig. 1). In contrast, the domains that have evolved simply to bind the phosphoinositides, such as PH domains, tend to form interactions with some or all of the phosphates but to leave one or both faces of the ring exposed (Fig. 2). Presumably, the tendency for the enzymes to more fully bury the headgroup arises from a necessity to exclude water from the active site or to more precisely position the reactive moieties in the active site. Within a family of domains, the affinity of phosphoinositide headgroup binding generally correlates with the number of hydrogen bonds between the

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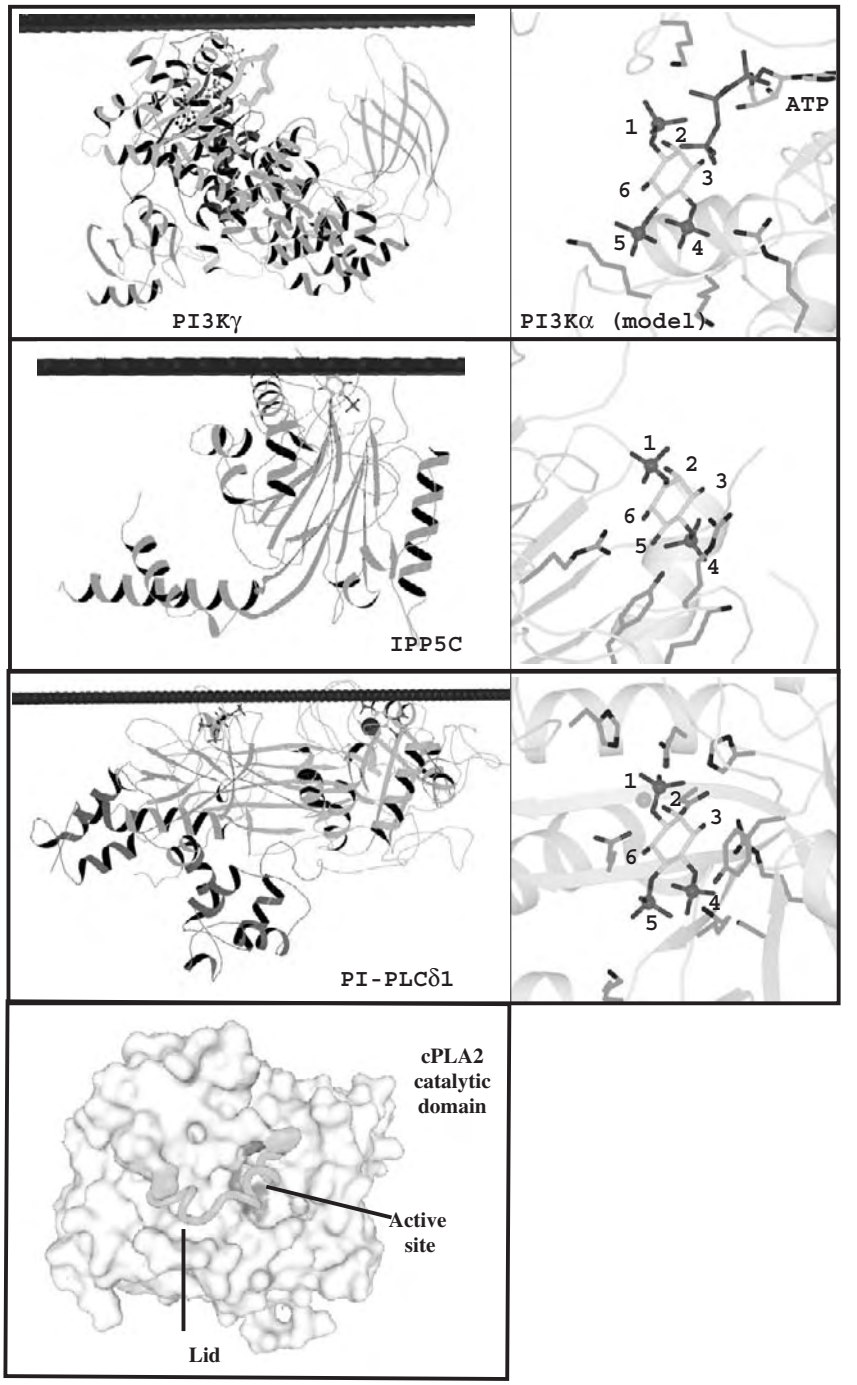


Figure 1 Lipid second messenger recognition by lipid-modifying enzymes. The left panels show the overall folds of the phosphoinositide-modifying enzymes with putative membrane-interacting regions placed in contact with a schematic membrane represented by a layer of spheres. The bound phosphoinositides are shown in stick representation. In the right panels, close-up views of the phosphoinositide/protein interactions are shown. The structures were optimally superimposed on the inositide moieties to present a common view. The molecular surface of $cPLA_2$'s catalytic domain is shown in the lower panel.

phosphoinositide and the protein. The enzymes generally have a lower affinity for the phosphoinositide headgroup than the highest affinity binding modules—as would be expected from the role of an enzyme to preferentially recognize the transition state rather than the substrate or the product.

Phosphoinositide 3-Kinase (PI3K). PI3Ks catalyze the phosphorylation of phosphoinositides at the 3-OH, giving rise to the second messengers PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3. The structure of PI3Kγ, representative of both PI 3- and PI 4-kinases, has a catalytic domain with an

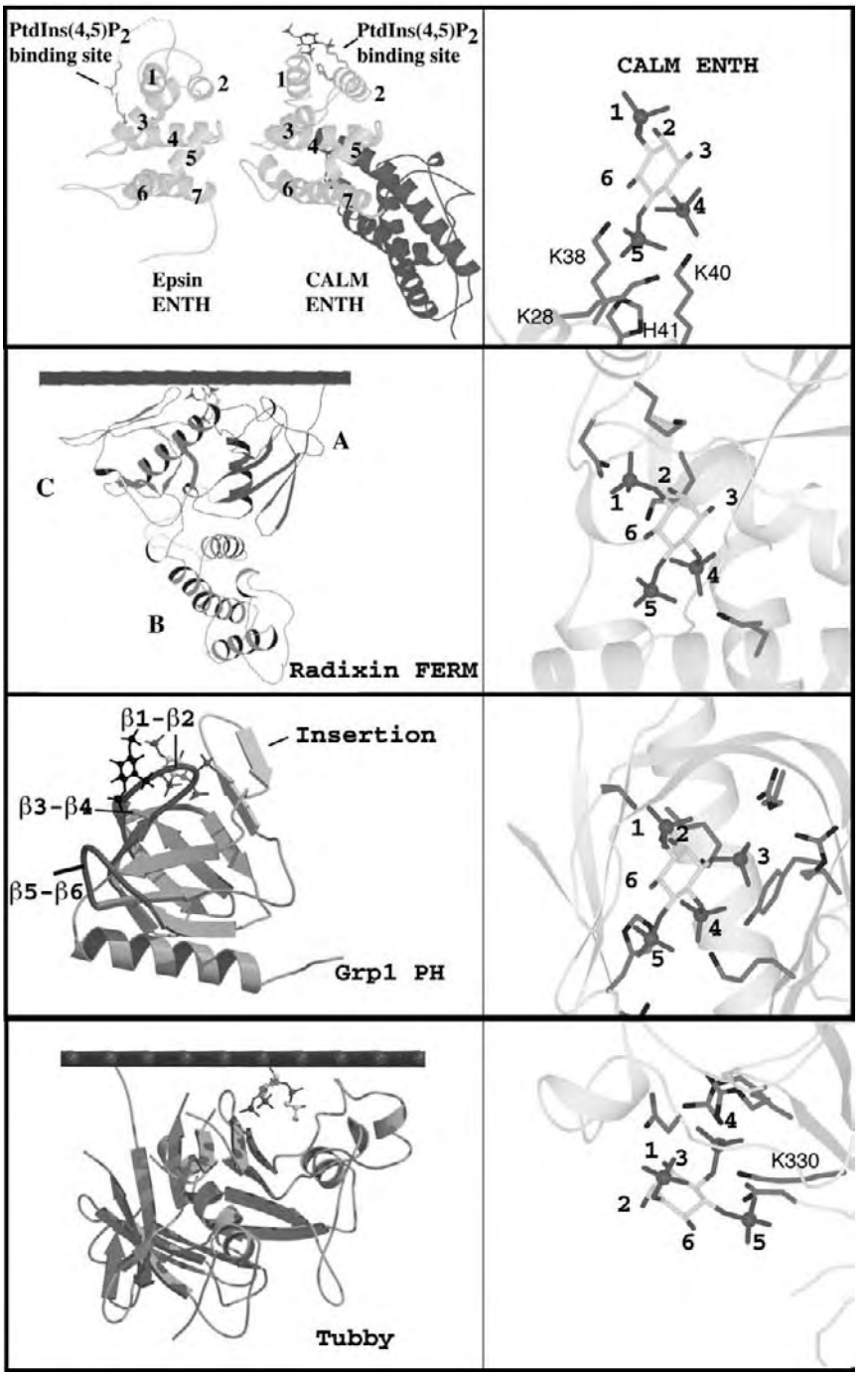


Figure 2 Recognition of polyphosphorylated phosphoinositides by specific binding modules. The representations are as in Fig. 1. In the first pair of panels, the PtdIns(4,5)P_2-binding sites of the epsin and CALM ENTH domains are illustrated. The structurally similar regions of the two domains are colored yellow. Part of the epsin PtdIns(4,5)P_2 site involves N-terminal residues that have been modeled (dotted lines). In the third pair of panels, Ins(1,3,4,5)P_4 bound to the Grp1 PH domain is shown (magenta phosphates). To illustrate the differences in the locations of the phosphoinositide binding pockets in β–spectrin and other PH domains such as Grp1, an Ins(1,4,5)P_3 (black) has been placed on the Grp1 domain in a location analogous to the β-spectrin binding pocket.

N-terminal lobe consisting of a five-stranded β-sheet closely related to protein kinases and a C-terminal lobe that is predominantly helical and more distantly related to protein kinases. The primary determinant of substrate preference for both PI3Ks is a region in the C-terminal lobe analogous to the activation loop of protein kinases [6,7]. Models of substrate binding proposed for PI3Ks place the phosphoinositide in a shallow pocket (Fig. 1) so that the 4- and 5-phosphates interact with basic residues in the activation loop, and the 1-phosphate contacts a Lys in a loop analogous to the

glycine-rich loop of protein kinases (but without glycines in PI3Ks) [2,7]. As with PLCδ1, the location of the active site and accessory domains for membrane binding suggest that the enzyme interacts with the membrane in such a manner that substrate lipids do not have to be removed from the lipid bilayer (Fig. 1).

Phosphatidylinositol Phosphate 4- and 5-Kinases (PIPkins). PtdIns(4,5)P_2-mediated signal transduction is essential for cytoskeletal organization and dynamics, membrane trafficking, and apoptosis. Synthesis of PtdIns(4,5)P_2 is catalyzed by PIPkins [8]. The type IIβ PIPkin has an N-terminal lobe with a seven-stranded antiparallel β-sheet structurally related to protein kinases and a C-terminal lobe consisting of a smaller five-stranded β-sheet [1]. The PIPkins have a requirement for phosphorylated phosphoinositides due to a cluster of four conserved, basic residues in a putative phosphoinositide-binding pocket. The binding pocket is surprisingly shallow and open and suggests that there are few or no contacts with the 2- and 3-OH of the headgroup. The specificity of the enzyme for PtdIns(4)P *versus* PtdIns(5)P is completely dictated by a loop in the C-terminal lobe analogous to the activation loop of PI3K and protein kinases, and a single point mutation in this loop can swap the specificity [9].

PTEN, a 3-Phosphoinositde Phosphatase. Essential to any signal transduction system is a mechanism to produce second messengers and a mechanism to eliminate them. PTEN has a critical role in cells to antagonize the action of PI 3-kinases by catalyzing the dephosphorylation of the 3-phosphate from PtdIns(3,4,5)P_3. The structure of PTEN has a fold and active site configuration similar to the dual-specificity protein phosphatases [3]. A model for substrate binding places His 93 and Lys 128 as ligands of the 5-phosphate [3]. Although the 4-phosphate is deeply buried in the PTEN active site, there is no basic residue present with which it would associate. This is consistent with the ability of the enzyme to dephosphorylate PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3. The 3-phosphate is also deeply buried, but, consistent with the presence of the scissile bond on this group, there is a basic residue, Arg 130, interacting with it.

Inositol Polyphosphate 5-Phosphatase (IPP5P). IPP5P plays an essential role in signaling by utilizing both inositol phosphates and phosphatidylinositol polyphosphates as substrates. The 5-phosphatases regulate the levels of both the soluble Ins(1,4,5)P_3 and the membrane-resident PtdIns(4,5)P_2. The structure of the catalytic domain of the synaptojanin IPP5P from *S. pombe* bound to the product of the reaction, Ins(4)P, shows an active site located at the bottom of a funnel-shaped depression containing the histidine essential for catalysis [4]. The catalytic mechanism is closely related to those of nucleases such as DNase I and DNase III. The 4-phosphate of the Ins(4)P interacts with three basic groups in the active site and makes water-mediated interactions with the divalent metal co-factor (Fig. 1). The product of the reaction binds in a catalytically nonproductive manner with the 4-phosphate remote from the catalytic histidine, thus showing why this family of enzymes is not able to use Ins(1,4)P_2 as a substrate.

Phosphoinositide-specific Phospholipase C (PI-PLC). PtdIns(4,5)P_2 is hydrolyzed by PI-PLC. The catalytic domain of the mammalian PLCδ1 consists of a $(\beta/\alpha)_8$ barrel [5], a common architecture for enzymes in general. Principles of PtdIns(4,5)P_2 headgroup recognition by PI-PLC have been inferred from a complex of PLCδ1 with the product of the reaction, Ins(1,4,5)P_3. With the exception of the 6-OH of the headgroup, all of the hydroxyls of the bound inositide are stereospecifically recognized by the enzyme. The PtdIns(4,5)P_2 headgroup lodges edge-on in the binding pocket with the 3-OH at the bottom and the 1-OH at the top. This places the 1-OH at the level of the putative membrane-binding surface, suggesting that the enzyme does not remove substrate from the membrane during the catalytic cycle, similarly to most of the phosphoinositide-recognizing enzymes and binding domains (Fig. 1).

Cytosolic Phospholipase A_2. The phospholipase A_2 (PLA_2) family of enzymes hydrolyzes the *sn-2* bond of phospholipids to generate free fatty acids and lysophospholipids. The cytosolic PLA_2 ($cPLA_2$) selectively hydrolyzes phospholipids with an *sn-2* arachidonic acid and therefore has a key role in supplying the precursor for eicosanoid biosynthesis. $cPLA_2$ has an N-terminal C2 domain that is important for Ca^{2+}-dependent membrane translocation and a catalytic domain. The enzyme has a central β-sheet with an active-site nucleophile located in a portion of the structure analogous to the nucleophilic elbow of other phospholipases having an α/β hydrolase fold [10]. Apart from this feature, however, $cPLA_2$ has a quite divergent fold. Residues in the active site that are buried by a flexible lid accomplish recognition of the substrate. Upon binding to the membrane interface, this lid undergoes a conformational change to expose a wide hydrophobic platform surrounding a funnel-shaped pocket that cradles the substrate (Fig. 1). Even though the structure suggests that the catalytic domain partially penetrates into the hydrophobic portion of the lipid membrane, the cleft leading to the active-site nucleophile is deep enough to require that the substrate be removed from the lipid bilayer [10].

Phosphoinositide-binding Domains

Polyphosphorylated Phosphoinositide-binding Domains

ENTH Domain. Several proteins involved in endocytosis have an N-terminal domain of about 140 residues known as the ENTH domain, which is necessary for binding to PtdIns (4,5)P_2. The ENTH domains of CALM [11], AP180 [12], and epsin [13,14] consist of helices wound into a solenoid reminiscent of other helical domains such as armadillo and TPR. The PtdIns(4,5)P_2 binding sites in the CALM and epsin ENTH domains differ significantly (Fig. 2). The unique

binding site of the CALM ENTH domain is on an exposed surface with the PtdIns(4,5)P_2 headgroup poised at the tips of three lysines (K28, K38, K40) and a histidine (H41) in helices α1 and α2 and the loop between them (Fig. 2). The residues involved in the interaction define a KX_9KX(K/R)(H/Y) motif that is present in other AP180 homologues but not in epsin [11]. The binding site in epsin involves basic residues in helices α3, α4 and a disordered N-terminal region that changes conformation upon lipid binding (Fig. 2) [14]. As was observed for PH domains, similarity in domain fold does not imply that the same region of the fold is used to interact with phosphoinositides.

The FERM Domain. The FERM domain is found in the ezrin/radixin/moesin (ERM) family of proteins as well as in talin, the erythrocyte band 4.1 protein, several tyrosine kinases and phosphatases, and the tumor suppressor merlin. Members of the ERM family of proteins have three structural domains, and the N-terminal FERM domain binds to PtdIns(4,5)P_2-containing membranes. Phospholipid binding is masked by an intra or intermolecular interaction between the C-terminal domain and the FERM domain. The FERM domain consists of three compact modules, A, B, and C [15–17]. Although the C module has an overall fold similar to PH domains that are known to bind PtdIns(4,5)P_2, the crystal structure of the radixin complex with the Ins(1,4,5)P_3 shows that the phosphoinositide binds between the A and C modules [18]. Two basic residues from the A module interact with the 4- and 5-phosphates and one from the C module interacts with the 1-phosphate (Fig. 2). The binding site is more open than most PtdIns(4,5)P_2 binding sites but less open than the ENTH-type PtdIns(4,5)P_2 binding site. PtdIns(4,5)P_2 binding causes conformational changes in the C module that prevent a self-association with the C-terminal tail of the protein and enable the N-terminal domain to interact with the cytosolic regions of integral membrane proteins. Mutagenesis suggests that the β5-β6 and β6-β7 loops in the C module may constitute a second PtdIns(4,5)P_2-binding site [19,20]. The phosphoinositide binding pocket defines part of a basic surface that is likely to be juxtaposed to the lipid bilayer, leaving an acidic groove between subdomains B and C free to interact with integral membrane adhesion proteins (Fig. 2) [18].

Tubby C-terminal DNA-binding Domain. A common feature among the tubby family proteins is the presence of a C-terminal DNA-binding domain with a unique fold consisting of a 12-stranded antiparallel β-barrel and a hydrophobic helix running through the barrel [21]. PtdIns(4,5)P_2 binding to the C-terminal domain causes Tubby to be localized to the plasma membrane until the levels of PtdIns(4,5)P_2 fall in response to receptor-mediated activation of PLC-β [21]. Loss of plasma-membrane localization is accompanied by nuclear translocation of the protein. The complex of the C-terminal domain of Tubby with glycerophosphoinositol 4,5-bisphosphate shows the PtdIns(4,5)P_2 headgroup in a shallow pocket that involves residues from three adjacent β-strands [21] and is located at one edge of the putative DNA-binding surface. The side chain of a single Lys (330) intercalates between the 4- and 5-phosphates in a manner that is unique to Tubby and the CALM-N ENTH domains (Fig. 2). In these domains, Lys side chain approaches the 4- and 5-phosphates approximately parallel to the plane of the inositol ring. In Tubby, the Lys makes an unusually close (2.1 Å) contact with the 5-phosphate. An additional Arg that coordinates the 4-phosphate is also positioned so that 3-phosphorylated lipids could interact with it, which may account for the PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 binding observed *in vitro* [22].

PH Domains. PH domains are among the most common phosphoinositide-binding modules present in mammalian genomes and show a wide range of phosphoinositide affinities and specificities. They consist of two orthogonal β-sheets curving to form a barrel-like structure closed off by a C-terminal α-helix [8,23]. High-affinity binding to phosphoinositides is achieved using residues in the β1-β2 (VL1), β3-β4 (VL2), and β6-β7 (VL3) loops. The PtdIns(4,5)P_2-specific PH domain of PLC-δ1 [24] differs from other PH domains in that the orientation of the bound inositide is flipped by 180° so that the position occupied by the 5-phosphate in PLC-δ1 is occupied by the 3-phosphate in the 3-phosphoinositide-specific PH domains that have been characterized (Fig. 2).

Among PH domains recognizing 3-phosphoinositides, three types of specificities are apparent: PtdIns(3,4,5)P_3-specificity such as the PH domain of Grp1 and Btk, dual PtdIns(3,4,5)P_3/PtdIns(3,4)P_2-specificity such as the PH domain of DAPP1 and PKB and PtdIns(3,4)P_2-specificity such as the C-terminal PH domain of TAPP1. The PtdIns(3,4,5)P_3 specificity is achieved by enveloping the 5-phosphate by using insertions in either the β6-β7 loop (as in GRP1 [25,26]) or in the β1-β2 loop (as in Btk, [27]). DAPP1 makes more interactions with the 4-phosphate while the 5-phosphate is largely exposed. The PtdIns(3,4)P_2 specificity of the TAPP1 PH domain arises from steric clashes of the 5-phosphate with residues in the β1-β2 loop [28]. The analogous region of the closely related DAPP1 PH domain has a Gly that makes space to accommodate the 5-phosphate of PtdIns(3,4,5)P_3. Basic and hydrophobic residues in the β1-β2 loop of Grp1 and Btk suggest that these PH domains may have additional, nonspecific interactions with lipid bilayers that enhance membrane avidity (Fig. 2) [26].

Other modes of phosphoinositide binding have been shown for PH domains. The PH domain of β-spectrin uses the β5-β6 loop and the side of the β1-β2 loop opposite that used by PLC-δ1 to interact with Ins(1,4,5)P_3 [29], showing that the same fold can be adapted to several different binding modes (Fig. 2). The PH domain from β-spectrin is an example of a PH domain with low affinity and little specificity for lipid binding. More recent analyses of the genome suggest that this may be characteristic of the vast majority of PH domains [23].

PtdIns(3)P-binding Domains

PtdIns(3)P is present in mammalian cells at fairly high concentrations relative to such transient lipid second

messengers as PtdIns(3,4,5)P_3. Its distribution in cells is restricted mainly to endosomal membranes. PtdIns(3)P levels can increase rapidly during certain processes such as receptor-mediated phagocytosis [30]. Two structurally unrelated domain types, FYVE and PX, are capable of specifically binding PtdIns(3)P [31].

FYVE Domains. The FYVE domains are found in many proteins involved in membrane transport [32]. The FYVE domains from Vps27 [33], Hrs [34], and EEA1 [35,36] consist of two small β-sheets stabilized by two Zn^{2+} ions and a C-terminal α-helix. The PtdIns(3)P forms hydrogen bonds with the protein by using the 1- and 3-phosphates and the 4-, 5-, and 6-OH groups [36]. The close approach of these hydrogen-bonding partners precludes polyphosphorylated phosphoinositides from binding (Fig. 3). The 3-phosphate forms a hydrogen bond with the last arginine in the (R/K) (R/K)HHCR signature motif characteristic of the FYVE domains. The 1-phosphate interacts with the protein *via* the first Arg of this motif. Like the PH domains, the FYVE domain buries only one face of the bound phosphoinositide. For EEA1, the face with the axial 2-OH is exposed to solution. The presence of the coiled-coil region preceding the EEA1 FYVE domain helps to unambiguously define the mode of membrane interaction and suggests that a loop flanking the PtdIns(3)P pocket, the "turret" loop, penetrates into the lipid bilayer (Fig. 3). Biophysical measurements indicate that this partial membrane penetration follows rather than precedes specific PtdIns(3)P binding [37].

PX Domains. PX domains are found in a wide range of proteins including many involved in lipid modification, intracellular signaling, and vesicle trafficking [38]. They consist of a three-stranded β-sheet subdomain and an α-helical subdomain that are joined by a conserved RR(Y/F) motif [39,40]. The structure of the PX domain from the p40 cytosolic subunit of the NADPH oxidase in a complex with PtdIns(3)P shows that the first Arg from the RR(Y/F) motif has a structural role in the core of the protein, while the second Arg and the Tyr residue interact with the 3-phosphate and the face of the inositide ring, respectively [40] (Fig. 3). The PX domain buries the face of the inositide adjacent to the axial 2-OH, leaving the opposite face largely exposed. The mode of membrane binding of the PX domain is suggested by the diacylglycerol moiety of the bound PtdIns(3)P and hydrophobic residues adjacent to the phosphoinositide binding pocket (Fig. 3).

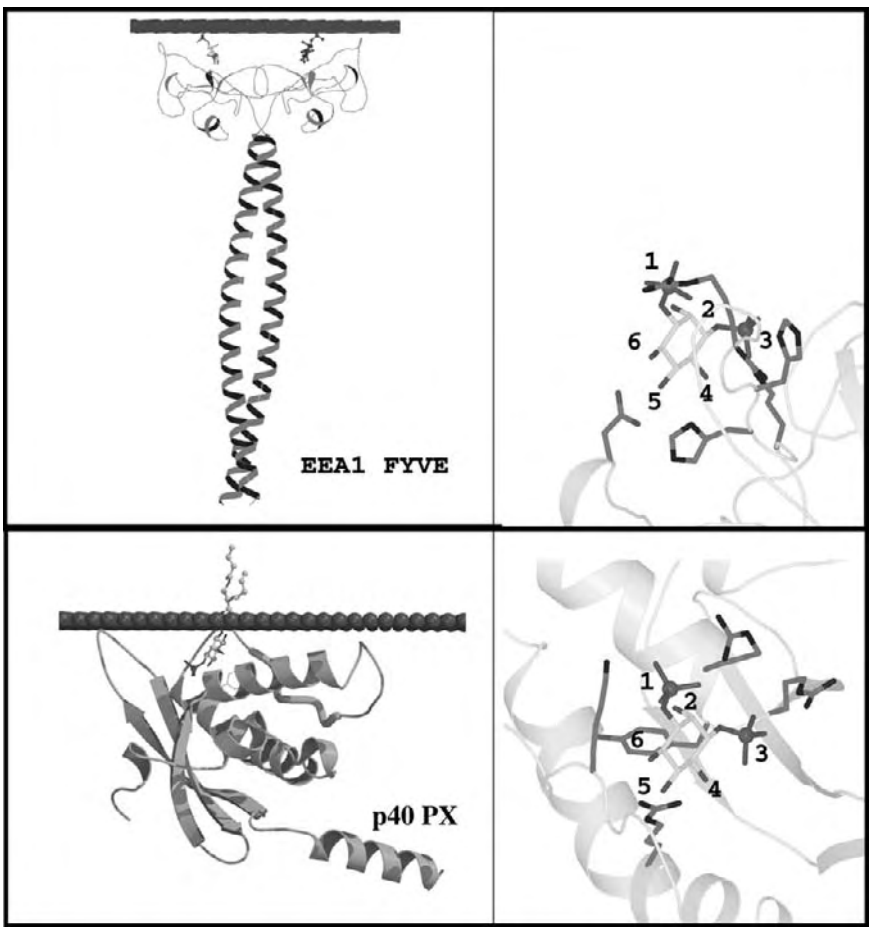


Figure 3 PtdIns(3)P recognition by specific binding modules. The representations are as in Fig. 1

Non-phosphoinositide Lipid Messenger Recognition

C1 Domains. The C1 domain is essential for membrane localization and activation of many proteins involved in signal transduction, including the protein kinase C isozymes [41]. The C1 domains are 50-residue modules containing two small β-sheets and a short C-terminal helix. The domains have been classified into two groups, the "typical" domains that fit a profile derived for phorbol ester or diacylglycerol (DAG) binding and the "atypical" domains that do not [42]. The phorbol ester sits in a groove that is formed by a splaying of adjacent β-strands in a sheet [43,44]. Hydrophilic groups on the phorbol ester intercalate between the strands and make backbone interactions with their exposed main-chain atoms. Once the phorbol ester is bound, the entire end of the domain presents a hydrophobic surface that penetrates into the lipid bilayer. Available binding data are consistent with a model in which the DAG fits into the same groove as the phorbol ester, forming hydrogen bonds with the main-chain atoms of the strands using its 3-OH.

Future Directions

Although much progress has been made in defining the nature of the interactions of lipid second messengers with proteins, many questions remain unanswered. Several lipid second messengers have been characterized for which there is no structural information about specific binding modules, e.g. PtdIns(3,5)P_2 and phosphatidic acid. A dimension of response to lipid-messenger recognition that remains largely unexplored is the effect of membrane binding on membrane structure during processes such as formation of multivesicular bodies. Many proteins use multiple weak interactions to bind to membranes in response to lipid second messengers, but an analysis of the energetics of the individual interactions is often lacking. Although membrane translocation in response to lipid second messengers is common, the nature and extent of allosteric responses mediated by membrane interactions are not clear. With methodologies that have emerged in the wake of genomic studies, we can look forward to answers to many of these questions in the near future.

Note Added in Proof

The details of the PtdIns(4,5) P_2-binding site of the epsin ENTH domain were described in the report of Ford, *et al* [45].

Acknowledgments

I apologize to colleagues whose work I was unable to cite given the wide scope of the review and the severe limitations on space. Marketa Zvelebil is thanked for coordinates of the PI3Kα model.

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CHAPTER 150

Pleckstrin Homology (PH) Domains

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Identification and Definition of PH Domains

The 100 to 120-amino acid pleckstrin homology (PH) domain was first named in 1993 [1–3] as a region of sequence similarity that occurs twice in pleckstrin [4] and is shared by a large number of other proteins. Levels of sequence identity between PH domains are generally low, lying between around 10 (or less) to 30 percent, and there is no conserved motif that identifies PH domains. Rather, PH domains are defined by a pattern of sequence similarity that suggests a common fold, and may therefore share structural similarity in the absence of functional relatedness. The majority of PH domain-containing proteins require membrane association for some aspect of their function. These proteins participate in cellular signaling, cytoskeletal organization, membrane trafficking, and/or phospholipid modification. Sequences encoding PH domains occur in some 252 genes in the first draft of the human genome sequence [5], making this the eleventh most populous domain family in humans. PH domains occur in 77 genes in *D. melanogaster*, 71 genes in *C. elegans*, and 27 in *S. cerevisiae* [5]. Understanding the functions of these common domains has therefore been a subject of considerable interest.

The Structure of PH Domains

Structures of 15 different PH domains have been determined by NMR and/or X-ray crystallography [6–19]. At the core of each PH domain is the same seven-stranded β-sandwich of two near-orthogonal β-sheets containing four- and three-strands respectively (Fig. 1). A characteristic C-terminal α-helix (αC) closes off one "splayed" or open corner [20] of the β-sandwich (top in Fig. 1), while three interstrand loops (the most variable in PH domains) close off the opposite splayed corner (abutting the membrane surface in Fig. 1). This core fold has also been seen in several other classes of domain that share no significant sequence similarity with PH domains [21]. These include the phosphotyrosine binding (PTB) domain [22,23], the Enabled/VASP homology 1 (EVH1) domain [24,25], a Ran binding domain [26], and the FERM domain (for band *f*our-point-one, *e*zrin, *r*adixin, *m*oesin homology domain) [27]. The basic β-sandwich structure has been termed the PH domain "superfold" by Saraste and colleagues [28]. The frequent occurrence of this fold probably reflects its adaptability to multiple functions by creating a stable structural scaffold that can bear loops with quite different recognition properties.

Beyond the conserved β-sandwich fold, one characteristic shared by all PH domains of known structure (except the *C. elegans* Unc89 PH domain [7]) is a marked electrostatic sidedness. Each PH domain is electrostatically polarized, with a positively charged face that coincides with the three most variable loops in the PH domain [9,29]. This positively-charged face abuts the membrane in Fig. 1, and its existence provided part of the motivation for initial tests of PH domain binding to (negatively charged) membrane surfaces.

PH Domains as Phosphoinositide-Binding Modules

The Fesik laboratory was the first to point out that PH domains can bind membranes containing phosphoinositides [30]. Specifically, they showed that the N-terminal PH domain

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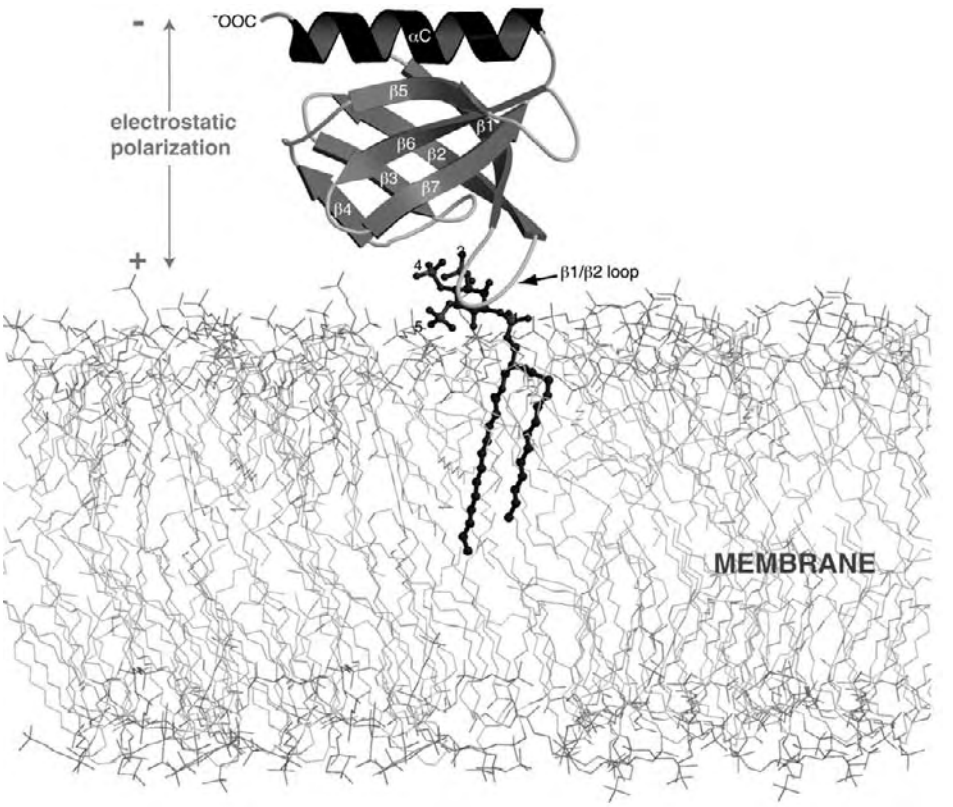


Figure 1 Hypothetical view of how the DAPP1 PH domain binds to PtdIns(3,4,5)P_3 in a membrane. The X-ray crystal structure of the DAPP1 PH domain [11] is shown in a ribbon representation with bound Ins(1,3,4,5)P_4. The β-sandwich structure of the PH domain can be seen, with strands β1 though β4 forming a sheet behind the plane of the paper, and strands 5 through 7 forming a β-sheet in front of the plane of the page. The characteristic C-terminal α-helix (αC) is also labeled (and caps the upper splayed corner of the β-sandwich). The direction of electrostatic polarization of PH domains is depicted schematically on the left. The positive face abuts the membrane in this orientation. A diacylglycerol molecule has been attached to the Ins(1,3,4,5)P_4 molecule to generate a hypothetical view of PtdIns(3,4,5)P_3 bound to the DAPP1 PH domain. The PtdIns (3,4,5)P_3 is embedded in a stick model of a phosphatidylcholine bilayer to guide thinking as to how the PH domain might bind the lipid headgroup in this context. MOLSCRIPT [98] was used to generate this figure.

from pleckstrin binds phosphatidylinositol-(4,5)-bisphosphate (PtdIns(4,5)P_2) with a K_D of approximately 30 μM. NMR analyses demonstrated that the positively charged face of the domain (shown to abut the membrane in Fig. 1) is the site at which the lipid binds [30]. A large number of subsequent studies have shown that phosphoinositide binding is a characteristic shared *in vitro* by nearly all PH domains, and a view has emerged that phosphoinositide binding is a conserved and likely physiologically relevant function for most PH domains [21,31,32]. For several PH domains, phosphoinositide binding has been convincingly demonstrated to be an important (and perhaps the only) function. In these cases the PH domain specifically recognizes the headgroup of a particular phosphoinositide, and this interaction plays an important role in targeting the PH domain-containing protein to cellular membranes [21,33]. However, PH domains in this category are rare. The majority—perhaps over 90 percent of PH domains—bind phosphoinositides with only low affinity and specificity [21,34–36]. How these PH domains participate in membrane targeting (if indeed they do) is not yet clear.

Highly Specific Recognition of Phosphoinositides (and Inositol Phosphates) by PH Domains

PH Domain Binding to Phosphatidylinositol-4,5-Bisphosphate

The phospholipase C-δ_1 (PLC-δ_1) PH domain was the first shown to recognize a specific phosphoinositide with high affinity [37–39]. The PLC-δ_1 PH domain recognizes both PtdIns(4,5)P_2 (which it binds with a K_D of approximately 2 μM) and its isolated soluble headgroup, inositol-(1,4,5)-trisphosphate (Ins(1,4,5)P_3), with which it forms a 1:1 complex (K_D=210 nM) [39]. An X-ray crystal structure of the Ins(1,4,5)P_3/PLC-δ_1 PH domain complex [10] showed that the three variable loops on the positively-charged face of the PH domain form the PtdIns(4,5)P_2/Ins(1,4,5)P_3 binding site. The detailed structure of this binding site also provided clear explanations for the strong Ins(1,4,5)P_3-specificity of the PLC-δ_1 PH domain (it binds Ins(1,4,5)P_3 at least 15-fold more strongly than any other inositol polyphosphate). When expressed as a green fluorescent

protein (GFP) fusion, or analyzed by indirect immunofluorescence, the PLC-δ_1 PH domain shows clear plasma membrane localization [40–43]. GFP fusion proteins of this PH domain have been used to identify the location of PtdIns(4,5)P_2 in living cells, and to monitor PtdIns(4,5)P_2 dynamics and/or Ins(1,4,5)P_3 accumulation in response to different agonists [41–45].

RECOGNITION OF PHOSPHATIDYLINOSITOL 3-KINASE PRODUCTS

Following the realization that some PH domains recognize specific phosphoinositides, it was found that protein kinase B (PKB, also known as Akt), a serine/threonine kinase with an N-terminal PH domain, is a downstream effector of phosphatidylinositol 3-kinase (PI 3-kinase) [46,47]. Mutations in the PKB PH domain prevent its PI 3-kinase-dependent activation, indicating that the PH domain itself plays a critical role in this step [47]. The PKB PH domain specifically recognizes both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, the major products of agonist-stimulated PI 3-kinase, but does not bind strongly to PtdIns(4,5)P_2 or other phosphoinositides [48–50]. As discussed elsewhere in this volume, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are all but undetectable in quiescent cells but accumulate transiently in the plasma membrane following stimulation of cells with a variety of agonists (to an estimated local concentration of 150 μM [51]). A PH domain that fails to bind PtdIns(4,5)P_2 (present constitutively in the plasma membrane), but which binds strongly to PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2, will be recruited to the plasma membrane specifically when these PI 3-kinase-generated lipid second messengers are present. The PH domain from PKB has these binding characteristics, and can be shown (as a GFP fusion protein) to be recruited efficiently to the plasma membrane of mammalian cells following growth factor stimulation [52,53]. As discussed elsewhere in this volume, once recruited by its PH domain to PI 3-kinase products at the plasma membrane, PKB is activated at this location by phosphorylation at two sites [54]. One phosphorylation event is performed by a serine/threonine kinase named PDK1 (for phosphoinositide-dependent kinase-1), which also has a PH domain that can recruit it to the plasma membrane in a PI 3-kinase-dependent manner [55,56].

Other PH domains that specifically recognize PI 3-kinase products include that from Bruton's tyrosine kinase (Btk) [34,57–59] and the PH domain from the Arf-guanine nucleotide exchanger Grp1 (general receptor for phosphoinositides-1) [60]. Both of these PH domains bind exclusively (and strongly) to PtdIns(3,4,5)P_3 or its headgroup Ins(1,3,4,5)P_4 [35,59,60]. A point mutation (at arginine-28) in the Btk PH domain, which leads to agammaglobulinemia in humans and mice [61,62], abolishes PtdIns(3,4,5)P_3/Ins(1,3,4,5)P_4 binding [34,57,58]. The effects of this Btk mutation on B-cell signaling provided the first clue that PH domains may play a role in signal transduction. Like the PKB PH domain, the Btk and Grp1 PH domains are recruited directly to the plasma membrane upon PI 3-kinase activation [53,63–65].

Skolnik and colleagues identified more than 12 different PH domains capable of driving PI 3-kinase-dependent plasma membrane recruitment using a novel yeast-based assay [66]. Where studied, each of these PH domains binds *in vitro* to PtdIns(3,4,5)P_3 (or Ins(1,3,4,5)P_4) with a K_D in the 10–100 nM range, and selects for PtdIns(3,4,5)P_3 over PtdIns(4,5)P_2 by a factor of 20 or more [21]. PH domains in this group share a sequence motif centered around the β1/β2 loop that links the first two β-strands of the PH domain sandwich. Several crystal structures of PH domains bound to Ins(1,3,4,5)P_4 have shown how this motif defines a specific binding site for the PtdIns(3,4,5)P_3 [6,11,67] (Fig. 2). The structural details of the binding site are remarkably well conserved across different structures and bear a strong resemblance (in structure and sequence) to the Ins(1,4,5)P_3 binding site of the PLC-δ_1 PH domain. The sequence motif identified by Skolnik and colleagues [66] serves as a strong and reliable predictor of which PH domains specifically recognize PI 3-kinase products.

PTDINS(3,4,5)P_3 VERSUS PTDINS(3,4)P_2

Among the PH domains with the sequence motif shown in Fig. 2, some bind equally well to both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 (e.g. the PKB and DAPP1 PH domains) while others bind only PtdIns(3,4,5)P_3 (e.g. the Grp1 and Btk PH domains). PH domains that recognize only PtdIns(3,4,5)P_3 tend either to have extended β1/β2 loops or (as in the Grp1 PH domain) insertions elsewhere in the structure that can make specific contacts with the 5-phosphate group. In the complex between the DAPP1 (dual-specific) PH domain and Ins(1,3,4,5)P_4 there are no hydrogen bonds between PH domain side chains and the 5-phosphate group [11], providing one explanation for why this PH domain binds equally well to Ins(1,3,4,5)P_4/PtdIns(3,4,5)P_3 and Ins(1,3,4)P_3/PtdIns(3,4,5)P_2.

There is no currently known PH domain that binds exclusively to PtdIns(3,4)P_2. Alessi and colleagues identified the C-terminal PH domain from TAPP1 (for *ta*ndem *P*H domain-containing *p*rotein-1) as a PH domain that prefers PtdIns(3,4)P_2 over other phosphoinositides according to protein-lipid overlay studies [68]. However, Ferguson *et al.* [11] showed clearly that this PH domain (called AA054961 in that study) binds with high affinity to the headgroups of both PtdIns(3,4)P_2 *and* PtdIns(3,4,5)P_3. In several other assays, using isolated headgroups and intact lipids, it has been shown that the C-terminal TAPP1 PH domain does prefer PtdIns(3,4)P_2, but binds to this phosphoinositide only four-fold more strongly than to PtdIns(3,4,5)P_3 [V. J. Sankaran and M. A. Lemmon, unpublished data]. In spite of this weak selectivity for PtdIns(3,4)P_2 over PtdIns(3,4,5)P_3, Alessi and colleagues have provided some evidence to suggest that the TAPP1 PH domain is recruited to the plasma membrane *in vivo* when PtdIns(3,4)P_2 production is stimulated but not when PtdIns(3,4,5)P_3 is thought to accumulate without PtdIns(3,4)P_2 production [69]. Whether other proteins exist that are regulated exclusively by PtdIns(3,4)P_2 remains to be seen.

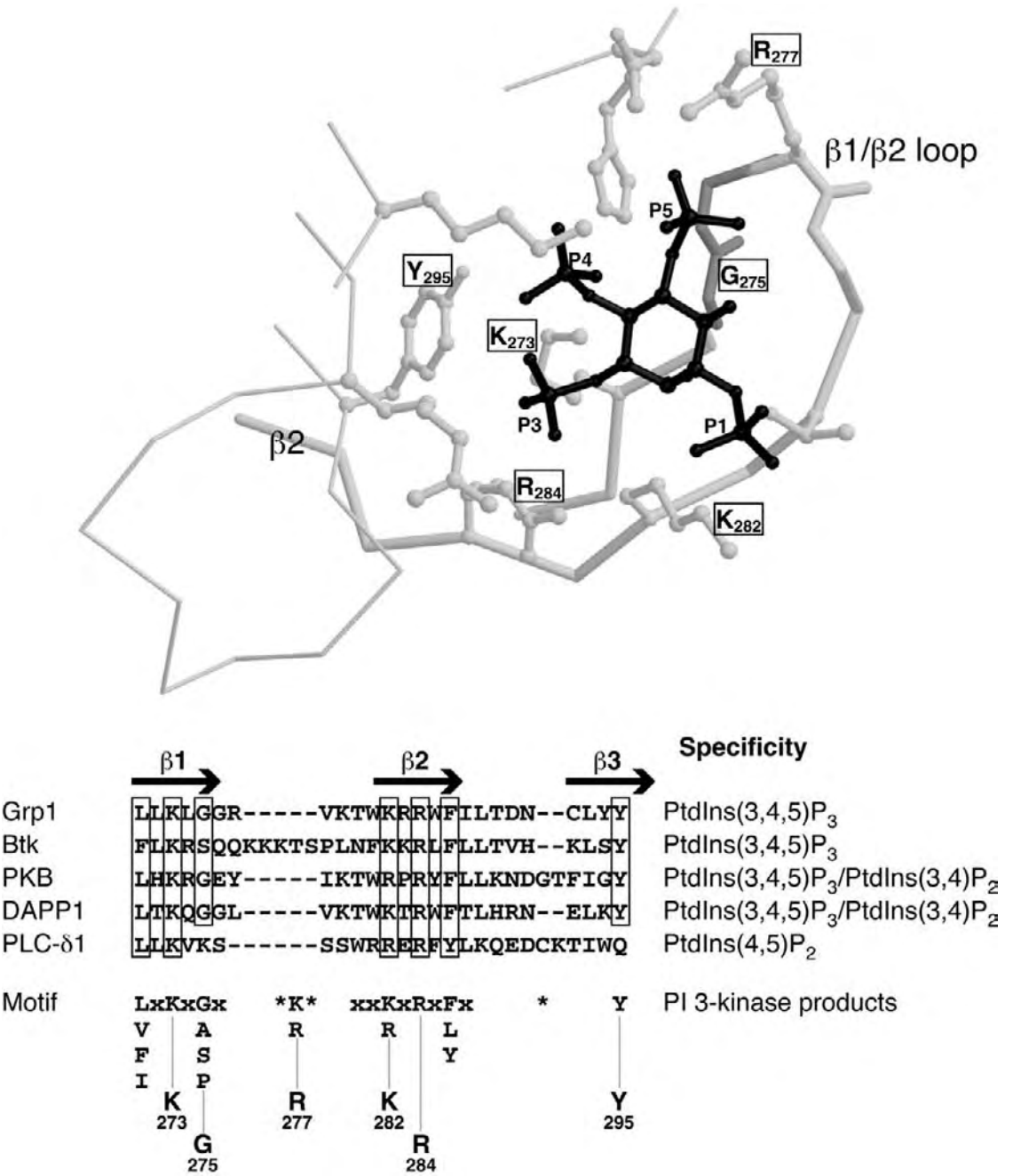


Figure 2 Close-up view of Ins(1,3,4,5)P_4 in the binding site of the Grp1 PH domain, depicting the side chains of residues in the sequence motif that predicts PI 3-kinase product specificity. The β1/β2 loop of the Grp1 PH domain is shown to "cradle" the Ins(1,3,4,5)P_4 molecule, with several residues marked forming side-chain hydrogen bonds with the bound lipid headgroup. The motif that predicts specificity for PI 3-kinase product binding is shown in the lower part of the figure, imposed upon the sequences of the N-terminal portions of the Grp1, Btk, PKB, and DAPP1 PH domains. The motif positions corresponding to the residues highlighted in the structural figure are also shown. K273, in strand β1 of Grp1, forms a hydrogen bond with both the 3- and 4-phosphates of Ins(1,3,4,5)P_4. G275 must be small in order to allow space for the inositol ring in this binding configuration. K282 forms a hydrogen bond with the Ins(1,3,4,5)P_4 1-phosphate. R284 forms a critical hydrogen bond with the 3-phosphate. This is equivalent to the arginine at which mutations in Btk cause agammaglobulinemias. Y295, in strand β3, is a conserved feature of PH domains that bind PI 3-kinase products, and its side chain forms a hydrogen bond with the 4-phosphate group. These 5 motif characteristics are conserved in all PH domains that recognize PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2, and several (but not all) are conserved in PtdIns(4,5)P_2 binding by the PLC-δ_1 PH domain [10]. Equivalents to the additional interaction of R277 with the 5-phosphate of Ins(1,3,4,5)P_4 are seen only in PtdIns(3,4,5)P_3-specific PH domains (Grp1 and Btk), and not those that also bind PtdIns(3,4)P_2 [11]. The extra long β1/β2 loop of the Btk PH domain contributes to 5-phosphate interactions, as does the β6/β7 loop insertion (not shown) of the Grp1 PH domain.

PH DOMAINS WITH OTHER PHOSPHOINOSITIDE-BINDING SPECIFICITIES

Dowler *et al*. [68] recently identified several PH domains that appear from protein-lipid overlay assays to have novel phosphoinositide specificities. Their strategy was to identify PH domains with sequences that match (or closely resemble) the PI 3-kinase product-binding motif presented in Fig. 2 and to assess phosphoinositide-binding specificity. The C-terminal TAPP1 PH domain was identified as a target for PI 3-kinase products with this approach, as were examples of PH domains that appear in overlay assays to recognize PtdIns-4-P, PtdIns-3-P, or PtdIns(3,5)P_2 specifically. It should be stressed that these phosphoinositide-binding specificities have not yet been confirmed via quantitative approaches *in vitro* or with localization studies *in vivo*, and that several of them appear from surface plasmon resonance (SPR) studies to have rather low affinities [68]. As well as PH domains with apparently novel specificities, Dowler *et al*. found several PH domains (with sequences related to the motif in Fig. 2) that interact with all phosphoinositides tested [68]. These results argue that the PtdIns(3,4,5)P_3-specific binding site depicted in Fig. 2 can be "remodeled" with only a handful of mutations to generate binding sites that instead recognize only the PtdIns(4,5)P_2 headgroup (the PLC-δ_1 PH domain), or perhaps only the PtdIns-4-P, PtdIns-3-P or PtdIns(3,5)P_2 headgroup. Remodeling of a different nature can alternatively generate a binding site that accommodates any phosphoinositide headgroup, so that binding is promiscuous.

Nonspecific Phosphoinositide Binding by PH Domains: The Majority Occupation?

Although this fact may not be immediately clear from a reading of the PH domain literature, by far the majority (>90 percent) of PH domains do *not* have a sequence that significantly resembles the motif shown in Fig. 2. Nonetheless, most PH domains lacking the motif do appear capable of phosphoinositide binding, although binding is weak and nonspecific in almost every case [21,35,36]. Where K_D values have been reported for phosphoinositide binding by this class of PH domains, they have ranged from around 30 μM to 4 mM or weaker [12,13,30,34,36,57,70–73]. In one case, that of the β-spectrin PH domain, a crystal structure of the PH domain with a weakly bound Ins(1,4,5)P_3 was reported [13]. Ins(1,4,5)P_3 binds to the surface of this electrostatically polarized PH domain, in the center of its positively charged face. NMR studies have similarly located the site of weak phosphoinositide binding in other PH domains to the variable loops on the positively-charged face [30,57,73,74], most likely driven by delocalized electrostatic attraction to the negatively charged ligand.

Although the physiological relevance of specific, high-affinity, phosphoinositide binding by PH domains has been well established in several cases, it remains unclear in most cases whether weak and promiscuous binding of phosphoinositides to the majority of PH domains plays any physiological role. It has been shown that the low-affinity, nonspecific binding of phosphoinositides to the PH domain of dynamin is essential for this protein's function in receptor-mediated endocytosis [75–77]. Similarly, the low affinity (and usually promiscuous) binding of PH domains from Dbl-family members to phosphoinositides [72] appears to be critical for their Rac/Rho exchange activity *in vivo* and their ability to transform cells [78–81]. Despite intensive study, and three crystal structures of DH/PH fragments from Dbl-family proteins [14,15,17], it remains unclear how low-affinity binding of phosphoinositides to the PH domains of these proteins influences the exchange activity of the adjacent DH (Dbl homology) domain. For many other proteins with PH domains in this class it has been demonstrated that the PH domain is critical for *in vivo* function, but it has not been established whether or not phosphoinositide binding is a physiologically relevant feature of the PH domain. Many more studies are required to address this question for other "promiscuous" PH domains.

Binding of PH Domains to Non-phosphoinositide Ligands

Since the first description of PH domains, many potential protein binding-partners have been reported. The first were βγ-subunits of heterotrimeric G-proteins, which were suggested to bind all PH domains [82] but now appear only to participate in membrane targeting of a small subset, which includes the PH domains from β-adrenergic receptor kinases (βARK's) [83,84]. Other reported protein targets for PH domains include protein kinase C (PKC) isoforms [85,86], the product of the *TCL1* (for T-cell leukemia) oncogene (which binds the PKB PH domain) [87–89], the receptor for activated PKC (RACK1) [90], $G_{12}\alpha$ [91], a protein called BAP-135 (reported to bind the Btk PH domain) [92], filamentous actin [93], acidic motifs found in proteins such as nucleolin (shown to bind the PH domains of IRS1 and IRS2) [94], and several others (reviewed in [21,95]). Although not all of these PH domain/protein interactions have been demonstrated to have physiological relevance, there is no doubt that some do, and that protein binding by PH domains cannot be ignored. Despite the relative wealth of reported protein targets, however, no common themes emerge from the described PH domain/protein interactions. This should not be surprising given the observed diversity in the modes of protein-target recognition by the structurally related EVH1, PTB, and Ran-binding domains [21].

Possible Roles of Non-phosphoinositide PH Ligands

PH domains for which protein targets have been reported include both examples that bind phosphoinositides weakly and promiscuously (e.g. the βARK, IRS-1, and dynamin PH domains), *as well as* PH domains that bind strongly and specifically to particular phosphoinositides (e.g. the Btk and PKB PH domains). It can therefore not be argued that the

protein targets described for PH domains are simply alternatives to, or surrogates for, the well-studied (but rare) specific phosphoinositide ligands. Rather, it appears likely that some PH domains bind multiple ligands.

Cooperation of Multiple Ligands in Membrane Recruitment of PH Domains

A requirement for simultaneous PH domain binding to two different ligands was first demonstrated for membrane targeting by the βARK PH domain [83]. The βARK PH domain binds very weakly to PtdIns(4,5)P_2 ($K_D > 200\,\mu M$) [12]. It also binds rather weakly to the βγ-subunits of heterotrimeric G-proteins [82]. Neither of these weak interactions alone is sufficient for high-affinity targeting of βARK to membranes, but the two interactions can cooperate to recruit βARK efficiently to relevant membrane surfaces [83].

Golgi Targeting of PH Domains by Multiple Ligands

The PH domain from oxysterol binding protein (OSBP), as well as several other related PH domains, is targeted specifically to the Golgi through interactions that appear to require both phosphoinositides and another unidentified (Golgi-specific) component [96]. These Golgi-targeted PH domains, which include those from FAPP1 and the Goodpasture antigen binding protein (GPBP), are highly promiscuous in their phosphoinositide binding (and are not PtdIns-4-P-specific) [34,96], arguing that phosphoinositide recognition alone cannot possibly determine their Golgi targeting. Phosphoinositide binding by these PH domains is several-fold weaker than PtdIns(4,5)P_2 binding by the PLC-δ_1 PH domain ([96] and D. Keleti, V. J. Sankaran, and M. A. Lemmon, unpublished), further suggesting that it may not be strong enough to drive membrane targeting of the OSBP PH domain independently. Studies in a series of yeast mutants have demonstrated that Golgi targeting of the OSBP and FAPP1 PH domains is dependent on PtdIns-4-P and not on PtdIns(4,5)P_2 production [96,97], but that the activity of Arf1p is also important [96]. It is therefore hypothesized that the presence of two binding partners in the Golgi is responsible for specific targeting of the OSBP, FAPP1, and GPBP PH domains to that organelle. On its own, phosphoinositide binding by these PH domains is not strong enough to drive membrane targeting *in vivo*, and would certainly not provide targeting specificity. The second (so far unidentified) target of these PH domains is thought to be Golgi-specific, but does not bind to the PH domains tightly enough to achieve Golgi targeting on its alone. Rather both phosphoinositide and this unknown component must be present in the same membrane (the Golgi) in order to recruit the OSBP and other PH domains to that compartment with high affinity and specificity. According to this model [96], PtdIns-4-P is implicated in Golgi targeting of the OSBP, FAPP1, and other PH domains not because of headgroup recognition, but because this happens to be the most abundant phosphoinositide in the membranes that contain the second PH domain ligand.

Conclusions

The eleventh most populous domain family in humans is now rather well understood structurally and lends its name to the PH domain superfold that includes proteins involved in binding to variety of phosphoinositide and protein ligands. Ligand binding by a small subgroup of PH domains—those that bind phosphoinositide headgroups with high affinity and specificity—is now understood rather well, although it remains possible that some PH domains from this class have additional, as yet unidentified, binding partners. PH domains that do *not* bind phosphoinositides with high affinity or specificity constitute the majority—perhaps 90 percent. The interactions driven by these PH domains are far less well understood. In many cases it even remains unclear whether phosphoinositide binding observed *in vitro* has any relevance *in vivo*. How weak and nonspecific phosphoinositide binding could contribute to membrane binding is a question that has yet to be fully addressed. It may do so though cooperation of multiple ligands that bind to a single PH domain (as discussed for the βARK and OSBP PH domains). Alternatively, the PH domain may be one of several domains within a multidomain protein or oligomer that cooperate with one another in driving membrane targeting. In these cases, specificity of membrane targeting may be defined not by the precise nature of the individual interactions (as with PH domains that bind PI 3-kinase products), but rather by the available combinations of interactions. Recruitment to a specific membrane may require that two or more PH domain targets coexist in that membrane.

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CHAPTER 151

PX Domains

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History and Overview of PX Domains

The Phox (*ph*agocyte *ox*idase) homology (PX) domain, containing ~100 amino acids, was initially identified by sequence profiling as a conserved region present in the C2 domain-containing class of PI 3-kinases and in the N-terminal region of the $p40^{phox}$ and $p47^{phox}$ subunits of the NADPH oxidase [1]. For nearly five years following their discovery, the function of PX domains remained obscure. A conserved polyproline motif conforming to the consensus sequence PXXP, where X denotes any amino acid, was noted within most PX domain sequences. This observation, coupled with the presence of one or more SH3 domains in numerous PX domain-containing proteins (Fig. 1), led to speculation that one function of PX domains might involve binding to SH3 domains [1].

PX domain-containing proteins are found in all eukaryotes from yeast to human, and can be loosely divided into two groups (c.f. Fig. 1). The first group includes a large family of cytoplasmic and/or para-membrane proteins known as sorting nexins (SNX), including SNX1 through SNX11 and SNX17 in higher eukaryotes [2–5], and Vam7p, Vps5p, Mvp1p, and Grd19p in yeast [6–9]. Most sorting nexins contain no other recognizable domain other than the PX domain (Fig. 1). Collectively, all members of the SNX family are believed to be involved in vesicular trafficking (Table I, top). A second group of PX domain-containing proteins all contain one or more domains of known function in addition to the PX domain (Fig. 1). These co-associating domains include protein-protein interaction domains such as SH3 domains, PDZ domains, and RGS domains; protein-lipid binding domains such as C2 domains and PH domains; and catalytic domains such as the lipid kinase domain of PI 3-kinase or the phospholipase domain of Phospholipase D. These additional domains play an important role in defining the functions of their constituent proteins, whose intracellular localization is determined, in part, by the PX domain (Table I, bottom).

Lipid-Binding Specificity and the Structure of PX Domain

The observation that many PX domain-containing proteins were membrane associated suggested that their ligands might be specific phospholipids. This was experimentally verified via protein overlay assays on solid-phase immobilized phospholipids and by solution-phase binding assays using phosphoplipid-containing synthetic liposomes. These experiments demonstrated that the ligands for many PX domains were specific phosphoinositide products of PI 3-kinase [10–13]. Different PX domains show distinct specificity for different phosphoinositides. The PX domains of $p47^{phox}$ and $p40^{phox}$, for example, bound to phosphatidylinositol-3,4-bisphosphate [PtdIns $(3,4)P_2$] and PtdIns(3)P, respectively [12,13]. The PX domains of Vam7p and SNX3 bind specifically to PtdIns(3)P [10,11,14] whereas the PX domain of cytokine-independent survival kinase (CISK) interacts with PtdIns$(3,5)P_2$, PtdIns $(3,4,5)P_3$, and PtdIns$(4,5)P_2$ [15]. It appears that the vast majority of PX domains, however, including all of those in the budding yeast *Saccharomyces cerevesiae*, interact primarily with PtdIns(3)P [16].

A sequence alignment of PX domains that shows specificity for PtdIns(3)P binding, including that of $p40^{phox}$, SNX3, SNX4, MVP1p, Vam7p, and MDM1p, is shown in Fig. 2. Insight into the structural basis of lipid-binding specificity for PX domains is beginning to emerge from a recent NMR structure of the $p47^{phox}$ PX domain without a bound ligand [17], and the X-ray crystal structure of the $p40^{phox}$ PX domain

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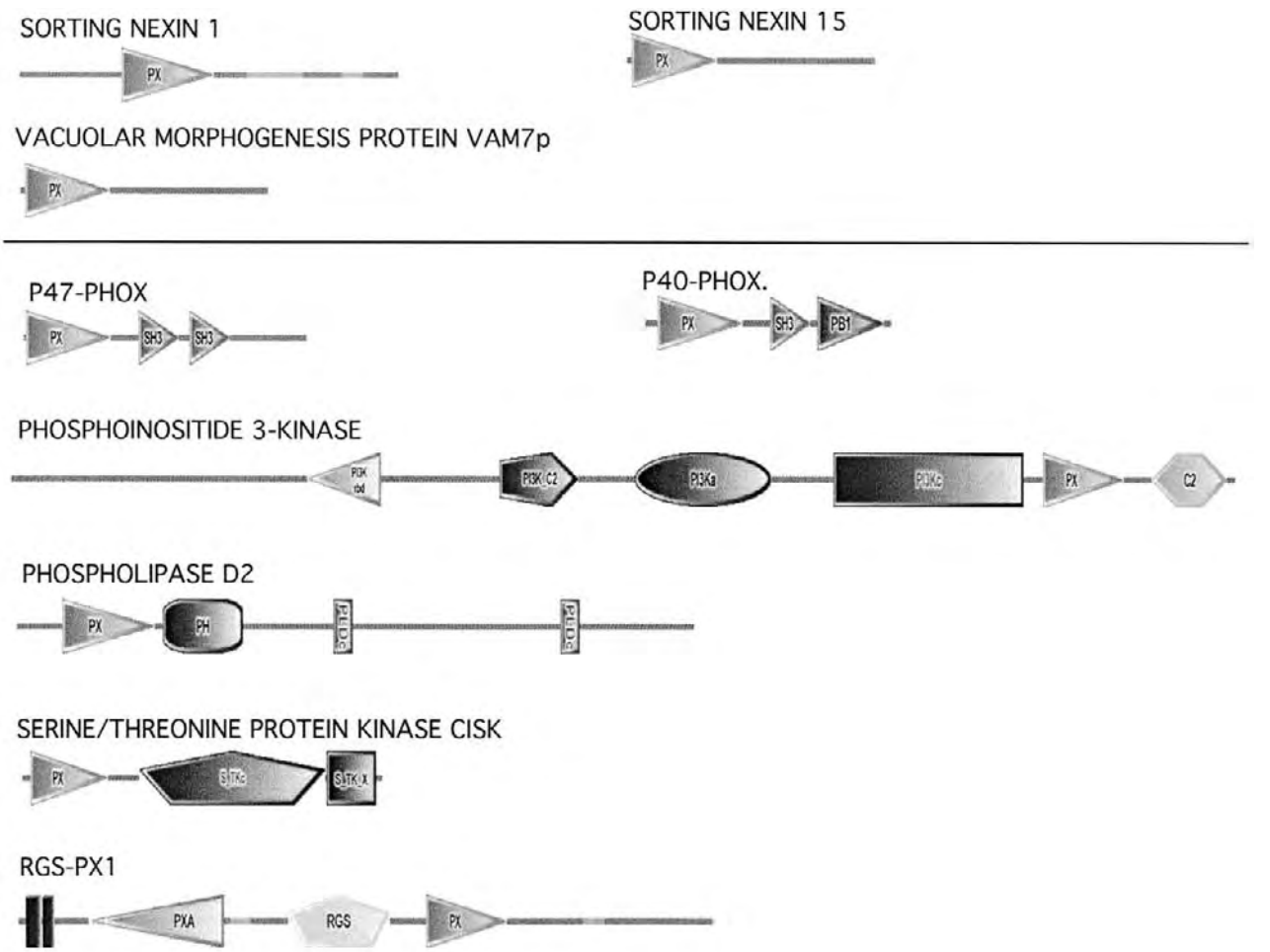


Figure 1 Domain architecture of PX domain-containing proteins. The upper panel shows the structures of sorting nexin proteins and their yeast homologues. The lower panel shows various PX domain-containing signaling molecules that contain additional co-associating domains.

Table I PX Domain-Containing Proteins

Protein	PX domain lipid target	Other domains	Protein function	Reference
SNX1	PtdIns (3)P	—	Binding to EGF receptor Targets EFGR to lysosome	4,7
SNX3	PtdIns (3)P	—	Regulates endosomal function	10
Vps5p/Mvp1p	PtdIns (3)P	—	Sorting carboxypeptidase Y to the vacuole	6
Vam7p	PtdIns (3)P	—	Golgi-to-vacuole transport	11
Grd19p	PtdIns (3)P	—	Retrieval of proteins from prevacuole to late Golgi	9
P40phox	PtdIns (3)P	SH3	Regulation of the NADPH oxidase	12,13
P47phox	PtdIns (3,4)P_2	SH3	Activation of NADPH oxidase	13
FISH	ND	SH3	Tyrosine kinase signaling	29
RGS-PX1	ND	RGS PXA	Gα-specific GAP Involved in vesicle trafficking	30
PLD1,2	ND	PH PLD Kinase	Cell division, signal transduction, and vesicle trafficking	31
Pi3K C2-γ	PtdIns(4,5)P_2	C2 Pi3 Kinase	EGF receptor signaling	14
CISK	PtdIns(3)P	Ser/Thr kinase	Cell survival	15
HS1BP3	ND	Leucine zipper	T and B cell development	32

The top section displays SNX proteins and their yeast homologs. The bottom section displays proteins containing other known modular signaling domains. (ND indicates Not Determined.)

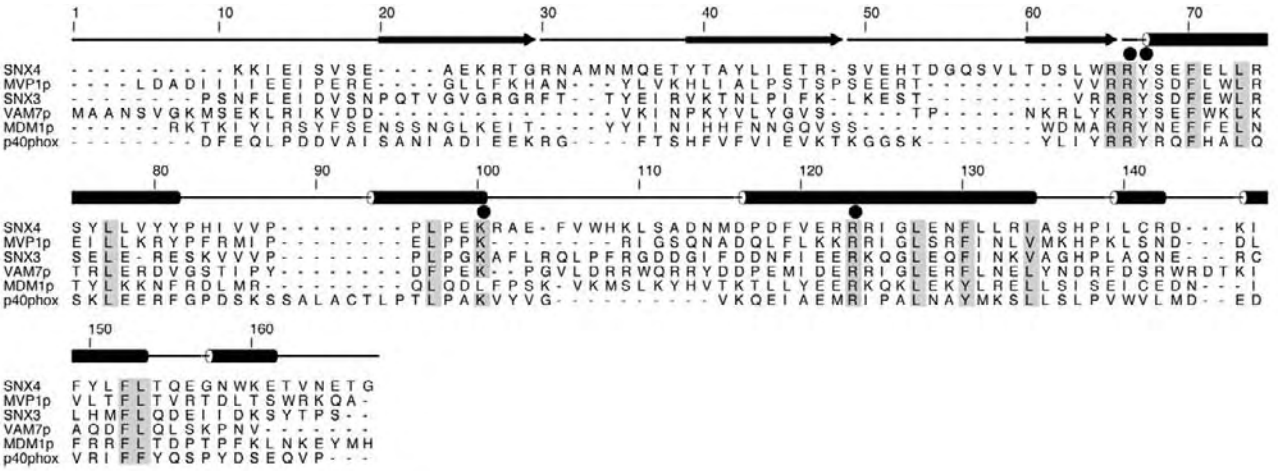


Figure 2 A structure-based sequence alignment of PX domains that bind to PtdIns(3)P. A general numbering scheme is indicated above the alignment, along with a cartoon indicating the positions of β-strands (arrows) and α-helixes (cylinders) based on the structure of the p40^{phox} PX domain. Conserved hydrophobic residues within the core of the domain are shaded in green, basic and aromatic residues that are involved in lipid binding are shaded in cyan and yellow, respectively. Sequence numbers for particular amino acid residues within individual PX domains are mentioned in the text.

bound to PtdIns(3)P [18]. The PX domain fold is a small three-stranded β-sheet packed against a helical subdomain containing four α helices and a short stretch of 3_{10} helix (Fig. 3). Both the p40^{phox} and p47^{phox} PX domain structures also contain a conserved PXXP motif that forms a type II polyproline (PP_{II}) helix. Since type II polyproline helices are well known to bind to SH3 domains, this structural observation suggests that some PX domains may, in fact, form intramolecular interactions with their co-associating SH3 domains.

Many PX domains contain a conserved Arg residue immediately preceding a conserved Tyr residue (Tyr-67 in the sequence alignment shown in Fig. 2). The preceeding Arg residue (Arg-66 in Fig. 2), which corresponds to R58 in the p40^{phox} PX domain, forms two salt bridges with the 3-phosphate of the lipid in the p40^{phox}PX:PtdIns(3)P crystal structure. The PX domain from the C2-containing PI 3-kinase, which binds to PtdIns(4,5)P_2, lacks an Arg residue at this position, suggesting that residues equivalent to R58 are specific to PX domains that bind to 3-phosphorylated phosphatidylinositols. The 4- and 5-hydroxyl groups of PtdIns(3)P form hydrogen bonds with another highly conserved residue corresponding to R105 in the p40phox structure (Fig. 3) and R123 in the alignment shown in Fig. 2. Phosphorylation on either the 4- or 5-hydroxyl would sterically impinge on the R105 side chain, rationalizing the PtdIns(3)P binding specificity of the p40^{phox} PX domain. However, R105 is also conserved in PX domains that bind phosphatidylinositols other than PtdIns(3)P, including those of CISK and p47phox. Presumably, alterations in the loops surrounding this residue relieve this steric clash and may allow direct interactions of R105 with the lipid phosphates in the 4- and 5- positions, in place of the interaction with the 4- and 5- hydroxyl groups seen in the p40^{phox} structure.

Tyrosine-59 in the p40^{phox}PX:PtdIns(3)P crystal structure (corresponding to Y67 in the alignment in Fig. 2) is another highly conserved amino acid, which forms the floor of the lipid-binding pocket through interactions between its aromatic side chain and the inositol ring. In the p40^{phox} PX:PtdIns(3)P structure, the 1-phosphate forms salt bridges with the side chains of K92 (position 100 in Fig. 2) and R60 (position 68 in Fig. 2) to stabilize the interaction between the domain and

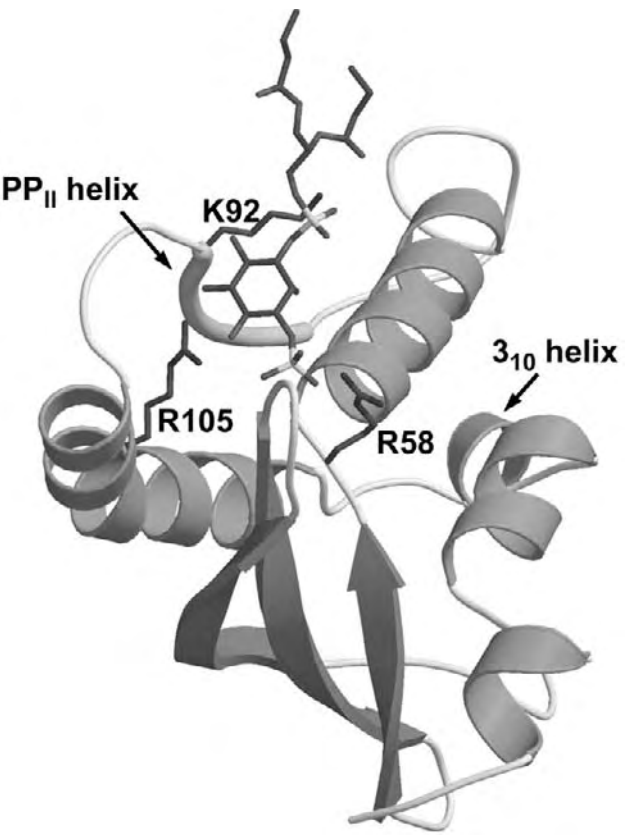


Figure 3 The structure of the p40^{phox}:PtdIns(3)P complex [18]. The lipid is depicted with the acyl chains at the top of the figure, as though it were protruding from a cell membrane. Amino acids that play key roles in PtdIns(3)P-binding are indicated, together with the polyproline-II helix and the 3_{10} helix.

the membrane proximal portion of the inositol lipid. Both of these residues are conserved in some, but not all, PX domains, suggesting that other residues also participate. Additional PX domain structures will clearly be necessary to fully understand the molecular determinants of phosphatidylinositol binding.

Function of PX Domain-containing Proteins

The function of several PX domain-containing proteins is reasonably well understood, although the exact role fulfilled by the PX domain is not yet well defined. The *phox* proteins, after which the PX domain was named, are subunits of the NADPH oxidase, the heme-containing enzyme responsible for superoxide production and killing of microorganisms by phagocytic cells. In resting phagocytes, the inactive NADPH oxidase is separated into a set of cytoplasmic subunits including $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ and a membrane-bound heme-containing flavocytochrome b_{558}, which consists of $gp91^{phox}$ and $p22^{phox}$. Upon phagocyte activation, the cytoplasmic components dock with the membrane-bound subunits to form a catalytically active enzyme that can transfer electrons from NADPH to oxygen to form reactive oxygen species (ROS), such as superoxide [19]. Production of superoxide in response to some stimuli requires the activity of PI 3-kinase, suggesting that specific PI 3-kinase lipid products may be directly involved in regulating oxidase assembly. The different lipid-binding specificities observed for the PX domains of $p47^{phox}$ and $p40^{phox}$ may target oxidase assembly to occur only within specific membrane compartments that contain the appropriate combination of PI 3-kinase-derived lipids [13,20,21].

Several PX domain-containing proteins in the budding yeast *Saccharomyces cerevesiae* participate in vesicular protein trafficking, including the Mvp1p and Vps1p proteins involved in vacuolar protein sorting [22] and the Vps17p and Vps5p proteins which translocate pre-vacuolar endosomes to the Golgi as part of the retromer protein complex [23]. In higher eukaryotes, SNX1, SNX2, SNX4, SNX6, and a splice variant of SNX1 (SNX1A) are known to associate with a variety of growth factor receptors, suggesting that they mediate receptor trafficking to vesicles [7,24].

CISK is a PX domain-containing Ser/Thr kinase, which functions in parallel with Ser/Thr kinase Akt/PKB to mediate IL-3 dependent cell survival in hematopoetic cells. CISK localizes to vesicular compartments, and this localization is dependent on the PX domain [15,25].

Phospholipase D (PLD) catalyzes hydrolysis of phospholipids to produce phosphatidic acid (PA) [26]. Both mammalian isoforms of PLD, PLD1 and PLD2, contain PX domains as well as PH domains that also bind to PI 3-kinase lipid products. The presence of two different lipid binding domains might target the lipase domain to specific phosphoinositide-containing regions of membranes, or might function as some type of lipid-regulated switch to control the activity of the lipase domain. The specificity of the PLD PX domain has not yet been reported.

Class II PI 3-kinases, defined by their *in vitro* usage of phosphatidylinositol and phosphatidylinositol 4-phosphate as substrates, also contain PX domains. The function of Class II PI 3-kinases is not well understood, though recent results suggest that they may participate in clathrin-mediated endocytosis [27,28].

In summary, PX domains join an expanding family of phosphoinositide-binding domains that includes C2 domains, PH domains, FYVE domains, ENTH domains, and tubby domains. The large differences in structure between these domains suggest that their lipid-binding function arose through the convergent evolution of different structures for the same biological function of lipid binding. It will be important for future work to further examine the structural foundation for lipid binding specificity by PX domains, and explore how their lipid-binding ability contributes to the overall function of PX domain-containing molecules.

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CHAPTER 152

FYVE Domains in Membrane Trafficking and Cell Signaling

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Introduction

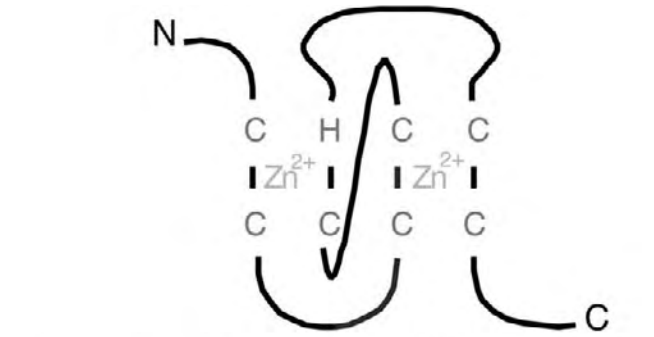
The recruitment of cytoplasmic proteins to specific membrane compartments is important for a diverse spectrum of cellular processes, including intracellular protein trafficking, cytokine and growth factor receptor signaling, actin cytoskeleton organization, and apoptosis [1–4]. Many proteins are localized to membranes through tightly regulated interactions with membrane-associated factors. Derivatives of phosphatidylinositol (PtdIns) that can be reversibly phosphorylated at different positions of the inositol ring are ideally suited for this function. Through the action of a set of well-conserved specific lipid kinases [5], different phosphoinositide (PI) species phosphorylated at the 3′, 4′, or 5′ positions of the inositol headgroup are generated, each of which can recruit or activate a specific subset of cytoplasmic effector proteins. The activity of these target proteins can then be attenuated through the action of PI-specific phosphatases and lipases [6–9]. Several studies have now identified multiple, well-conserved PI-binding motifs, each of which can recognize particular PI isoforms with a high degree of specificity [10]. In this chapter, we discuss the structural basis of a novel zinc finger that binds PtdIns 3-phosphate [PtdIns(3)P], termed the FYVE domain, and the roles played by several proteins harboring this motif in membrane trafficking and cell signaling.

Role for PtdIns(3)P in Membrane Trafficking and Identification of the FYVE Domain

A role for PtdIns(3)P in vesicular transport was first discovered in the study of Golgi to vacuole transport in yeast [11]. *Saccharomyces cerevisiae* expresses one PtdIns 3-kinase isoform, Vps34 [5]. Deletion of the *VPS34* gene resulted in a lack of PtdIns(3)P synthesis and defects in endosomal membrane trafficking from the Golgi and plasma membrane to the lysosome-like vacuole [12]. Likewise, PtdIns (3)P has been shown to play important roles in several membrane trafficking pathways to mammalian lysosomes [13]. The fungal metabolite wortmannin, an inhibitor of PI 3-kinase activity, has been shown to impair homotypic endosome fusion *in vitro* and the transport of enzymes such as cathepsin D to lysosomes *in vivo* [14–16]. Accordingly, the human homolog of the yeast Vps34 PtdIns 3-kinase has been identified and found to be sensitive to wortmannin [5].

Several proteins have been implicated as downstream effectors of PtdIns(3)P in vesicle transport. One of these, mammalian EEA1 (early endosome antigen 1), has been shown to localize to endosomal membranes in a wortmannin-sensitive manner [17,18]. Consequently, deletion of the FYVE domain of EEA1 was shown to diminish its endosomal association, suggesting that this domain may directly bind PtdIns(3)P [19]. The FYVE domain, named after the first four proteins found to contain this motif (*F*ab1, *Y*OTB,

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Fab1	WMK DESSKE CFS C GKT FNTFR RKHH C RI Cx4 Cx2 Cx15 RV CYN CYE
YDR313	WQA DEEAHS CFQ C KTN FSFLV RRHH C RI Cx4 Cx2 Cx27 RT CNE CYD
Vac1	WRD DRSVLF C NI C SEP FGLLL RKHH C RL Cx4 Cx6 Cx32 RL C SH C ID
EEA1	WAE DNEVQN CMA CGKG FSVTV RRHHC RQ Cx4 Cx2 Cx14 RV CDA CFN

Figure 1 The FYVE domain is a conserved RING finger domain. (Top) Schematic cartoon of the RING finger FYVE domain. The conserved cysteine/histidine residues that coordinate two Zn^{2+} atoms are shown. The highly conserved basic patch surrounding the third cysteine is indicated in blue. (Bottom) Sequence alignment of the FYVE domains of Fab1, Pib1 (YDR313c), Vac1, and EEA1. Identical residues are shown in boldface. The conserved cysteine residues are highlighted in gray. The highly conserved basic patch, R(R/K)HHCR, found in all FYVE domains is shown. The conserved hydrophobic region adjacent to the basic patch is indicated in red.

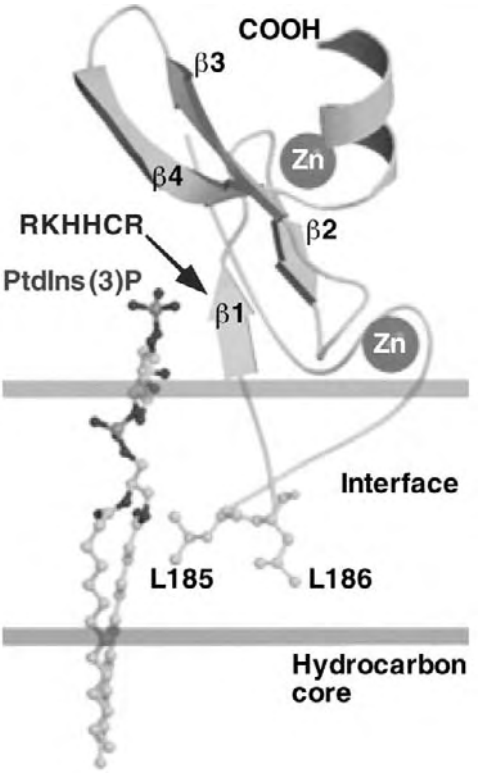


Figure 2 The FYVE domain is a modular PtdIns(3)P-binding motif. The crystal structure of the FYVE domain of yeast Vps27 [26]. The ribbon depicts four β strands followed by a carboxy-terminal α-helix. The two Zn^{2+} atoms are shown. The highly conserved positively charged residues (RKHHCR) in the ß1 strand predicted to make contacts with the 3′-phosphate group of PtdIns(3)P are indicated. In addition, residues in a hydrophobic loop upstream of the first β-sheet predicted to penetrate into the membrane bilayer are indicated [26]. The membrane layer is divided into an interfacial region (lipid headgroups and the hydrophobic interface) and a hydrocarbon core (lipid acyl chains). (Reprinted from Hurley, Cell, 97, 657–666, 1999. With permission).

*V*ac1 and *E*EA1), was originally identified as a RING-finger family member that coordinates two Zn^{2+} ions through eight cysteine/histidine residues spaced in a conserved manner [$CX_2CX_{9-39}CX_{1-3}(C/H)X_{2-3}CX_2CX_{4-48}CX_2C$] (Fig. 1) [19,20]. An important finding of subsequent studies was the demonstrated ability of FYVE domains to specifically bind PtdIns-3-P *in vitro*, as recombinant EEA1 FYVE sedimented with liposomes containing PtdIns(3)P but not other PI species [21–23]. The identification of modular protein domains that bind PtdIns(3)P with high affinity and specificity, such as the FYVE domain, has been a crucial step in further understanding the roles of this lipid in membrane trafficking events, as described in greater detail below.

Structural Basis for the FYVE Domain

Insight into the molecular mechanisms that mediate the interaction between FYVE domains and PtdIns(3)P is provided by several structural studies on this motif [24,25]. The FYVE domain, as mentioned, is an approximately 80-amino-acid sequence containing eight conserved cysteine/histidine residues that coordinate two Zn^{2+} ions. In addition, several other residues are conserved, most notably a highly basic R(R/K)HHCR patch adjacent to the third cysteine residue, an amino-terminal WxxD motif, and a conserved hydrophobic region upstream of the basic patch (Fig. 1). As first determined from the crystal structure of the yeast Vps27 FYVE domain, the basic patch is localized within β1 of two double-stranded antiparallel β-sheets (composed of β1/β2 and β3/β4), which are stabilized by the two zinc ions and a C-terminal α-helix [26]. Molecular modeling suggested that the inositol head group of PtdIns(3)P fits into a pocket created by the backbone of the first β-sheet, and the 3′-phosphate group contacts side groups of the final histidine and arginine residues found in the basic patch (Fig. 2) [26]. In addition, from this model, the 1′-phosphate of PtdIns(3)P is poised to form a salt bridge with the first arginine in the conserved basic patch [26]. In combination, these interactions are specific for PtdIns(3)P as additional or other phosphate groups on the inositol ring would prohibit interaction with the FYVE domain due to spatial constraints, consistent with previous *in vitro* binding studies indicating this motif does not bind to other phosphoinositides.

Although a similar structure was proposed for the FYVE domain of *Drosophila* Hrs, a homologue of Vps27, a different model for PtdIns(3)P binding was suggested [27]. The major difference involved an anti-parallel association of two Hrs FYVE monomers to generate a homodimer with two ligand-binding pockets. Residues from β1, including the conserved basic patch, together with a hydrophobic strand from β4 of the opposite FYVE monomer, line each pocket.

In addition, this model differed from that of the Vps27 FYVE structure in regard to the orientation of the FYVE domain with respect to the membrane. Thus, even though both models were consistent in regard to the overall structure of the FYVE domain, further studies were required to help resolve the true nature of the interaction between the FYVE domain and PtdIns(3)P containing membranes.

To gain further insight into the interaction of the FYVE domain with PtdIns(3)P, NMR studies of the EEA1 FYVE domain were performed [28,29]. As expected, these studies highlighted the importance of the basic residues found in the first β-sheet as they displayed large chemical shift changes in the presence of PtdIns(3)P. In addition, residues in a hydrophobic loop upstream of the first β-sheet displayed chemical shifts, but only in the presence of micelle-embedded PtdIns (3)P, suggesting that these residues contact the membrane nonspecifically. Similar to the membrane orientation predicted by the Vps27 FYVE structure (see Fig. 2), this hydrophobic loop may extend into the cytoplasmic side of the membrane bilayer, perhaps directly interacting with hydrophobic acyl chains.

However, when fused to GFP or GST, the EEA1 FYVE domain alone failed to efficiently localize to cellular membranes [21–23,30]. Residues from an additional coiled-coil region adjacent to the FYVE domain were required. Most recently, the crystal structure of the EEA1 FYVE domain including these additional residues has revealed the formation of stable homodimers that could bind two molecules of inositol 1,3-bisphosphate, a soluble mimic of PtdIns(3)P [31]. However, unlike the model proposed from studies of the Hrs FYVE domain, each EEA1 FYVE domain independently bound inositol 1,3-bisphosphate. Dimerization of the EEA1 FYVE domains was mediated primarily through interactions between the coiled-coil domains. Taken together, these data suggest that dimerization enhances binding of individual FYVE domains to membrane-restricted PtdIns(3)P. However, while the EEA1 FYVE structures provide an accurate model for PtdIns(3)P binding, additional factors may be involved in targeting and/or stabilization of FYVE domains at cellular membranes. It is interesting that residues adjacent to the EEA1 FYVE domain required for membrane localization are required for EEA1 to bind Rab5, a small GTPase that functions on membranes in the endocytic pathway [18,30]. Together, these data suggest that a combination of protein-PtdIns(3)P and protein-protein interactions is essential for specific and stable localization of FYVE domain-containing proteins to particular cellular membranes.

Conservation of the FYVE Domain and Localization of PtdIns(3)P

To date, analysis of the human genome has uncovered a total of approximately 30 FYVE domain-containing proteins, while the *Caenorhabditis elegans* genome contains 15 and the *S. cerevisiae* genome harbors 5. Thus, while the FYVE domain itself has been well conserved through the course of evolution, there appears to have been a significant expansion in the roles played by this lipid-binding motif. As described earlier, the major role for PtdIns(3)P, and by extension its FYVE domain-containing effectors, involves endocytic membrane transport. However, recent findings also show that PtdIns(3)P may have roles in growth factor signaling and actin cytoskeleton organization through the recruitment/activation of other FYVE domain-containing proteins. However, before further examining the role of the FYVE domain in cell signaling, localization of PtdIns(3)P itself must first be explored.

Initial studies of PtdIns(3)P localization were carried out via a GFP fusion to the FYVE domain of EEA1 in yeast [21]. Results indicated that the fusion co-localized with prevacuolar endosomes and weakly labeled the vacuolar membrane. An important finding is that this localization was dependent on Vps34 PtdIns 3-kinase activity, demonstrating a requirement for PtdIns(3)P in mediating membrane association *in vivo* [21]. Two FYVE domains in tandem fused to GFP similarly localized to endosomal structures in fibroblasts [32]. However, due to the limitations of light microscopy, a more detailed analysis of PtdIns(3)P localization required more extensive studies of the recombinant FYVE domain dimer. Using an electron microscopic labeling approach, PtdIns(3)P was found to be highly enriched on endosomes as expected from previous work, but the lipid was also observed in the nucleolus and in the internal vesicles of multivesicular bodies (MVBs) [32]. Consistent with the presence of PtdIns(3)P on vesicles inside the lumen of MVBs, the efficient turnover of PtdIns(3)P in yeast was shown to be dependent on hydrolase-mediated degradation in the vacuole [33]. It is likely that most, if not all, PtdIns(3)P effectors are recruited to and/or activated at endosomal/vacuolar membranes, the major sites of PtdIns(3)P accumulation in cells.

FYVE Domains in Membrane Trafficking

Studies of EEA1 have been instrumental in defining the localization of PtdIns(3)P. Closer examination of the protein reveals that it is a large coiled-coil protein (Fig. 3A) that can bind to the GTP-bound form of Rab5, a GTPase required for endosomal membrane fusion [18,34]. Together with PtdIns(3)P, Rab5-GTP recruits EEA1 to endosomal membranes where it functions in membrane fusion. Consistent with this hypothesis, depletion of EEA1 inhibits homotypic endosome fusion *in vitro*, while excess EEA1 stimulates fusion [18,34]. Furthermore, studies suggest that EEA1 may engage in oligomeric complexes during membrane fusion, which may tether Rab5-positive endosomes, thus facilitating pairing of SNARE proteins to drive membrane fusion [34,35].

Similar to EEA1, another FYVE domain-containing protein, Rabenosyn-5 (Table I), is an effector of Rab5-GTP. Its localization to the endosome is dependent on PtdIns(3)P binding and is required for endosome fusion [36]. While EEA1 appears to interact with specific SNARES including syntaxin-13 [35], Rabenosyn-5 directly interacts with the

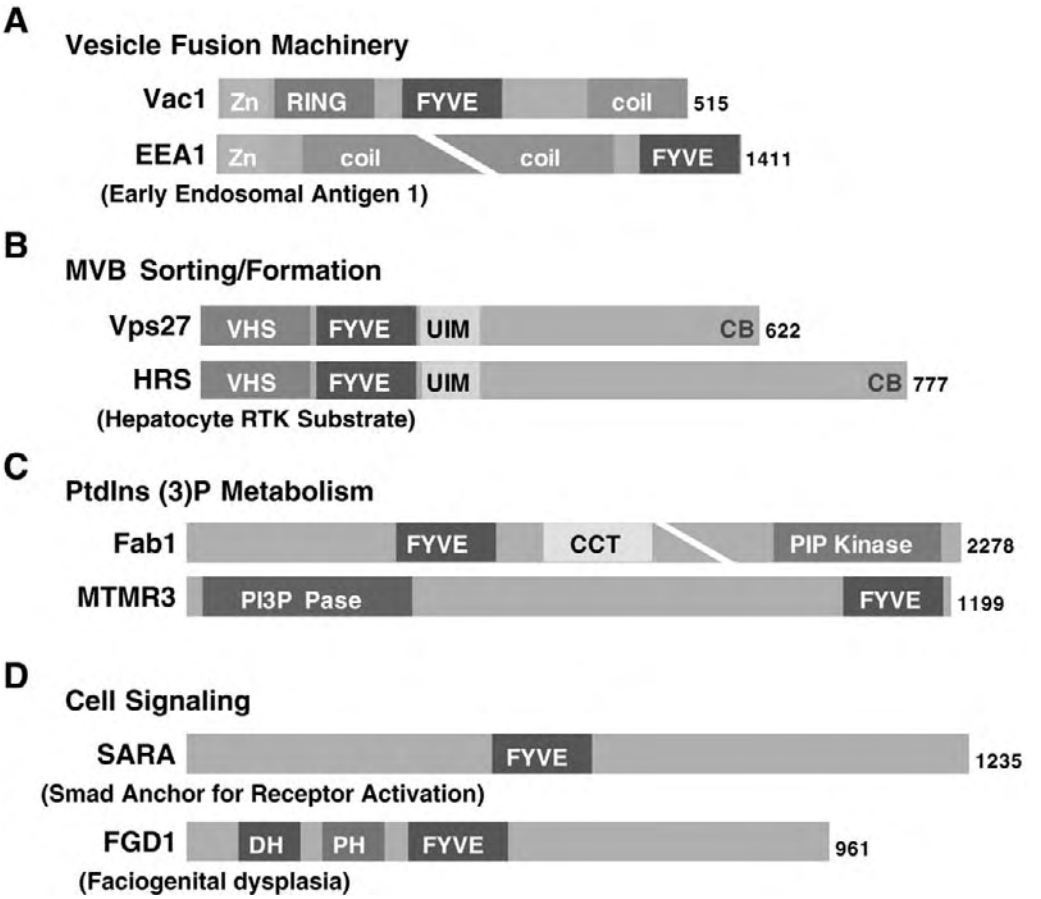


Figure 3 Schematic representation of protein motifs found within FYVE domain proteins. Several FYVE domain proteins have additional domains that bind protein targets. Together, these PtdIns(3)P-protein and protein-protein interactions define the specific function of each FYVE domain protein. Examples of yeast and mammalian FYVE domain proteins that act in various cellular processes are shown. (A) FYVE domain proteins implicated in vesicle targeting and fusion events. (B) FYVE domain proteins involved in endosomal/MVB sorting. (C) FYVE domain proteins containing enzymatic activities implicated in PI synthesis and turnover. (D) FYVE domain proteins involved in cell signaling. Other abbreviations: Zn, Zn^{2+} finger domain; RING, Zn^{2+} finger domain; coil, coiled-coil domain; VHS, conserved domain found in Vps27, Hrs, and STAM; SH3, Src homology 3 domain; UIM, ubiquitin-interacting motif; CB, clathrin box binding motif; CCT, chaperonin-like region; PIP kinase, PtdIns(3)P 5-kinase catalytic domain; PI3P Pase, myotubularin-related PtdIns(3)P phosphatase catalytic domain; DH, Dbl homology domain; PH, pleckstrin homology domain.

Sec1-like protein hVps45, suggesting distinct roles for these Rab5/PtdIns(3)P effectors in endosome fusion [36]. Similarly, the yeast homolog of Rabenosyn-5, Vac1, also contains a FYVE domain (Fig. 3A) and interacts with Vps21 and Vps45, homologs of Rab5 and Sec1 [37–39]. Deletion of *VAC1* results in an accumulation of vesicles destined for the endosome and a defect in protein sorting to the lysosome-like vacuole, suggesting that Vac1 is evolutionarily conserved and required for endocytic docking and/or fusion [38]. Substitutions in the FYVE domain of Vac1 also result in defects in vacuolar protein sorting, indicating a requirement for this domain in Vac1 function. However, these Vac1 mutants can still associate with membranes, suggesting that additional factors are involved in Vac1 localization [38]. Nevertheless, the FYVE domains found in EEA1 and Vac1/Rabenosyn-5 may still be required for concentration of these proteins on PtdIns(3)P-rich endosomes, while Rab binding may further drive specificity of these interactions. Once on endosomal membranes, EEA1 and Vac1/Rabenosyn-5 appear to intimately participate in the machinery that drives endosome fusion (Fig. 4).

In addition to Rab5, another small GTPase (Rab4) that has been implicated in the recycling of internalized receptors back to the plasma membrane regulates a FYVE domain-containing effector, Rabip4 (Table I). Like EEA1 and Vac1/Rabenosyn-5, Rabip4 localizes to endosomes and can affect endosomal morphology [40]. Moreover, overproduction of Rabip4 leads to the intracellular retention of normally recycled transporters such as Glut1[40]. These data suggest that FYVE domains and thus PtdIns(3)P are not only involved in anterograde trafficking to lysosomes but also endosomal membrane recycling to the plasma membrane.

Another well conserved FYVE domain-containing protein that has been implicated in membrane trafficking is Hrs (Fig. 3B), a *h*epatocyte growth factor *r*eceptor tyrosine

Table I FYVE Domain-Containing Proteins Discussed in the Review

Yeast				
Protein	Cellular function	Domains	Targets	Ref.
Vac1	Golgi to endosome transport	FYVE, RING, COIL	PtdIns(3)P, Vps21, Vps45, Pep12	[37–39]
Vps27	MVB sorting/formation	VHS, FYVE, UIM	PtdIns(3)P, ubiquitin	[42–44]
Fab1	PtdIns(3)P 5-synthesis, MVB sorting	FYVE, PtdIns(3)P 5-kinase	PtdIns(3)P	[44, 51]
Pib1	Ubiquitin ligase	FYVE, E3 ubiquitin ligase	PtdIns(3)P, unknown	[48]
Pib2	unknown	FYVE	PtdIns(3)P?	[21]
Mammalian				
Protein	Cellular function	Domains	Targets	Ref.
EEA1	Endosome fusion	FYVE, COIL	PtdIns(3)P, Rab5, syntaxins	[17–23,35]
Rabenosyn-5	Endosome fusion	FYVE	PtdIns(3)P, Rab5, and hVps45	[36]
Rabip4	Endosomal membrane recycling	FYVE	PtdIns(3)P, Rab4	[40]
Hrs	Endosomal/MVB Sorting	VHS, FYVE, UIM, CB	PtdIns(3), ubiquitin, Clathrin	[45–47]
PIKfyve	Endosome morphology, PtdIns(3,5)P_2 synthesis	FYVE, PtdIns(3)P 5-kinase	PtdIns(3)	[52]
MTMR4	PtdIns(3)P turnover	FYVE, PtdIns(3)P phosphatase	PtdIns(3)P	[6,55]
endofin	Unknown	FYVE	PtdIns(3)P	[59]
Frabin	Actin cytoskeleton	FYVE, PH, GEF	PI isoforms, Cdc42, Rac	[61,62]
DFCP1	Unknown	FYVE-like	PtdIns(3)P?	[63]

As well as binding to PtdIns(3)P, FYVE domain-containing proteins have other domains that interact with protein targets. Together, these interactions likely play important roles in defining protein function.

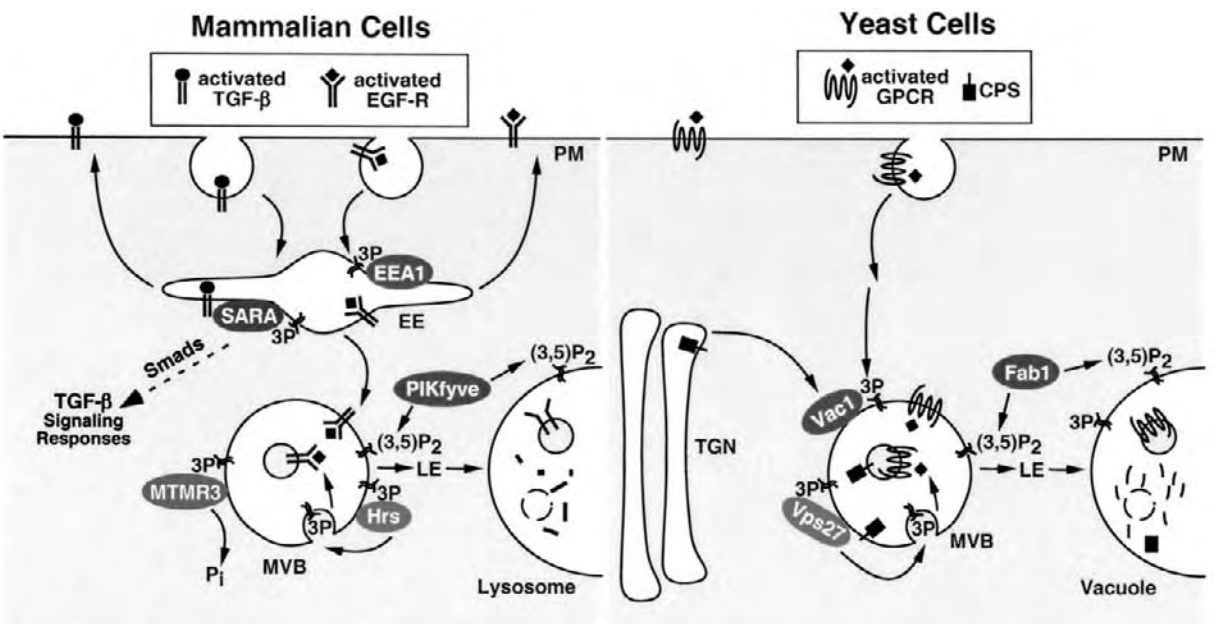


Figure 4 Cellular localization and functions of FYVE domain-containing proteins in mammalian and yeast cells. In mammalian and yeast cells, PtdIns(3)P (designated as 3P) recruits the Rab effectors EEA1 or Vac1 to endosomes where they participate in vesicle fusion in Golgi (TGN) to vacuole transport and endocytic trafficking. The FYVE domain-containing orthologs Hrs and Vps27 are required for MVB sorting pathways that transport PtdIns(3)P and cargo proteins, such as carboxypeptidase S (CPS) and internalized cell surface receptors, to the vacuole lumen where they are degraded. The PtdIns(3)P 5-kinases Fab1/PIKfyve and the PtdIns(3)P-specific phosphatase MTMR3 terminate PtdIns(3)P signaling by converting PtdIns(3)P to PtdIns(3,5)P_2 or PtdIns, respectively. PtdIns(3)P 5-kinase signaling via PtdIns(3,5)P_2 (designated as 3,5P_2) is also required for MVB sorting. In mammalian cells, the FYVE domain containing-protein SARA recruits Smad proteins, effectors of transforming growth factor beta (TGF-β) signaling, to the endosome.

kinase *s*ubstrate [41]. Studies of its yeast homolog Vps27 demonstrate a requirement for this PtdIns(3)P effector in protein trafficking, functioning after Vac1 in the endocytic pathway [42]. Specifically, inactivation of Vps27 results in a defect in the generation of intralumenal vesicles within MVBs and the vacuole [43,44]. Similarly, mouse embryos that lack Hrs exhibit defects in endosomal morphogenesis [45]. It is striking that Hrs and EEA1 are localized to different regions on endosomes. Specifically, Hrs colocalizes with clathrin and can bind to clathrin via a carboxy-terminal clathrin interacting motif [46]. Disruption of the PtdIns(3)P-FYVE interaction in Hrs by treatment with the PtdIns 3-kinase inhibitor wortmannin results in loss of both Hrs and clathrin localization to endosomes, again demonstrating the importance of the FYVE domain in endosomal function [47]. Further studies are required to precisely determine what other requirements may be necessary for Hrs/Vps27 to associate with endosomes in addition to PtdIns(3)P.

In addition to Vac1 and Vps27, yeast harbor another FYVE domain-containing protein that localizes to the endosome and vacuole, Pib1 (Table I) [21]. Localization of Pib1 to these structures is dependent on its FYVE domain through an interaction with PtdIns(3)P [48]. In addition to its FVYE domain, Pib1 contains a RING domain that possesses E2-dependent ubiquitin ligase activity *in vitro* [48]. In light of recent studies indicating a role for ubiquitin modification in the sorting of proteins into multivesicular bodies [43,49,50], the finding that Pib1 is an E3 RING-type ubiquitin ligase that localizes to the endosome via a PtdIns(3)P-FYVE interaction is especially interesting. However, deletion of *PIB1* fails to result in a defect in sorting of known ubiquitinated substrates through the MVB pathway [48], suggesting that other E3 ubiquitin ligases may act together with Pib1 in this process. Alternatively, Pib1 may be responsible for ubiquitination of a specific subset of cargo that have not yet been examined.

FYVE Domains Involved in PtdIns(3)P Metabolism

Additional FYVE domain-containing proteins found in both yeast and mammalian cells that appear to be involved in the formation of MVBs are the PtdIns(3)P 5-kinases, Fab1 and PIKfyve (Fig. 3C). Deletion of *FAB1* results in a loss of intralumenal vesicles and drastically enlarged vacuoles [44,51]. This abnormal vacuole morphology may also be in part due to defects in the recycling and/or turnover of membranes deposited at the vacuole. However, this remains to be demonstrated, since effectors of PtdIns(3,5)P_2 generated by Fab1 have yet to be identified. It is interesting that Fab1 has been shown to localize to both prevacuolar and vacuolar membranes, similar to the distribution of PtdIns(3)P, suggesting that its amino-terminal FYVE domain may have a role in localization and/or activity of Fab1 [51]. However, deletion of an amino-terminal fragment of Fab1 including its FYVE domain fails to significantly perturb its function, since this form of Fab1 can rescue a temperature-sensitive *fab1* mutant, suggesting that other determinants for Fab1 localization exist [51]. In contrast, the FYVE domain of mammalian PIKfyve is absolutely critical for its localization to membranes of the late endocytic pathway [52]. These studies highlight a surprising difference between certain yeast members of the FYVE domain family and their mammalian counterparts. Specifically, Vac1, Vps27, and Fab1 contain FYVE domains that are not entirely essential for membrane binding of the intact proteins. Nonetheless, this does not exclude a role for PtdIns(3)P-FYVE interactions for protein localization in yeast, but instead may emphasize the role of additional protein-protein interactions in precise subcellular targeting.

In addition to PtdIns kinases, recent studies have uncovered a set of PI phosphatases that contain a FYVE domain, such as MTMR3 (Fig. 3C) and MTMR4 (Table I). Both are members of the myotubularin family of phosphatases that were originally shown to dephosphorylate serine/threonine and tyrosine residues *in vitro* but have subsequently been shown to act upon PtdIns(3)P as their primary substrate [6,53]. It is interesting that various myotubularin members have been implicated in multiple disorders, including myotubular myopathy [53], which involves defects in muscle differentiation and Charcot-Marie-Tooth disease [54], a condition caused by defects in myelin development. MTMR3 and MTMR4 (also named FYVE-DSP1 and FYVE-DSP2) are localized in membrane fractions [54], but further studies are required to determine whether this localization is dependent on their FYVE domains. What is more important, however, the identification of both PtdIns(3)P 5-kinases and PtdIns(3)P phosphatases that contain FYVE domains raises the possibility that the FYVE domain may serve a regulatory role in the control of these enzyme activities when lipid is bound (Fig. 4). Recruitment of either a PtdIns(3)P 5-kinase or PtdIns(3)P-specific phosphatase could play a role in terminating PtdIns(3)P signaling by converting PtdIns(3)P to PtdIns(3,5)P_2 or PtdIns, respectively. Future work on these members of the FYVE domain family will be informative in shedding light on this question.

FYVE Domains in Signaling

In addition to membrane trafficking and phosphoinositide metabolism, FYVE domains are also found in proteins required for other cellular processes, such as growth factor signaling. The FYVE domain containing-protein SARA (Fig. 3D) recruits Smad proteins, effectors of transforming growth factor beta (TGF-beta) signaling, to the endosome (Fig. 4) [56]. There, bound TGF-beta receptors can phosphorylate Smad2 and Smad3 via their cytoplasmic serine/threonine kinase domain [56]. Phosphorylated Smads can then bind to Smad4, and this resulting complex is able to translocate to the nucleus and activate transcription of target genes [56]. SARA provides an excellent example in which the trafficking of cell-surface receptors is intimately coupled to intracellular signaling [56,57]. In this case, the FYVE

domain of SARA spatially regulates TGF-beta signaling, restricting it to endosomes that contain both PtdIns(3)P and activated TGF-beta receptors. This spatial control permits the cell to prevent inappropriate activation of Smad signaling and allows for a large range of separation between the on and off states of this pathway. Consistent with this, treatment with the PtdIns 3-kinase inhibitor wortmannin results in mislocalization of SARA and leads to defects in Smad phosphorylation and downstream transcriptional activation [58]. Similar to SARA, a largely uncharacterized protein named endofin (Table I) also localizes to endosomes in a PtdIns(3)P-dependent manner [59]. Although 50% identical to SARA, endofin fails to interact with Smad2 and does not play a role in TGF-beta signaling [59]. This suggests that other yet to be defined signaling pathways may be regulated at the level of the endosome in a PtdIns(3)P-dependent manner.

Although less clear, FYVE domains are also found in proteins that regulate the actin cytoskeleton. These include Fgd1, a *f*acio*g*enital *d*ysplasia gene product implicated in the developmental disease Aarskog-Scott syndrome (Fig. 3D), and Frabin (Table I), which act as guanine nucleotide exchange factors (GEFs) for the small GTPases Cdc42 and Rac. Fgd1 specifically activates Cdc42, which in turn regulates actin cytoskeleton organization [60]. Frabin, through the action of Cdc42-dependent and independent pathways, has been implicated in filopodia and lamellipodia formation, respectively [61]. However, these events are not likely to involve endocytic trafficking, since early studies have indicated that this family of FYVE domain-containing proteins does not localize to the endosome [62]. Closer examination of these GEFs shows they also contain PH domains that can bind other PI species [61], and their FYVE domains lack a well-conserved tryptophan residue that is conserved in most other FYVE domains. Further studies are required to determine whether these regulators of actin cytoskeleton organization actually bind PtdIns(3)P through their FYVE domains or bind another ligand that may be structurally related to PtdIns(3)P.

FYVE-like Domains

In addition to the highly conserved FYVE domain, several other FYVE-like domains have recently been uncovered. For example, a protein identified from a human bone marrow cDNA library named DFCP1 contains two FYVE-like domains (Table I), but in both cases, the first conserved arginine in the conserved basic R(R/K)HHCR patch is replaced with a serine or threonine [63]. It remains to be determined how this might effect PtdIns(3)P binding or whether DFCP1 localization is dependent on PtdIns(3)P *in vivo*. Initial studies indicate that DFCP1 localizes to the endoplasmic reticulum, Golgi, and other intracellular vesicles, unlike what has been seen with bona fide PtdIns(3)P effectors, such as EEA1 and Hrs [63]. Future studies aimed at determining the ligand binding specificities of FYVE-like domains should help resolve these apparent discrepancies.

Conclusions

Within the span of a few years, the identification of the FYVE domain as a specific PtdIns(3)P binding motif has had a significant impact on the field of vesicular trafficking and has shed light on additional cellular functions of PtdIns(3)P. Through localization studies of the FYVE domain via both conventional light microscopy and high-resolution electron microscopy, PtdIns(3)P has been found to exist in endosomal membranes, on vesicles contained within endosomes, and in vacuolar/lysosomal membranes. More recently, GFP-FYVE fusions have been used to observe PtdIns(3)P on phagosomes [64]. However, additional PtdIns(3)P interacting proteins are involved in this process, as recent studies indicate that another lipid binding motif, the PX domain, specifically recognizes PtdIns(3)P [65]. Hence, it is likely that new effectors of this lipid will continue to emerge.

The question still remains whether the FYVE domain alone provides sufficient specificity for protein localization. Studies in yeast would favor a significant but not singular role for the FYVE domain in this regard. Instead, PtdIns(3)P-FYVE interactions coupled with protein-protein interactions are likely to ensure specific membrane recruitment of these proteins. This level of specificity would help ensure appropriate membrane-restricted responses and functions. Further studies are required to determine the validity of this concept and whether this is a general principle or may only apply to a certain subset of FYVE domain-containing proteins.

Acknowledgments

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CHAPTER 153

Protein Kinase C: Relaying Signals from Lipid Hydrolysis to Protein Phosphorylation

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Introduction

Protein kinase C (PKC) has been in the spotlight since the discovery a quarter of a century ago that, through its activation by diacylglycerol, it relays signals from lipid hydrolysis to protein phosphorylation [1]. The subsequent discovery that PKCs are the target of phorbol esters resulted in an avalanche of reports on the effects on cell function of phorbol esters, nonhydrolyzable analogs of the endogenous ligand, diacylglycerol [2–4]. Despite the enduring stage presence of PKC and tremendous advances in understanding the enzymology and regulation of this key protein, an understanding of the function of PKC in biology is still the subject of intense pursuit. Its uncontrolled signaling wreaks havoc in the cell, as epitomized by the potent tumor-promoting properties of phorbol esters. In fact, the pluripotent effects of phorbol esters, compounded with the existence of multiple isozymes of PKC, has made it difficult to uncover the precise cellular function of this key enzyme [5]. Studies with knockout mice have underscored the problem, with knockouts of most isozymes having only subtle phenotypic effects [6]. This chapter summarizes our current understanding of the molecular mechanisms of how protein kinase C transduces information from lipid mediators to protein phosphorylation.

Protein Kinase C Family

The 10 members of the mammalian PKC family are grouped into three classes based on their domain structure, which, in turn, dictates their cofactor dependence (Fig. 1). All members comprise a single polypeptide that has a conserved kinase core carboxyl-terminal to a regulatory moiety. This regulatory moiety contains two key functionalities: an autoinhibitory sequence (pseudosubstrate) and one or two membrane-targeting modules (C1 and C2 domains). The C1 domain binds diacylglycerol and phosphatidylserine specifically and is present as a tandem repeat in conventional and novel PKCs (C1A and C1B); the C2 domain nonspecifically binds Ca^{2+} and anionic phospholipids such as phosphatidylserine. Non-ligand-binding variants of each domain exist: atypical C1 domains do not bind diacylglycerol and novel C2 domains do not bind Ca^{2+}.

Conventional PKC isozymes (α, γ, and the alternatively spliced βI and βII) are stimulated by diacylglycerol and phosphatidylserine (C1 domain) and Ca^{2+} (C2 domain); novel PKC isozymes (δ, ε, η/L, θ) are stimulated by diacylglycerol and phosphatidylserine (C1 domain); and atypical PKC isozymes (ζ, ι/λ) are stimulated by phosphatidylserine (atypical C1 domain) [5–7]. (Note that PKC μ and ν were considered to constitute a fourth class of PKCs but are now generally regarded as members of a distinct family

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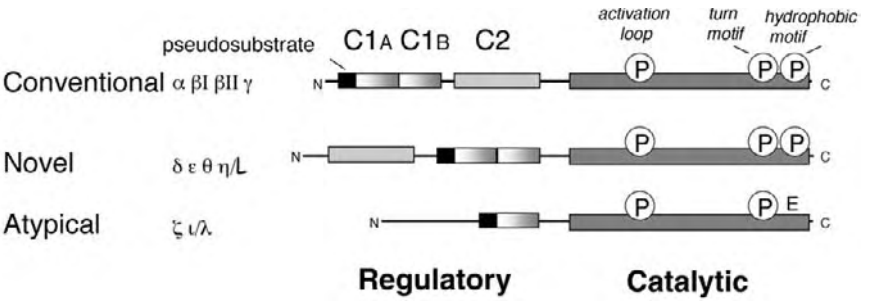


Figure 1 Domain composition of protein kinase C family members showing autoinhibitory pseudosubstrate, membrane-targeting modules (C1A and C1B and C2 domains), and kinase domain of the three subclasses: conventional, novel, and atypical isozymes. Also indicated are the positions of the three processing phosphorylation sites, the activation loop and two carboxyl-terminal sites, the turn motif, and hydrophobic motif. (Adapted from Newton, A. C. and Johnson, J. E., *Biochem. Biophys. Acta*, 1376, 155–172, 1998.)

called protein kinase D.) The role of the novel C2 domain in novel PKCs and that of the atypical C1 domain in atypical PKCs is not clear, but each may regulate the subcellular distribution of these isozymes through protein–protein interactions.

Regulation of Protein Kinase C

The normal function of PKC is under the coordinated regulation of three major mechanisms: phosphorylation/ dephosphorylation, membrane targeting modules, and anchor proteins. First, the kinase must be processed by a series of ordered phosphorylations to become catalytically competent. Second, it must have its pseudosubstrate removed from the active site to be catalytically active, a conformational change driven by engaging the membrane-targeting modules with ligand. Third, it must be localized at the correct intracellular location for unimpaired signaling. Perturbation at any of these points of regulation disrupts the physiological function of PKC [7].

Phosphorylation/ Dephosphorylation

The function of PKC isozymes is controlled by phosphorylation mechanisms that are required for the maturation of the enzyme. In addition to the processing phosphorylations, the function of PKC isozymes is additionally fine-tuned by both Tyr and Ser/Thr phosphorylations [8,9]. The conserved maturation phosphorylations are described below.

PHOSPHORYLATION IS REQUIRED FOR THE MATURATION OF PROTEIN KINASE C

The majority of PKC in tissues and cultured cells is phosphorylated at two key phosphorylation switches: a loop near the active site, referred to as the *activation loop*, and a sequence at the carboxyl terminus of the kinase domain [10,11]. The carboxyl-terminal switch contains two sites: the turn motif, which by analogy with protein kinase A is at the apex of a turn on the upper lobe of the kinase domain, and the hydrophobic motif, which is flanked by hydrophobic residues (note that, in atypical PKCs, a Glu occupies the phospho-acceptor position of the hydrophobic motif). It is the phosphorylated species that transduces signals. While it had been appreciated since the late 1980s that PKC is processed by phosphorylation [12], the mechanism and role of these phosphorylations are only now being unveiled [7,9].

The first step in the maturation of PKC is phosphorylation by the phosphoinositide-dependent kinase, PDK-1, of the activation loop. This enzyme was originally discovered as the upstream kinase for Akt/protein kinase B [13] and was subsequently shown to be the activation loop kinase for a large number of AGC kinases, including all PKC isozymes [14–16]. The name PDK-1 was based on the phosphoinositide-dependence of Akt phosphorylation and is an unfortunate misnomer because the phosphorylation of other substrates (for example, the conventional PKCs) has no dependence on phosphatidylinositol 3-kinase (PI3K) lipid products [17]. Rather, PDK-1 appears to be constitutively active in the cell, with substrate phosphorylation regulated by the conformation of the substrate [18–20].

Completion of PKC maturation requires phosphorylation of the two carboxyl-terminal sites, the turn motif and hydrophobic motif. In the case of conventional PKCs, this reaction occurs by an intramolecular autophosphorylation mechanism [21]. Autophosphorylation also accounts for the hydrophobic motif processing of the novel PKCε [22]; however, it has been suggested that another member of this family, PKCδ, may be the target of a putative hydrophobic motif kinase [23].

Research in the past few years has culminated in the following model for PKC phosphorylation. Newly synthesized enzyme associates with the plasma membrane, where it adopts an open conformation with the pseudosubstrate exposed, thus unmasking the PDK-1 site on the activation loop [17,24]. It is likely held at the membrane by multiple weak interactions with the exposed pseudosubstrate, the C1 domain, and the C2 domain (because the C1 and C2 ligands are absent, these domains are weakly bound via their interactions with anionic phospholipids). PDK-1 docks onto the carboxyl terminus of PKC, where it is positioned to phosphorylate the activation loop [25]. This phosphorylation is

the first and required step in the maturation of PKC; mutation of the phospho-acceptor position at the activation loop to Ala or Val prevents the maturation of PKC and results in accumulation of unphosphorylated, inactive species in the detergent-insoluble fraction of cells [26,27].

Completion of PKC maturation requires release of PDK-1 from its docking site on the carboxyl terminus. Physiological mechanisms for this release have not yet been elucidated, but it is interesting that over-expression of peptides that have a high affinity for PDK-1 promotes the maturation of PKC [25]. One such peptide is PIF, the carboxyl-terminus of PRK-2, which has a hydrophobic phosphorylation motif with a Asp at the phospho-acceptor position [28]. Release of PDK-1 unmasks the carboxyl terminus of PKC, allowing phosphorylation of the turn motif and the hydrophobic motif [7,10].

DEPHOSPHORYLATION: DEACTIVATION SIGNAL

While the phosphorylation of conventional PKCs is constitutive, the dephosphorylation appears to be agonist stimulated [29]. Both phorbol esters and ligands such as tumor necrosis factor α (TNFα) result in PKC inactivation and dephosphorylation [29–31]. In addition, serum selectively promotes the dephosphorylation of the activation loop site in conventional PKCs, thus uncoupling the phosphorylation of the activation loop from that of the carboxyl-terminal sites [17]. The hydrophobic site of PKCε has also been reported to be selectively dephosphorylated by a rapamycin-sensitive phosphatase [32]. It is likely that the uncoupling of the dephosphorylation of these sites has contributed to confusion as to whether the hydrophobic site is regulated by its own upstream kinase rather than autophosphorylation [23].

Membrane Translocation

The translocation from the cytosol to the membrane has served as the hallmark for PKC activation since the early 1980s [33,34]. The molecular details of this translocation have emerged from abundant biophysical, biochemical, and cellular studies showing that diacylglycerol acts like molecular glue to recruit PKC to membranes, an event that, for conventional PKCs, is facilitated by Ca^{2+} [35–37].

Both *in vitro* and *in vivo* data converge on the following model for the translocation of conventional PKC in response to elevated Ca^{2+} and diacylglycerol [35,38]. In the resting state, PKC bounces on and off membranes by a diffusion-limited reaction. However, its affinity for membranes is so low that its lifetime on the membrane is too short to be significant. Elevation of Ca^{2+} results in binding of Ca^{2+} to the C2 domain of this soluble species of PKC. This Ca^{2+}-bound species has a dramatically enhanced affinity for the membrane, with which it rapidly associates. The membrane-bound PKC then diffuses in the two-dimensional plane of the membrane, searching for the much less abundant ligand, diacylglycerol. This search for diacylglycerol is considerably more efficient from the membrane than one initiated from the cytosol. Following collision with, and binding to, diacylglycerol, PKC is bound to the membrane with sufficiently high affinity to allow release of the pseudosubstrate sequence and activation of PKC. Decreases in the level of either second messenger weaken the membrane interaction sufficiently to release PKC back into the cytosol. Note that if PMA is the C1 domain ligand, PKC can be retained on the membrane in the absence of elevated Ca^{2+} because this ligand binds PKC two orders of magnitude more tightly than diacylglycerol [39]. Similarly, if Ca^{2+} levels are elevated sufficiently, PKC can be retained at the membrane in the absence of a C1 ligand.

Novel PKC isozymes translocate to membranes much more slowly than conventional PKCs in response to receptor-mediated generation of diacylglycerol because they do not have the advantage of pre-targeting to the membrane by the soluble ligand, Ca^{2+} [40]. Atypical PKC isozymes do not respond directly to either diacylglycerol or Ca^{2+}.

Anchoring Proteins

The control of subcellular localization of kinases by scaffold proteins is emerging as a key requirement in maintaining fidelity and specificity in signaling by protein kinases [41]. PKC is no exception, and a battery of binding partners for members of this kinase family have been identified [42–45]. These proteins position PKC isozymes near their substrates, near regulators of activity such as phosphatases and kinases, or in specific intracellular compartments. Disruption of anchoring can impair signaling by PKC, and *Drosophila* photoreceptors provide a compelling example. Mislocalization of eye-specific PKC by abolishing its binding to the scaffold protein, ina D, disrupts phototransduction [46].

Unlike protein kinase A binding proteins (AKAPs) [47], there is no consensus binding mechanism for interaction of PKC with its anchor proteins. Rather, each binding partner identified to date interacts with PKC by unique determinants and unique mechanisms. Some binding proteins regulate multiple PKC isozymes, while others control the distribution of specific isozymes. There are binding proteins for newly synthesized unphosphorylated PKC, phosphorylated but inactive PKC, phosphorylated and activated PKC, and dephosphorylated, inactivated PKC [43,45]. Anchoring proteins for PKC have diverse functions—some positively regulate signaling while others negatively regulate it. An emerging theme is that many scaffolds bind multiple signaling molecules in a signaling complex; for example, AKAP 79 binds PKA, PKC, and the phosphatase calcineurin [48]. The physical coupling of kinases and phosphatases underscores the acute regulation that each must be under to maintain fidelity in signaling.

Model for Regulation of Protein Kinase C by Phosphorylation and Second Messengers

Figure 2 outlines a model for the regulation of PKC by phosphorylation, second messengers, and anchoring proteins. Newly synthesized PKC associates with the membrane in a conformation that exposes the pseudosubstrate

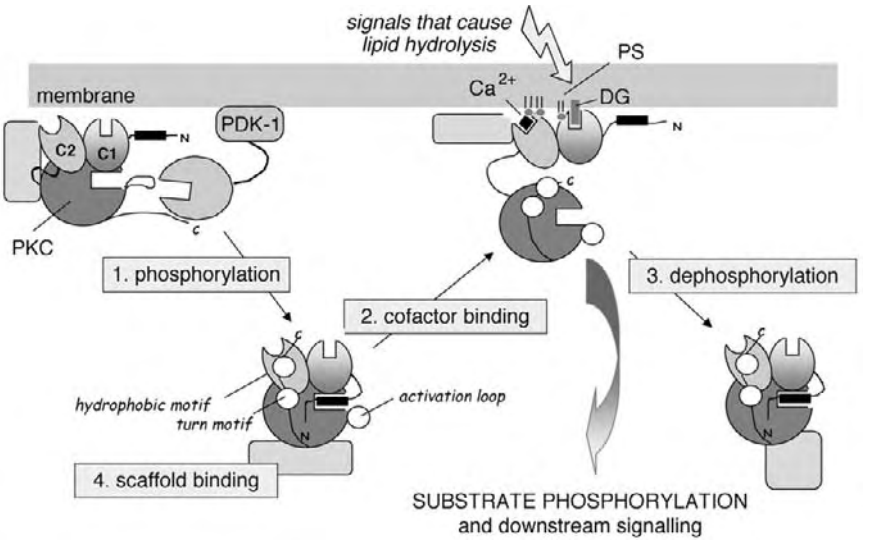


Figure 2 Model showing the major regulatory mechanisms for PKC function: (1) processing by phosphorylation, (2) activation by lipid mediators, (3) deactivation by dephosphorylation, and (4) spatial control by scaffold proteins. See text for details. (Adapted from Newton, A. C., *Chem. Rev.*, **101**, 2353–2364, 2001.)

(black rectangle), allowing access of the upstream kinase, PDK-1, to the activation loop. PDK-1 docks onto the carboxyl terminus of PKC. Following its phosphorylation of the activation loop and release from PKC, the turn motif and hydrophobic motif are autophosphorylated. The mature PKC is released into the cytosol, where it is maintained in an auto-inhibited conformation by the pseudosubstrate (middle panel), which has now gained access to the substrate-binding cavity (open rectangle in the large circle representing the kinase domain of PKC). It is this species that is competent to respond to second messengers. Generation of diacylglycerol and, for conventional PKCs, Ca^{2+} mobilization provide the allosteric switch to activate PKC. This is achieved by engaging the C1 and C2 domains on the membrane (Fig. 2, right panel), thus providing the energy to release the pseudosubstrate from the active site, allowing substrate binding and catalysis. In addition to the regulation by phosphorylation and cofactors, anchoring/scaffold proteins (stippled rectangle) play a key role in PKC function by positioning specific isozymes at particular intracellular locations [43,45]. Following activation, PKC is either released into the cytosol or, following prolonged activation, dephosphorylated and downregulated by proteolysis.

Function of Protein Kinase C

Despite over two decades of research on the effects of phorbol esters on cell function, a unifying mechanism for the role of PKC in the cell has remained elusive. An abundance of substrates have been identified, and the reader is referred to reviews summarizing these and potential signaling pathways involving PKC [5,6,49–52]. However, a unique role for PKC in defining cell function is lacking. This is epitomized by the finding that there is no severe phenotype associated with knocking-out specific PKC isozymes in mice.

Closer analysis of the phenotypes of knockout animals of various PKC isozymes does suggest a common theme: animals deficient in PKC are deficient in adaptive responses. For example, PKCε $^{-/-}$ mice have reduced anxiety and reduced tolerance to alcohol [53], PKCγ$^{-/-}$ mice have reduced pain perception [54], and PKCβII$^{-/-}$ mice have reduced learning abilities [55]. This theme carries over to the molecular level, where many of the substrates of PKC are receptors that become desensitized following PKC phosphorylation.

Summary

PKC plays a pivotal role in cell signalling by relaying information from lipid mediators to protein substrates. The relay of this information is under exquisite conformational, spatial, and temporal regulation, and extensive studies on the molecular mechanisms of this control have provided much insight into how PKC is regulated. With novel approaches in chemical genetics, analysis of crosses of PKC isozyme knockout mice, and proteomics, the PKC signaling field is poised to move to the next level of making headway into the *raison d'être* of this ubiquitous family of kinases.

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CHAPTER 154

Role of PDK1 in Activating AGC Protein Kinase

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Introduction

Stimulation of cells with growth factors, survival factors, and hormones leads to recruitment to the plasma membrane of a family of lipid kinases known as class 1 phosphoinositide 3-kinases (PI 3-kinases, [1]). In this location PI 3-kinases phosphorylate the glycerophospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), at the D-3 position of the inositol ring, converting it to PtdIns(3,4,5)P_3, which is then converted to PtdIns(3,4)P_2 through the action of the SH2-containing inositol phosphatases (SHIP1 and SHIP2) or back to PtdIns(4,5)P_2 via the action of the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10).

PtdIns(3,4,5)P_3 and perhaps PtdIns(3,4)P_2 play key roles in regulating many physiological processes, including controlling cell apoptosis and proliferation, most of the known physiological responses to insulin, and cell differentiation and cytoskeletal organization [2]. PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 exert their cellular effects by interacting with proteins that possess a certain type of pleckstrin homology domain (PH domain). A number of types of PH domain containing proteins that interact with PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 have now been identified. These include the serine/threonine protein kinases protein kinase B (PKB; also known as Akt) [3], tyrosine kinases of the Tec family [4,5], numerous adaptor molecules such as the Grb2-associated protein (GAB1 [6]), the dual adaptor of phosphotyrosine and 3-phosphoinositides (DAPP1 [7–10]), and the tandem PH-domain-containing proteins (TAPP1 and TAPP2 [11]), as well as guanosine triphosphate (GTP)/guanosine diphosphate (GDP) exchange [12–14] and GTPase-activating proteins [15,16] for the ARF/Rho/Rac family of GTP binding proteins (Fig 1). This chapter focuses on research aimed at understanding the mechanism by which PtdIns(3,4,5)P_3 regulates one branch of its downstream signaling pathways, namely enabling PDK1 to phosphorylate and activate a group of serine/threonine protein kinases that belong to the AGC subfamily of protein kinases. These include isoforms of PKB [3,17], p70 ribosomal S6 kinase (S6K) [18,19], serum- and glucocorticoid-induced protein kinase (SGK) [20], p90 ribosomal S6 kinase (RSK) [21], and protein kinase C (PKC) isoforms [22]. Once these diverse AGC kinase members are activated, they phosphorylate and change the activity and function of key regulatory proteins that control processes such as cell proliferation and survival as well as cellular responses to insulin [2,3,23].

Mechanism of Activation of PKB

The three isoforms of PKB (PKBα, PKBβ, and PKBγ) possess high sequence identity and are widely expressed in human tissues [17]. Stimulation of cells with agonists that activate PI 3-kinase induce a large activation of PKB isoforms within a few minutes. The activation of PKB is downstream of PI 3-kinase, as inhibitors of PI 3-kinase such as wortmannin or LY294002, or the over-expression of a dominant-negative regulatory subunit of PI 3-kinase inhibit the activation of PKB in cells by virtually all agonists tested [24–26]. Over-expression of a constitutively active mutant of PI 3-kinase induces PKB activation in unstimulated cells [27],

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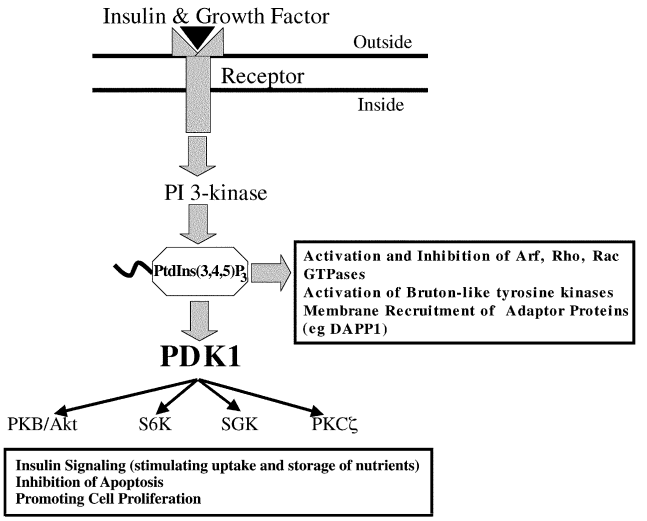


Figure 1 Overview of the PI 3-kinase signaling pathway. Insulin and growth factors induce the activation of PI 3-kinase and generation of PtdIns(3,4,5)P_3. In addition to leading to the activation of PKB/Akt, S6K, SGK, and atypical PKC isoforms such as PKCζ, PtdIns(3,4,5)P_3 also recruits a number of other proteins (outlined in the text) to the plasma membrane to trigger the activation of non-PDK1/AGC-kinase-dependent signaling pathways. Key challenges for future experiments are not only to define the specific cellular roles of the individual AGC kinase but also to understand the function and importance of other branches of signaling pathways activated by PI 3-kinase.

as does deletion of the PTEN phosphatase which also results in increased cellular levels of PtdIns(3,4,5)P_3 [28–32].

All PKB isoforms possess an N-terminal pleckstrin homology (PH domain) that interacts with PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 followed by a kinase catalytic domain and then a C-terminal tail. Stimulation of cells with agonists that activate PI 3-kinase induces the translocation of PKB to the plasma membrane, where PtdIns(3,4,5)P_3 as well as PtdIns(3,4)P_2 are located and, consistent with this, translocation of PKB is prevented by inhibitors of PI 3-kinase or by the deletion of the PH domain of PKB [33–35]. These findings strongly indicate that PKB interacts with PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 *in vivo*. The binding of PKB to PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 does not activate the enzyme but instead recruits PKB to the plasma membrane where it becomes phosphorylated at two residues at this location, namely Thr308 and Ser473. Inhibitors of PI 3-kinase and dominant-negative PI 3-kinase prevent phosphorylation of PKB at both residues following stimulation of cells with insulin and growth factors [17]. Thr308 is located in the T-loop (also known as *activation loop*) between subdomains VII and VIII of the kinase catalytic domain, situated at the same position as the activating phosphorylation sites found in many other protein kinases. As discussed later, Ser473 is located outside of the catalytic domain in a motif that is present in most AGC kinases and which has been termed the *hydrophobic motif*. The phosphorylation of PKBα at both Thr308 or Ser473 is likely to be required to activate PKBα maximally, as mutation of Thr308 to Ala abolishes PKBα activation, whereas mutation of Ser473 to Ala reduces the activation of PKBα by approximately 85%. The mutation of both Thr308 and Ser473 to Asp (to mimic the effect of phosphorylation by introducing a negative charge) increases PKBα activity substantially in unstimulated cells, and this mutant cannot be further activated by insulin [3]. Attachment of a membrane-targeting domain to PKBα results in it becoming highly active in unstimulated cells and induces a maximal phosphorylation of Thr308 and Ser473 [33,36]. These observations indicate that recruitment of PKB to the membrane of unstimulated cells is sufficient to induce the phosphorylation of PKBα at Thr308 and Ser473. Furthermore, there must be sufficient basal levels of PtdIns(3,4,5)P_3/PtdIns(3,4)P_2, T308 kinase, and Ser473 kinase located at the membrane to stimulate phosphorylation and activation of membrane-targeted PKB. PKBβ and PKBγ are activated by phosphorylation of the equivalent residues in their T-loops and hydrophobic motifs [37,38].

PKB Is Activated by PDK1

A protein kinase was purified [39,40] and subsequently cloned [41,42] that phosphorylated PKBα at Thr308 only in the presence of lipid vesicles containing PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2. Because of these properties it was named 3-phosphoinositide-dependent protein kinase 1 (PDK1) and is composed of an N-terminal catalytic domain and a C-terminal PH domain which, like that of PKB, interacts with PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 [42,43]. The activation of PKB by PDK1 is stereospecific for the physiological D-enantiomers of these lipids, and neither PtdIns(4,5)P_2 nor any inositol phospholipid other than PtdIns(3,4)P_2 can replace PtdIns(3,4,5)P_3 in the PDK1-catalyzed activation of PKB [39,42].

Although co-localization of PKB and PDK1 at the plasma membrane through their mutual interaction with 3-phosphoinositides is likely to be important for PDK1 to phosphorylate PKB, the binding of PKB to PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 is also postulated to induce a conformational change in PKB, exposing Thr308 for phosphorylation by PDK1. This conclusion is supported by the observation that in the absence of 3-phosphoinositides, PDK1 is unable to phosphorylate wild-type PKB under conditions where it is able to efficiently phosphorylate a mutant form of PKB that lacks its PH domain, termed ΔPH-PKB [40,41]. Consistent with this, a PKB mutant in which a conserved Arg residue in the PH domain is mutated to abolish the ability of PKB to bind PtdIns(3,4,5)P_3 cannot be phosphorylated by PDK1 in the presence of lipid vesicles containing PtdIns(3,4,5)P_3 [40]. Moreover, artificially promoting the interaction of PDK1 with wild-type PKB and ΔPH-PKB by the attachment of a high-affinity PDK1 interaction motif to these enzymes is sufficient to induce maximal phosphorylation of Thr308 in ΔPH-PKB but not in wild-type PKB in unstimulated cells [44].

More recently, the three-dimensional structure of the isolated PH domain of PKB complexed with the head group of

PtdIns(3,4,5)P_3 has been solved [45]. Interestingly, the structure of the PH domain of PKB complexed to the inositol head group of PtdIns(3,4,5)P_3 revealed that the 3- and the 4-phosphate groups form numerous interactions with specific basic amino acids in the PKB PH domain, but in contrast the 5-phosphate group does not make any significant interaction with the protein backbone and is solvent exposed, thus providing the first structural explanation of why PKB interacts with both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 with similar affinity [45].

The interaction of PDK1 with PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 is thought to be the primary determinant in enabling PDK1 and PKB to colocalize at membranes and permitting PDK1 to phosphorylate PKB efficiently. These conclusions are supported by the finding that the rate of activation of PKBα by PDK1 *in vitro*, in the presence of lipid vesicles containing PtdIns(3,4,5)P_3, is lowered considerably if the PH domain of PDK1 is deleted. Furthermore, the mutant of PKB that lacks its PH domain is also a very poor substrate for PDK1, compared to wild-type PKB, as it is unable to interact with lipid vesicles containing PtdIns(3,4,5)P_3.

Activation of Other Kinases by PDK1

The finding that the T-loop residues of PKB are very similar to those found on other AGC kinases suggested that PDK1 might phosphorylate and activate these members [46,47]. An alignment of the T-loop sequences of insulin and growth-factor-stimulated AGC kinases is shown in Fig. 2. It was found that the AGC kinases activated downstream of PI 3-kinase (namely, S6K1 [48,49], SGK isoforms [50–52], and atypical PKC isoforms [53,54]) were phosphorylated specifically at their T-loop residue by PDK1 *in vitro* or following the over-expression of PDK1 in cells. Moreover, AGC kinases that were not activated in a PI 3-kinase-dependent manner in cells—such as the p90 ribosomal S6K (p90RSK) isoforms [55,56], conventional and related PKC isoforms [57–60], PKA [61], and the non-AGC Ste20 family member PAK1 [62]—were also proposed to be physiological substrates for PDK1, as they could all be phosphorylated by PDK1 at their T-loop residue *in vitro* or following overexpression of PDK1 in cells.

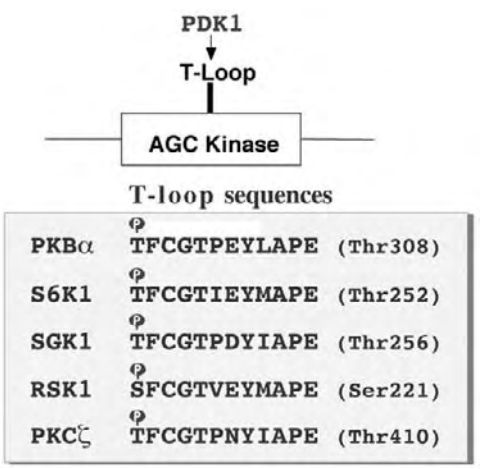


Figure 2 Alignment of the amino acid sequences surrounding the T-loop of insulin and growth-factor-stimulated AGC kinases.

Genetic evidence for the central role that PDK1 plays in mediating the activation of these AGC kinases was obtained from the finding that in PDK1$^{-/-}$ ES cells, isoforms of PKB, S6K, and RSK could not be activated by agonists that switch on these enzymes in wild-type cells [63]. In ES cells lacking PDK1, the intracellular levels of endogenously expressed PKCα, PKCβI, PKCγ, PKCδ, PKCε, and PRK1 are also vastly reduced compared to wild-type ES cells [64], consistent with the notion that PDK1 phosphorylation of these enzymes plays an essential role in post-translational stabilization of these kinases [65,66]. The levels of PKCζ were only moderately reduced in the PDK1$^{-/-}$ ES cells and PKCζ in these cells is not phosphorylated at its T-loop residue [64], providing genetic evidence that PKCζ is a physiological substrate for PDK1. In contrast, PKA was active and phosphorylated at its T-loop in PDK1$^{-/-}$ ES cells, to the same extent as in wild-type ES cells [63], thus arguing that PDK1 is not rate limiting for the phosphorylation of PKA in ES cells. It is possible that PKA phosphorylates itself at its T-loop residue *in vivo*, as it has been shown to possess the intrinsic ability to phosphorylate its own T-loop when expressed in bacteria. Thus far, we have no genetic data in PDK1-deficient cells as to whether or not PAK1 is active, but it should be noted that PAK1 can also phosphorylate itself at its T-loop in the presence of Cdc42-GTP or Rac-GTP, stimulating its own activation in the absence of PDK1 [67].

Phenotype of PDK1 PKB- and S6K-Deficient Mice and Model Organisms

PDK1$^{-/-}$ mouse embryos die at day E9.5, displaying multiple abnormalities that include a lack of somites, forebrain, and neural-crest-derived tissues, although the development of the hind- and midbrain proceeds relatively normally [68]. Other eukaryotic organisms also possess homologs of PDK1 that activate homologs of PKB and S6K in these species [69]. As in mice, knocking out PDK1 homologs in yeast [70–72], *Caenorhabditis elegans* [73], and *Drosophila* [74,75] results in nonviable organisms, confirming that PDK1 plays a key role in regulating normal development and survival of these organisms. Elegant genetic analysis of the PI 3-kinase/PDK1/AGC kinase pathway in *Drosophila* has demonstrated that this pathway plays a key role in regulating both cell size and number [76,77]. For example, the over-expression of dPI 3-kinase [78,79] or inactivation of the PtdIns(3,4,5)P_3 3-phosphatase dPTEN [80–82] results in an increase in both the cell number as well as the cell size of *Drosophila*. Moreover, loss-of-function mutants of Chico, the fly homolog of insulin receptor substrate adaptor protein [83], dPI 3-kinase, or over-expression

of dPTEN results in a decrease in cell size and number. More recently, a partial loss-of-function mutation in dPDK1 was shown to cause a 15% reduction in fly body weight and a 7% reduction in cell number [74]. Loss of function mutants of dS6K1 [84] or dPKB reduce *Drosophila* cell size without affecting cell number [82,85]. PKB and S6K have also been knocked out in mice, but these studies are complicated by the presence of two isoforms of S6K (S6K1 and S6K2) and three isoforms of PKB (PKBα, PKBβ, and PKBγ) encoded for by distinct genes, in contrast to *Drosophila*, which have one isoform of these enzymes. Mice lacking S6K1 were viable, but adult mice were 15% smaller and possessed 10 to 20% reduced organ masses [86]. It was subsequently shown that S6K1 knockout mice possessed a reduced pancreatic islet β-cell size but the size of other cells types investigated was apparently unaffected [87]. Mice lacking PKBα were also reported to be 20% smaller than wild-type animals, but it was not determined whether the lack of PKBα resulted in a reduction of cell size or cell number [88,89]. In contrast, deletion of PKBβ caused insulin resistance without affecting mouse size [90].

PDK1 hypomorphic mutant mice that express only ≈10% of the normal level of PDK1 in all tissues have been generated [68]. These mice are viable and fertile, and despite the reduced levels of PDK1, injection of these mice with insulin induces the normal activation of PKB, S6K, and RSK in insulin-responsive tissues. Nevertheless, these mice have a marked phenotype, being 40 to 50% smaller than control animals. The volumes of the kidney, pancreas, spleen, and adrenal gland of the PDK1 hypomorphic mice are reduced proportionately. Furthermore, the volume of adrenal gland zona fasciculata cells is 45% lower than control cells, whereas the total cell number and the volume of the nucleus remains unchanged. Cultured embryonic fibroblasts from the PDK1 hypomorphic mice are also 35% smaller than control cells but proliferate at the same rate. Embryonic endoderm cells completely lacking PDK1 from E7.5 embryos were 60% smaller than wild-type cells [68]. These results establish that, as in *Drosophila*, PDK1 plays a key role regulating cell size in mammals. However, the finding that AGC kinases tested are still activated normally in the PDK1 hypomorphic mice may suggest that PDK1 regulates cell size by a pathway that is independent of PKB, S6K, and RSK, although this hypothesis requires further investigation. In this regard, Tian *et al.* [91] have recently reported that PDK1 can interact via its noncatalytic N terminus with the PI 3-kinase-regulated Ral GTP exchange factor, leading to its activation. The Ral GTPase has not been implicated in regulating cell size, but it will be important to investigate whether activation of Ral GTPases is defective in PDK1 hypomorphic or knockout cell lines or mice tissues.

Hydrophobic Motif of AGC Kinases

All insulin and growth-factor-activated AGC kinases, in order to become maximally activated, require phosphorylation of a residue located in a region of homology to the hydrophobic motif of PKBα that encompasses Ser473. This is located ≈160 amino acids C-terminal to the T-loop residue lying outside the catalytic regions of these enzymes. This hydrophobic motif is characterized by a conserved motif: Phe–Xaa–Xaa–Phe–Ser/Thr–Tyr/Phe (where Xaa is any amino acid and the Ser/Thr residue is equivalent to Ser473 of PKB). Atypical PKC isoforms (PKCζ, PKCλ, PKCτ) and the related PKC isoforms (PRK1 and PRK2), instead of possessing a Ser/Thr residue in their hydrophobic motifs, have an acidic residue. PKA, in contrast, possesses only the Phe–Xaa–Xaa–Phe moiety of the hydrophobic motif, as the PKA amino acid sequence terminates at this position [92]. PDK1 is the only AGC kinase member that does not appear to possess an obvious hydrophobic motif [92], and the implications of this are discussed below. A major outstanding challenge is to characterize the mechanism by which PKB and other AGC kinases are phosphorylated at their hydrophobic motifs. In spite of considerable effort to discover the kinases responsible for the phosphorylation of AGC kinase members, no convincing evidence has thus far been obtained. The extensive literature and considerable controversy in this area have been extensively reviewed [93]. The only exception is for RSK and conventional PKC isoforms. For RSK, the phosphorylation of the C-terminal non-AGC kinase domain of this enzyme by ERK1/ERK2 triggers this domain to phosphorylate the N-terminal AGC kinase domain at its hydrophobic motif [21]. In the case of conventional PKC isoforms, there is good evidence that these enzymes can autophosphorylate themselves at their hydrophobic motifs following phosphorylation of their T-loops by PDK1 [22].

Mechanism of Regulation of PDK1 Activity

An important question is to determine the mechanism by which the ability of PDK1 activity to phosphorylate its AGC kinase substrates is regulated by extracellular agonists. When isolated from unstimulated or cells stimulated with insulin or growth factors, PDK1 possesses the same activity toward PKB or S6K1 [41,49,94]. Furthermore, although PDK1 is phosphorylated at 5 serine residues in 293 cells, insulin or insulin-like growth factor 1 (IGF1) did not induce any change in the phosphorylation state of PDK1 [95]. Only one of these phosphorylation sites (namely, Ser241) was essential for PDK1 activity. Ser241 is located in the T-loop of PDK1, and, because PDK1 expressed in bacteria is stoichiometrically phosphorylated at Ser241, it is likely that PDK1 can phosphorylate itself at this residue [95]. Although PDK1 becomes phosphorylated on tyrosine residues following stimulation of cells with peroxovanadate (a tyrosine phosphatase inhibitor) or over-expression with a Src-family tyrosine kinase [96–98], no tyrosine phosphorylation of PDK1 has been detected following stimulation of cells with insulin [95,96].

Taken together, these observations suggest that PDK1 might not be activated directly by insulin/growth factors.

Instead, one possibility that might explain how PDK1 could phosphorylate a number of AGC kinases in a regulated manner is that PDK1, instead of being activated by an agonist, is constitutively active in cells and that it is the substrates that are converted into forms that can interact with PDK1 and thus become phosphorylated at their T-loops. In the case of PKB as discussed above, it is the interaction of PKB with PtdIns(3,4,5)P_3 that converts it into a substrate for PDK1. In the case of other AGC kinases that are activated downstream of PI 3-kinase, such as S6K, SGK, and PKC isoforms, which do not possess a PH domain and thus do not interact with PtdIns(3,4,5)P_3 and whose phosphorylation by PDK1 *in vitro* is not enhanced by PtdIns(3,4,5)P_3, it is not obvious how PtdIns(3,4,5)P_3 can regulate the phosphorylation of these enzymes *in vivo*. Recent studies indicate that a conserved motif located C-terminal to the catalytic domains of isoforms of most AGC kinases (the hydrophobic motif of S6K1,or SGK1 [44]) and atypical (PKCζ) and related PKC (PRK2) isoforms [57] can interact with a hydrophobic pocket in the kinase domain of PDK1 (the PIF pocket) [92]. Evidence indicates that this results in a docking interaction, which is required for the efficient T-loop phosphorylation of AGC kinases that do not interact with PtdIns(3,4,5)P_3/PtdIns(3,4)P_2. These experiments indicate that the interaction of S6K and SGK with PDK1 is significantly enhanced if these enzymes are phosphorylated at their hydrophobic motifs in a manner equivalent to that of the Ser473 phosphorylation site of PKB [44]. It is therefore possible that PtdIns(3,4,5)P_3 does not activate PDK1 but instead induces phosphorylation of S6K and SGK isoforms at their hydrophobic motifs, thereby converting these enzymes into forms that can interact with PDK1 and hence become activated. Consistent with this notion, the expression of mutant forms of S6K1 and SGK1 in which the hydrophobic motif phosphorylation site is altered to Glu to mimic phosphorylation is constitutively phosphorylated at their T-loop residues in unstimulated cells [50,99,100]. It is currently not clear how PtdIns(3,4,5)$_3$ could stimulate the phosphorylation of the hydrophobic motif, but it is possible that it could either activate the hydrophobic motif kinases or inhibit the hydrophobic motif phosphatases.

Frodin *et al.* [101] demonstrated that phosphorylation of the hydrophobic motif of p90RSK (which is induced following phosphorylation of p90RSK by ERK1/ERK2 [21]) strongly promotes its interaction with PDK1, therefore enhancing the ability of PDK1 to phosphorylate p90RSK at its T-loop motif. Thus, the phosphorylation of p90RSK by ERK1/ERK2 converts RSK into a form that can interact with and be activated by PDK1. Thus, the mechanism by which PDK1 recognizes isoforms of RSK is analogous to that by which it recognizes SGK/S6K, the only difference being the mechanism regulating phosphorylation of the hydrophobic motifs of these enzymes. The model of how isoforms of PKB, S6K, SGK, and RSK are activated by PDK1 is summarized in Fig. 3.

Related PKC isoforms (PRK1 and PRK2) and atypical PKC isoforms (PKCζ and PKCτ) possess a hydrophobic motif in which the residue equivalent to Ser473 is Asp or Glu, and these enzymes can in principle interact with PDK1 as soon as they are expressed in a cell [57]. However, it is possible that the interaction of related PKC isoforms and atypical PKC isoforms with PDK1 could be regulated through the interaction of these enzymes with other molecules. For example, the interaction of PRK2 with Rho-GTP [60] or PKCζ with hPar3 and hPar6 [102] might induce a conformational change in these enzymes that controls their interaction with PDK1.

PDK1 would be expected to activate PKB at the plasma membrane and its other non-3-phosphoinositide binding substrates in the cytosol. Consistent with this finding, PDK1 has been found to be localized in mainly the cytosol and plasma membrane of both stimulated and unstimulated cells [43,94]. It is controversial as to whether or not PDK1 translocates to the plasma membrane of cells in response to agonists that activate PI 3-kinase. Three reports [43,94,96] indicate that a small proportion of PDK1 is associated with the membrane of unstimulated cells, and they do not report any further translocation of PDK1 to membranes in response to agonists that activate PI 3-kinase and PKB. However, other groups have reported that PDK1 translocates to cellular membranes in response to agonists that activate PI 3-kinase [103,104]. Indeed, as mentioned earlier, there is evidence that at least some PDK1 is likely to be located at cell membranes of unstimulated cells as the expression of a membrane-targeted PKB construct in such cells is active and fully phosphorylated at Thr308 [33,36].

Structure of the PDK1 Catalytic Domain

Further insight into the mechanism by which PDK1 interacts with its AGC kinase substrates has been obtained recently from the high-resolution crystal structure of the human PDK1 catalytic domain. The structure defines the location of the PIF pocket on the small lobe of the catalytic domain—a marked hydrophobic pocket in the small lobe of the kinase domain [105] that corresponds to the region of the catalytic domain predicted from previous modeling and mutational analysis to form the PIF pocket [92]. Interestingly, mutation of several of the hydrophobic amino acids that make up the surface of this pocket abolish or significantly inhibit the ability of PDK1 to interact and activate S6K1 and SGK1 [44], indicating that this hydrophobic pocket does indeed represent the PIF pocket. As phosphorylation of the hydrophobic motif of S6K1 and SGK1 promotes the binding of S6K1 and SGK1 with PDK1, this suggests that a phosphate-interacting site is located near the PIF pocket. Interestingly, close to the PIF pocket in the PDK1 crystal structure, an ordered sulfate ion was interacting with four surrounding side chains (Lys76, Arg131, Thr148, and Gln150). Mutation of Lys76, Arg131, or Q150 to Ala reduces or abolishes the ability of PDK1 to interact with a phospho-peptide that encompasses the phosphorylated residues of the hydrophobic motif of S6K1, thereby

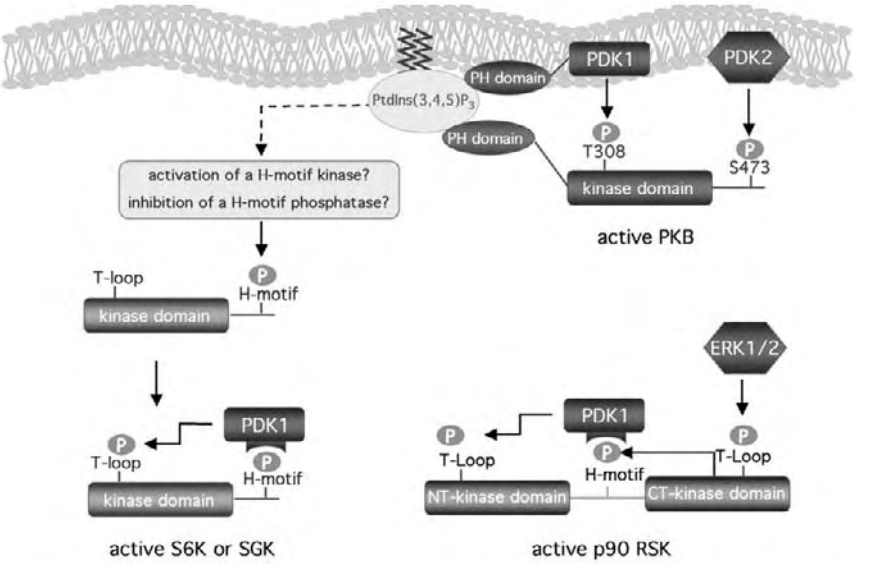


Figure 3 The mechanism by which phosphorylation of PKB, S6K SGK, and RSK by PDK1 is regulated. It should be noted that in this model of how PKB, S6K, SGK, and RSK are phosphorylated at their T-loop, PDK1 activity is not directly activated by insulin or growth factors, consistent with the experimental observation that PDK1 is constitutively active in cells. Instead, it is the substrates of PDK1 that are converted into forms that can be phosphorylated. In the case of PKB, it is the interaction of PKB with PtdIns(3,4,5)P_3 at the plasma membrane that colocalizes PDK1 and PKB and also induces a conformational change in PKB that converts it into a substrate for PDK1. In the case of S6K and SGK, which do not possess PH domains and cannot interact with PtdIns(3,4,5)P_3, this is achieved by the phosphorylation of these enzymes at their hydrophobic motif (H-motif) by an unknown mechanism, which thereby generates a docking site for PDK1. RSK isoforms possess two catalytic domains: an N-terminal AGC-kinase-like kinase domain and a C-terminal non-AGC kinase domain. The activation of RSK isoforms is initiated by the phosphorylation of these enzymes by the ERK1/ERK2 classical MAP kinases, which phosphorylate the T-loop of the C-terminal kinase domain. This activates the C-terminal kinase domain, which then phosphorylates the hydrophobic motif of the N-terminal AGC kinase. This creates a binding site for PDK1 to interact with RSK isoforms, leading to the phosphorylation of the T-loop of the N-terminal kinase domain and activating it. Phosphorylation of all RSK substrates characterized thus far is mediated by the N-terminal kinase domain; however, it is possible that the C-terminal domain of this enzyme will phosphorylate distinct substrates that have not as yet been identified.

suggesting that this region of PDK1 does indeed represent a phosphate docking site [105]. The only other AGC kinase for which the structure is known (namely, PKA) also possesses a hydrophobic pocket at a region of the kinase catalytic domain equivalent to that of PKA which is occupied by the four C-terminal residues of PKA(FXXF) and resembles the first part of the hydrophobic motif phosphorylation site of S6K and SGK (FXXFS/TY) in which the Ser/Thr is the phosphorylated residue [92]. Occupancy of this pocket of PKA by the FXXF residues is likely to be essential to maintaining PKA in an active and stable conformation, as mutation of either Phe residue drastically reduces PKA activity toward a peptide substrate, as well as reducing PKA stability [106,107]. In contrast to the PIF-pocket in the PDK1 structure, PKA does not possess a phosphate docking site located next to the hydrophobic FXXF binding pocket. Sequence alignments of the catalytic domains of AGC kinases, including PDK1, indicate that all AGC kinases possess a PIF pocket, and kinases such as isoforms of RSK, PKB, S6K, and SGK possess a phosphate docking site next to this pocket. The role of these pockets of the AGC kinases is probably to interact with their own hydrophobic motifs, and this interaction may account for the ability of these kinases to be activated following the phosphorylation of their hydrophobic motif. However, unlike other AGC kinases, PDK1 does not possess a hydrophobic motif C-terminal to its catalytic domain and therefore utilizes its empty PIF/phosphate binding pocket to latch onto its substrates that are phosphorylated at their hydrophobic motifs, thereby enabling PDK1 to phosphorylate these enzymes at their T-loop residue and activate them.

Concluding Remarks

Elucidation of the mechanism by which PKB was activated by PDK1 in cells provided the first example of how the second messenger PtdIns(3,4,5)P_3 could activate downstream signaling processes. However, there remain many major unsolved questions for future research to address. A major challenge will be to clarify the mechanism by which PtdIns(3,4,5)P_3 induces the phosphorylation of the

hydrophobic motif of PKB and other AGC kinases members, which is a key trigger for the activation of these enzymes. The results discussed in this chapter also provide a framework within which drugs could be developed to inhibit the PDK1/AGC kinase pathway to treat forms of cancers in which this pathway may be constitutively activated. Indeed, it is now estimated that PTEN is mutated in up to 30% of all human tumors, resulting in elevated PtdIns(3,4,5)P_3 levels and hence PKB and S6K activity which are likely to contribute to the proliferation and survival of these tumors [108]. It could be envisaged that a PDK1 inhibitor would be effective at reducing the PKB and S6K activities that contribute to growth and survival of these tumors.

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CHAPTER 155

Modulation of Monomeric G Proteins by Phosphoinositides

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Introduction

Most, if not all, membranes in eukaryotic cells carry low mole percent phosphoinositides. These lipids act as regulatable scaffolds, dictating the localization and functions of many proteins on the membrane surface. A clear example of this principle is at the inner leaflet of the plasma membrane, where PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are rapidly generated following cell-surface receptor activation of type I phosphoinositide 3OH-kinases (PI3K; Chapter 25 by D. Fruman) and act to recruit several PH domain containing proteins. These translocations are driven by the high specificity and affinity of specific PH domains for PtdIns(3,4,5)P_3 and PtdIns-(3,4)P_2 (Chapter 29 by M. Lemmon). Many of the proteins that harbor PH domains are enzymes that regulate the activity of monomeric (small) GTPases: the guanine nucleotide exchange factors (GEFs), which promote GTP-loading of the GTPase, and GTPase activating proteins (GAPs), which enhance the endogenous GTPase activity of the GTPase. Small GTPases are considered to be active or "on" in their GTP-bound form and inactive or "off" in their GDP-bound form. Thus, historically, GEFs are generally considered to be activators and GAPs to be inactivators. More recent work suggests this is too simplistic. It appears that monomeric GTPases often need to cycle between on/off states to function effectively and that the GAPs can themselves be the target or effector of the monomeric GTPase or act as scaffolds to bring the monomeric GTPase together with targets and hence dictate the context of its activation. We summarize here recent evidence on the modulation of GEFs and GAPs by phosphoinositides with a focus on events regulated by PI3K.

Rho Family Small GTPases

Rho family GTPases are best known for their ability to modulate the actin cytoskeleton (where Rho regulates the formation of stress fibers, Rac regulates membrane ruffles, and Cdc42 regulates filopodia) but they are also involved in the control of such diverse processes as NADPH oxidase, transcriptional activation, G1 cell cycle progression, cell transformation, and secretion [1].

Direct Interactions of Rho family GTPases with Phosphoinositides

Both Rac and Cdc42 interact directly with PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 with an apparent affinity of 4.5 μM [2]. Lipid binding has been mapped to two sites containing abundant hydrophobic and positively charged sites on the GTPase, and leads to the enhanced dissociation of Rac-associated guanine nucleotides but not association of GTP and hence activation.

Modulation of Rac GEFs by Phosphoinositides

PI3K is thought to be involved in the activation of Rac by stimulating the following Dbl family GEFs: SOS, Vav1, Tiam-1, P-Rex, and possibly PIX. Like all Rho family GEFs [3] they contain the characteristic Dbl homology (DH)-PH domain module (see Fig. 1).

Apart from its well-characterized function as a Ras GEF, SOS functions also as a Rac GEF. The SOS PH domain translocates to the plasma membrane and preferentially to

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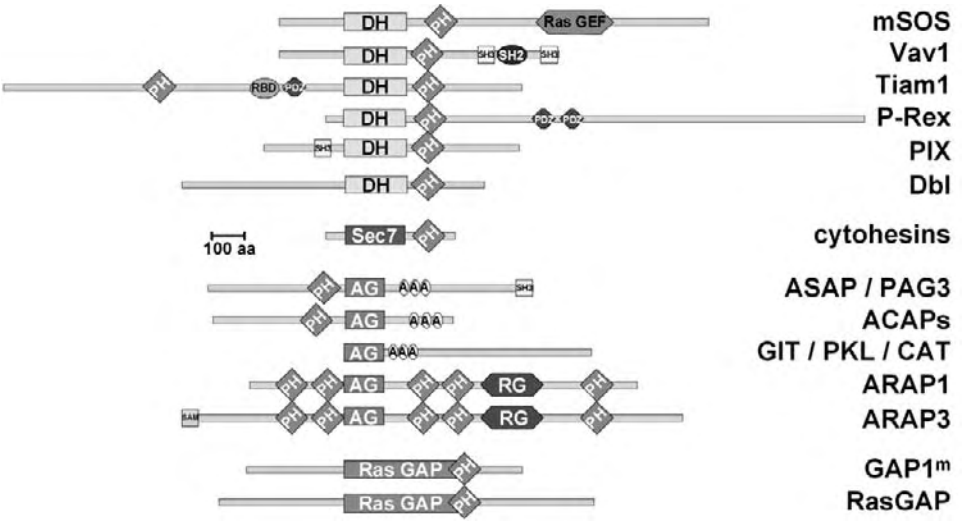


Figure 1 Alignment of all GEF and GAP proteins discussed along their relevant catalytic domains. Domains are drawn as determined via the SMART program. Abbreviations: A, ankyrin repeat; RBD, Ras binding domain; RG, Rho GAP; AG, Arf GAP domains.

leading edges after serum stimulation [4], and this is PI3K dependent. This phenomenon depends on the presence of key residues within the SOS PH domain mediating the interaction with PtdIns(3,4,5)P_3. The SOS DH domain alone functions as a Rac GEF both *in vitro* and *in vivo* [5], but a DH-PH construct is active only in the presence of an activating signal for PI3K. Current evidence suggests PtdIns(3,4,5)P_3-binding to the PH domain relieves an autoinhibitory constraint on the DH domain leading to an increase in catalyic activity toward Rac [6,7].

Vav proteins (Vavs 1–3) are essential for cytoskeletal, proliferative, and developmental pathways in lymphoid cells. Vav-1 is the only family member known to be regulated by PI3K; its regulation has been studied in-depth *in vitro*. Based on structural studies of Vav1 [8], the current model is that GEF activity of Vav1 is autoinhibited by the binding of its N-terminus to the DH domain. Phosphorylation of Y174 by Src-type tyrosine kinases [9] causes the release of the N-terminal inhibitory peptide and this transition is facilitated by PtdIns(3,4,5)P_3, but not PtdIns(4,5)P_2, binding to the PH domain [6,10]. The evidence is less clear-cut *in vivo*, but Gringhuis *et al.* [11] demonstrated convincingly, that in CD5 receptor signaling in T lymphocytes, Vav lies upstream of Rac and downstream of PI3K.

Tiam-1 is a broadly expressed, PI3K-regulated Rac GEF involved in cell adhesion and migration. It contains two PH domains. The N-terminal one binds with high affinity to PtdIns(3,4,5)P_3, is crucial for activation of Rac (ruffling), and plays a role in, but does not dictate, membrane localization [12,13]. Both *in vivo* and *in vitro* evidence suggests that Tiam-1 is regulated by threonine phosphorylation by CaMKII [14] and by binding of PtdIns(3,4,5)P_3 to the Tiam1 N-terminal PH domain [15], both of which moderately activate Tiam-1 catalytic activity, in a cooperative fashion [15]. Neither interactions with nor activation by PtdIns(3,4,5)P_3 of a Tiam1 DH–PH domain construct could be reproduced in a recent study [16].

P-Rex (PtdIns(3,4,5)P_3-dependent Rac exchanger) was purified from porcine neutrophils as a PtdIns(3,4,5)P_3-activated Rac GEF that would be responsible for regulating Rac downstream of activation of heterotrimeric G proteins [17]. Analysis of recombinant P-Rex1 shows that it interacts with lipid vesicles in a PtdIns(3,4,5)P_3-dependent fashion. P-Rex1 RacGEF activity is directly and substantially activated by G protein $\beta\gamma$ subunits and PtdIns(3,4,5)P_3 in a synergistic fashion both *in vivo* and *in vitro*.

PIX was identified as a Pak-binding protein with GEF activity toward Rac and Cdc42 involved in the recruitment of PAK to focal adhesions. PIX binds directly to GIT, a potential PtdIns(3,4,5)P_3-stimulated Arf GAP ([18]; see s "PtdIns(3,4,5)P_3-regulated Arf GEFs: The Cytohesin Family," below). PIX interacts with the p85 regulatory subunit of type IB PI3K, and PIX GEF activity is very weakly stimulated by PtdIns(3,4,5)P_3 [19], but the mechanism is unclear.

Phosphoinositide Binding to Cdc42 GEFs

The PH domain of Dbl is reported to bind to PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. Both phosphoinositides inhibit Dbl GEF activity. Dbl is partially localized to the plasma membrane in a PH domain dependent fashion [20]. It is interesting that Dbl PH domain point mutants that do not bind phosphoinositides and confer increased Cdc42 GEF activity do not promote focus formation. Another study reports binding of PtdIns(4,5)P_2 to DH–PH constructs of Dbs (Dibl's big sister) and intersectin, but neither GEF activity was found to be affected by its inclusion into assays *in vitro* [16].

Arf Family GTPases

Arf family G proteins [21] differ from other small G proteins in that they do not have any detectable intrinsic GTPase activity; therefore they have an absolute requirement for both GEFs and GAPs to cycle rapidly between GTP- and GDP-bound states. The closely related Arf family members are grouped into class I (Arfs 1–3), class II (Arfs 4 and 5) and Class III (Arf 6). Class I Arfs are primarily involved in trafficking in the ER-Golgi and endosomal systems. Little is known about class II Arfs. Arf 6 functions in endosomal and plasma membranes to regulate secretion and coordinate cytoskeletal changes (ruffling) in collaboration with Rac, which it transports to the plasma membrane [22]. Phosphoinositides have regulatory inputs into Arfs 6 and 1 by acting on both their GEFs and GAPs.

PtdIns(3,4,5)P_3-regulated Arf GEFs: The Cytohesin Family

Out of the growing number of Arf GEFs [23] only the cytohesin family (comprising the highly homologous Cytohesin 1 and 4, ARNO and Grp1) are regulated by phosphoinositides. Cytohesins share with other Arf GEFs the catalytic Sec7 domain and contain further a characteristic C-terminal PH domain (Fig. 1), which selectively binds specific phosphoinositides. The cytohesins exhibit characteristically dramatic translocations from the cytosol to the plasma membrane that are both dependent on PI3K activity and a functional PH domain. This translocation probably represents their main mode of activation. The field is littered with controversy regarding cytohesin family lipid-binding specificities, an issue that may be explained by the presence of allelic variants of all cytohesins bar cytohesin 4 [24]. The variants are distinguished by the insertion of a single glycine residue into the lipid-binding specificity-determining region of the PH domain. Binding and selectivity of diglycine PH domains to PtdIns(3,4,5)P_3 over PtdIns(4,5)P_2 or PtdIns(3,4)P_2 is exquisite; tri-glycine PH domains are less selective and bind less tightly. Hence, isolated diglycine PH domain constructs can translocate to membranes containing PtdIns(3,4,5)P_3, whereas triglycine PH domains translocate only in the context of the full-length GEFs [25], when a polybasic stretch C-terminally adjacent of the PH domain enhances membrane association [26]. ARNO is phosphorylated by PKC on serine 392, which lies in the polybasic stretch adjacent to the PH domain. The negative charge conferred by the phosphate reduces catalytic activity and binding to membranes, suggesting that PKC may act to switch off ARNO GEF activity [27]. It is interesting that 80% of GRP-1 is expressed in the diglyine version whereas Arno and cytohesin-1 are predominently in the triglycine form [24], possibly conferring differential sensitivity to PI3K activation.

A second issue of controversy concerns Grp1 and ARNO substrate specificities. Their abilities to use Arf6 as a substrate *in vitro* appear to depend on the precise assay conditions used, whereas Arfs 1 and 5 act as more robust substrates [28,29]. *In vivo*, all cytohesins have been shown very convincingly to translocate from a cytoplasmic location to the plasma membrane in a PI3K-dependent fashion ([30] and references therein). This coincides with Arf6 distribution, which cycles between endosomal compartments and plasma membrane, but not Arf1, which is confined to intracellular membranes. Indeed, Arf6 and ARNO have been shown to co-localize in ruffles in a PtdIns(3,4,5)P_3-, GEF activity-, and PH domain-dependent fashion [30].

PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3-regulated Arf GAPs

Arf GAPs are characterized by a Zn-finger containing Arf GAP domain and adjacent ankyrin repeats. Our knowledge of Arf GAPs has expanded dramatically over the last few years [23]. Many Arf GAPs are regulated by phosphoinositides. The Pap/ASAP/ACAP family is activated by binding to PtdIns(4,5)P_2 whereas the GIT/CAT/PKL and ARAP families bind to and are activated by PtdIns(3,4,5)P_3 (Fig. 1). Both Pap (PAG3)/ASAP/ACAPs and GIT/CAT/PKLs function in cytoskeletal remodeling and associate with paxillin (reviewed in [31]).

The Pap, ASAP, and ACAPs ([31] and references therein; [32,33]) are all robustly activated in their Arf GAP activities by PtdIns(4,5)P_2 (in the context of phosphatidic acid), which they bind to using their PH domains. ASAP and Pap/PAG3 function as Arf 1 and 5 GAPs *in vitro*, but there is some *in vivo* evidence for Pap/PAG3 acting on Arf 6 also. ACAPs function as PtdIns(4,5)P_2-stimulated Arf 6 GAPs *in vitro* and *in vivo*. There is an intriguing link between PDGF and ASAP/ACAP Arf GAPs, which are reported to localize to PDGF-induced dorsal ruffles ([34]; although this has not been demonstrated by video imaging) and inhibit their formation when overexpresssed [35,36]. The role of the PH domain has been investigated further in the context of ASAP [34]. It doesn't bind preferentially to PtdIns(3,4,5)P_3 and supports but is not vital for the observed translocation; it is crucial for catalytic Arf GAP activity, leading to a model of ASAP regulation, where Arf GAP and PH domains interact until lipid binding by the PH domain allows freeing of the Arf GAP catalytic site.

GIT family proteins have been identified in various screens as binding partners for paxillin [see 31], the Rho GEF PIX [18,35], and G-protein coupled receptor kinases (GRK; [36]). Several family members exist in addition to multiple spliced variants, which appear to be expressed tissue-specifically [36]. GIT1 and 2 were analyzed in terms of their GAP activities and both were found to use Arfs 1, 5, and 6 as substrates. Their catalytic activity was enhanced moderately by high concentrations (200 μM) of PtdIns(3,4,5)P_3 but not PtdIns5P, PtdIns(4,5)P_2, or diacylglycerol [37]. We do not know how PtdIns(3,4,5)P_3 interacts with GIT proteins, since they lack domains known to mediate interactions with lipids.

The second class of PtdIns(3,4,5)P_3 regulated Arf GAPs are ARAPs. Their unusual domain structure comprising 5 PH domains, as well as Arf- and Rho GAP domains, predicts that these proteins are ideally suited to mediate cross-talk

between small G-protein families. ARAP1 and 2 were identified by homology-based cloning [38] and ARAP3 on the basis of its binding to PtdIns(3,4,5)P_3 [39]. ARAP1 is a PtdIns-(3,4,5)P_3-dependent Arf GAP specific for Arf 1 and Arf 5 *in vitro*. *In vivo*, ARAP1 localizes to Golgi structures and regulates cell spreading and the formation of filopodia via the regulation of Arf 1/5 and Cdc42. In contrast, ARAP3 is a promiscuous Rho GAP *in vitro* and is a very specific, PtdIns-(3,4,5)P_3-dependent Arf 6-GAP *in vitro* and *in vivo*. *In vivo*, ARAP3 causes dynamic remodeling of the actin cytoskeleton and a striking loss of adhesion in a PI3K dependent manner.

Modulation of Ras Family GTPases by PI3K

The best understood pathway regulated by Ras family G proteins is the Raf-Erk-MAPK cascade. In addition, Ras has a well-established role in the activation of class I PI3K [40], but there are now numerous, cell-type specific examples in which PI3K has a regulatory role upstream of Ras. One hypothesis is that low amounts of PtdIns(3,4,5)P_3 (generated in most attached cells via integrin-engagement) may fulfill a permissive role in the activation of Ras, possibly allowing the recruitment of Shc-Grb2-SOS RasGEF complexes to the plasma membrane in the absence of significant recruitment via the phosphorylated tails of activated growth-factor receptors [41]. Alternatively, PI3K might modulate some RasGAPs (p120RasGAP, GAP1^m) as they clearly possess PH domains capable of binding PtdIns(3,4,5)P_3. Insulin-stimulation of Swiss 3T3 adipocytes causes a PI3K-dependent inhibition of RasGAP, leading to activation of Ras [42]. Similarly, in U937 cells, PI3K inhibitors act to inhibit Ras, which is mediated via an increase in GTP hydrolysis on Ras, indicating negative control of a RasGAP by PI3K [43]. GAP1^m has been shown to be recruited to the plasma membrane in a PH-domain and PI3K-dependent fashion [44]. Curiously, neither PtdIns-(3,4,5)P_3 nor its soluble headgroup Ins(1,3,4,5)P_4 could significantly influence GAP1^m RasGAP activity *in vivo* or *in vitro* [44,45], thus raising the question as to whether it may influence an effector function of the RasGAP rather than the GAP activity.

Conclusion

Monomeric GTPases are molecular switches that drive many complex processes involving cellular membranes. They are characteristically lipid modified in their active states and hence retained at the lipid bilayer. Their activities are controlled by the actions of "GTP-loading" GEFs and "GTP-hydrolizing" GAPs whose activities appear to be regulated by the presence of phosphoinositides in the lipid surface in which they reside. In some cases, it is clear that the phosphoinositides act predominantly to localize and hence concentrate the GEF/GAP with the GTPase (usually via direct binding to an appropriate PH domain); perhaps the clearest example is in PtdIns(3,4,5)P_3-dependent recruitment of cytohesin family Arf GEFs from the cytosol to the plasma membrane. In other examples of GEF/GAP regulations by phosphoinositides, bulk translocation is probably irrelevant or only part of the regulatory mechanism; in these cases phosphoinositide binding allows some form of allosteric change leading to an increase in catalytic activity. In the best-studied examples, phosphoinositide binding to a PH domain relieves an autoinhibitory constraint on a neighboring catalytic domain, and given the almost universal positioning of PH domains adjacent to GEF and GAP catalytic modules (see Fig. 1), this may prove to be a general principle. Both of these types of mechanisms can be seen as "acutely regulatory" (where the levels of the appropriate phosphoinositides are changed rapidly, e.g. PtdIns(3,4,5)P_3 synthesis via receptor regulated PI3Ks) or more "permissive" (where the levels of the phosphoinositides are more constant, e.g. arguably in examples of PtdIns(4,5)P_2 regulation). Clearly more focused work needs to be done before we have a satisfactory explanation of how phosphoinositides regulate the activity of individual GEFs and GAPs, but it is already clear just how universal this form of regulation appears to be and hence how important phosphoinositides are for coordinating the regulation of this class of molecules.

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CHAPTER 156

Phosphoinositides and Actin Cytoskeletal Rearrangement

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Historical Perspective

Cytoskeletal proteins were the first proteins shown to be regulated by phosphoinositides (PPIs), beginning with the report by Lassing and Lindberg that PIP2 dissociated profilin-actin complexes *in vitro* and promoted actin polymerization [1]. This finding suggested that increases in cellular PIP2 would drive the polymerization of cytoskeletal actin. At that time products of the PI3-kinase pathway had not yet been implicated in cell signaling, and it was thought that PI(4,5)P2 was the primary lipid responsible for direct effects on profilin, a hypothesis that is largely supported by many subsequent studies of actin-binding proteins. Since that time dozens of actin-binding proteins have been found to be either activated or inhibited by PPIs *in vitro,* usually by PIP2, and studies in the last few years confirm that at least some of these reactions occur in a similar way in cytoplasm. The fundamental predictions that increased PPI synthesis leads to actin assembly and depletion of PPIs triggers actin depolymerization have been borne out in studies in which PPI levels are altered in cells either by manipulation of expression of lipid kinases [2–5] or phosphatases [3,6–10] or by introduction of constructs such as PH domains [8,11] or PIP2-binding peptides [12–15] that sequester the lipids and may mimic the effects of endogenous proteins whose cellular role appears to involve sequestration of membrane phosphoinositides [16–18].

In the last several years the number of PPI-binding proteins has increased greatly, and actin-binding proteins are now a minority of the total ligands proposed for these lipids. Many of the newly reported proteins were identified by their possession of PH, FYVE, PX, or other PPI-binding motifs and the lipid-binding potential measured after this identification. Often these protein modules bind specifically to PPIs generated by PI3-kinases rather than to PI(4)P or PI(4,5)P2. In contrast, most PPI-binding cytoskeletal proteins were first identified biochemically to interact with PIP2 and the specific binding sites sought only afterward. It is noteworthy that the structures of PPI binding sites in cytoskeletal proteins are less well characterized than the motifs listed above, and it is perhaps not a coincidence that the PPI-binding domains common to proteins involved in vesicle traffic or spatial localization of signaling are conspicuously missing from most actin-binding proteins.

This review will focus on recent advances that demonstrate how PPIs are involved in stimulation of actin polymerization *in vivo*, or activation of proteins involved in the formation of cytoskeleton and membrane links, and how binding of cytoskeletal proteins to membrane PPIs may relate to the lateral or transverse movement of lipids to affect raft formation or lipid asymmetry.

Stimulating Cellular Actin Polymerization

There is increasing evidence for a localized increase in PIP2 at sites of actin polymerization and remodeling using the fluorescent chimera GFP-PH-PLCδ1 as a PIP2 reporter [19] (see section entitled Relation of Actin Assembly to

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Phosphoinosite-containing Lipid Rafts). Localized PIP2 increase may depend on small GTPases in the Rho family (Rac and Rho) and the ADP ribosylation factor family (Arf6, Arf1). These GTPases have profound effects on the actin cytoskeleton, and can either alter the activity of type I phosphatidylinositol 4 phosphate 5 kinases (PIP5K), the enzymes that convert PI4P to PIP2, or recruit them to sites of actin polymerization [5]. For example, Rac and Rho bind PIP5Ks and recruit them to the plasma membrane [20] while Arf6 acts downstream of Rac to activate PIP5Ks [21].

The Mechanisms of Actin Polymerization

Since actin polymerization *in vivo* occurs primarily through the rapid growing end (+) of actin nuclei, the mechanisms by which (+) ends are generated are of intense interest. Three mechanisms have been proposed (reviewed in [22,23]): first, *de novo* actin nucleation by the Arp2/3 complex as a result of activation by the Wiskott-Aldrich syndrome family proteins (WASP, N-WASP etc.) or other proteins; second, severing of preexisting filaments by cofilin/actin depolymerizing protein (cofilin/ADF) or gelsolin family proteins; third, uncapping of the (+) end by capping proteins such as CapZ. Once the (+) ends are liberated, actin monomer delivery is accelerated by profilin and funneling of actin monomers to the favored sites by (+) end capping at other sites. The supply of actin monomer is sustained by severing and facilitated depolymerization from the (−) end by cofilin/ADF.

PIP2 alters *in vitro* the activity of critical proteins in each of these steps. It activates N-WASP, synergistically with Cdc42 [24] or with SH3 adapters such as Nck independently of Cdc42 [25], to promote *de novo* nucleation from the Arp2/3 complexes by an unmasking mechanism depicted in Fig. 1C. In contrast, PIP2 inactivates cofilin/ADF, CapZ, profilin and gelsolin-related severing and capping proteins (reviewed in [18]). The mechanism for PIP2 inhibition of these proteins is not completely understood but may involve the changes depicted in Figs. 1A and B (see section entitled Different Mechanisms of PPI-Actin Binding Protein Regulation).

Several recent studies implicate PIP2 in control of the cytoskeleton *in vivo*. Genetic disruption of *Mss4m*, which encodes the single PIP5K in yeast, or *skittles* (one of two PIP5K) in *Drosophila* produce cytoskeletal defects. Since mammalian cells have multiple PIP5Ks, and knockout animals are not yet available, other approaches have been used to examine the role of PIP5K *in vivo*. These include microinjection of an anti-PIP2 antibody [26], introduction of a cell-permeant gelsolin PIP2-binding peptide [13,15], addition of PIP2 or a PIP2-binding peptide to semi-intact cells [5], overexpression of actin regulatory proteins with defective PIP2 binding [27], and manipulation of the expression levels of PIP5Ks [9] and the phosphoinositide phosphatases that dephosphorylate PIP2 [10].

PIP5K overexpression induces dramatic actin phenotypes, establishing a causal relation between PIP2 and actin cytoskeletal dynamics. The responses vary depending on the cell types used and most likely the extent of overexpression. They include NWASP-dependent actin comet tail formation [9], Rho and Rho-kinase dependent actin stress fiber formation [28], and the arrest of Arf6-regulated plasma membrane-endosome recycling [29]. The multiple phenotypes are not surprising, given that PIP5Ks may be regulated by several small GTPases that induce distinct actin structures in a sometimes sequential and at other times mutually exclusive manner. Furthermore, the site of PIP2 generation as well as the subset of actin regulatory proteins at those sites will dictate the dominant response. The actin-modulating proteins that contribute to these phenotypes have been identified in the first two cases, thus providing mechanistic insight into how PIP2 regulates the actin cytoskeleton *in vivo*.

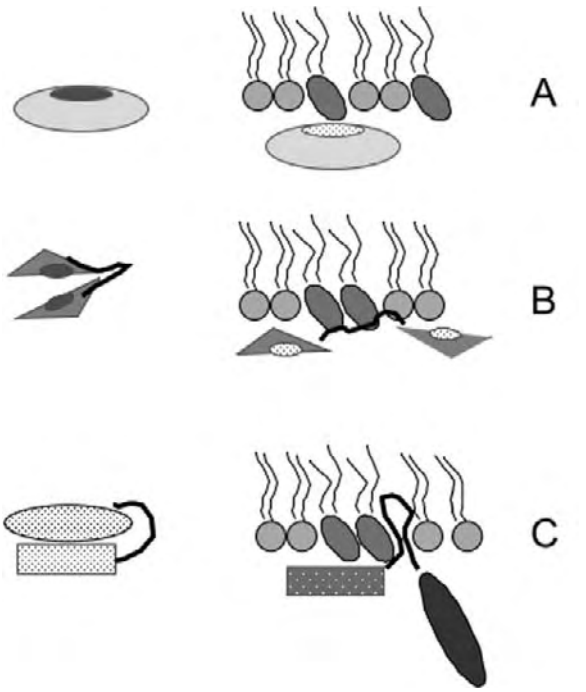


Figure 1 Three models for regulation of protein function by membrane-bound phosphoinositides. Actin-binding sites are shown in blue. Solid patches denote active sites and dotted patches inhibited sites. PIP2 is shown as large-headed lipids within one leaflet of a bilayer composed of neutral lipid. (A) The actin site is occluded by the lipid without other structural change. (B) PIP2 binding reorients two protein domains such that structures required for actin binding can no longer function cooperatively. (C) Protein binds and inserts in membrane to simultaneously stabilize membrane association and expose sites for actin and membrane proteins.

PIP5K Overexpression Induces Actin Comet Formation

Actin comets formed around pathogens, such as *Listeria*, *Shigella,* and vaccinia, have contributed significantly to our understanding of the mechanism of cellular actin polymerization because they either introduce their own membrane protein or hijack the host's N-WASP to initiate actin assembly by the same mechanism used at the cell membrane. Since N-WASP also stimulates actin comet formation around

intracellular vesicles and lipid vesicles in cell extracts [30] and in *Xenopus* eggs [31], the possibility that PIP2 promotes vesicle trafficking by generating actin comets is particularly attractive. Indeed, tiny comets that form spontaneously have been sighted, and much more robust comets are found in cells that overexpress PIP5K [9]. Overexpressed PIP5K is enriched at the head of the comets, establishing that the *in situ* generation of PIP2 at the vesicle may recruit and activate N-WASP to promote *de novo* actin nucleation by Arp2/3. Dynamin, another PIP2-activated protein that is involved in vesicle trafficking, is also recruited to the head of the PIP5K-induced comets, and overexpression of dominant negative dynamin mutants inhibits comet formation [4,32].

Actin comets are formed from endocytic and Golgi-derived exocytic vesicles, particularly those with cholesterol and sphinogolipid-enriched membrane microdomains (rafts), and raft disruption reduces the number of comets dramatically [9]. Rafts have previously been shown to contain an agonist-sensitive pool of phosphoinositides that responds to PLC signaling, and they are the primary sites of PIP2 synthesis [17]. The preferential formation of actin comets in raft domains establishes that rafts are platforms for the integration of PIP2 signaling and actin polymerization, reinforcing the concept that specialized regions of the membrane are closely related to specific cytoskeletal structures discussed in the section on mechanisms of PPI-actin binding protein regulation.

PIP5K Overexpression Induces Actin Stress Fiber Formation

PIP5K overexpression in CV1 cells induces robust actin stress fiber formation and inhibition of membrane ruffling in response to growth factors due to an inability to generate (+) end actin nuclei [28]. These two effects are consistent with activation of Rho and inhibition of Rac-dependent cytoskeletal pathways, respectively, and are remarkably similar to that observed in fibroblasts isolated from gelsolin knockout animals [34]. Gelsolin binding to actin is inhibited in PIP5K-overexpressing cells, suggesting that inhibition of severing by gelsolin and perhaps by cofilin/ADF may account for the formation of long actin filaments and the inability to generate (+) end nuclei to mount an actin polymerization response. Furthermore, profilin and CapZ are also inhibited, while ezrin/radixin/moesin, the membrane-linker proteins (see below), are activated. Together, these changes can amplify the consequences of severing inhibition. In conclusion, these studies show that several PIP2-sensitive actin modulating proteins behave *in vivo* as predicted from their well-characterized behavior *in vitro*.

Relation Among PIP5K, Arf6, and Actin Remodeling

Arf6 overexpression induces actin comets [35] and stimulates membrane ruffling [29]. However, although one study finds comet formation is not inhibited by an antibody to PIP2 [35], other studies show that Arf6 activates PIP5K *in vitro* and *in vivo* [21,29]. Overexpression of a constitutively active Arf6 or PIP5K induces PIP2 generation and actin polymerization around recycling endosomes, eventually trapping them into an aggregate that cannot recycle back to the plasma membrane [29]. The phenotype suggests that cycling of Arf6 between the GTP- and GDP-bound forms is important for actin regulation , and this is likely to be achieved through activation and inactivation of PIP5K. Sustained high-level PIP2 production due to constitutive Arf6 overexpression or PIP5K overexpression promotes polymerization and inhibits depolymerization to generate abnormal actin structures around the vesicles. The abnormally large actin comets found in other cellular contexts [9] are another manifestation of sustained actin polymerization. The requirement for transient changes in PIP2 levels highlights the importance of dissipating PIP2 in a spatially and temporally defined manner. Although PIP2 is hydrolyzed by PLC during agonist signaling, PIP2 is likely to be cleared primarily by phosphoinositide phosphatases.

Effects of Manipulating the Level of Phosphoinositide Phosphatases

Phosphatases that dephosphorylate phosphoinositides or inositol polyphosphates at the 5′ position are classified into four groups, according to their substrate specificity [10]. The type II phosphatases that hydrolyze PIP2 have been used to study the effect on the actin cytoskeleton. Overexpression of synaptojanin or other type II phosphatases decreases actin stress fibers [6,36] or induces actin arborization [37]. However, it is not known whether the effects are due specifically to a decrease in PIP2 or water-soluble inositol phosphates.

The most definitive evidence is obtained by disruption of the synaptojanin 1 gene [38], which results in an accumulation of clathrin-coated vesicles and polymerized actin in the endocytic zones of nerve terminals. These changes are correlated with an increase in PIP2 concentration. Synaptojanin and PIP5K are both concentrated at synapses, and they antagonize each other in the recruitment of clathrin coats to lipid membranes *in vitro* [3]. These results strongly suggest that the PIP2 level at the synapse is critically dependent on the balance of phosphoinositide kinase and phosphatases, and that PIP2 has a pivotal role in the regulation of actin and endocytic vesicle formation at the synapse [39].

Actin-Membrane Linkers Localized or Activated by PIP2

In contrast to actin monomer-binding or severing proteins that are generally inactivated by PPIs, proteins that crosslink actin filaments to each other or link them to the cell membrane are usually activated to bind actin or directed to link actin to transmembrane receptors by the lipids [40]. Evidence from mutational analyses suggests how this activating switch occurs, and interfering with PPI binding disrupts this linking process in cells.

Alpha Actinin

Recent studies of alpha actinin provide a good example of how activation of actin or other ligand binding may occur. In this case, an actin- and titin-binding motif of the antiparallel alpha actinin dimer is occluded in the inactive state because it binds a complementary domain within the same homodimer [41]. When PIP2 binds to alpha actinin, self-association is disrupted, exposing the actin- and titin-binding motifs so that they can bind their targets (see Fig. 1C). Similar activation switches have been proposed for band 4.1/ezrin/radixin/moesin (FERM) protein family members and for the focal adhesion proteins talin and vinculin.

Ezrin/Radixin/Moesin

ERM proteins are among the best currently characterized PPI-activated proteins. Both actin and membrane protein binding sites are inactive in the dormant state of the protein because of self-association between the two domains responsible for these separate activities, and the structural basis for this self-inactivation is now clear from protein structures determined by crystallography for radixin [42] and moesin [43,44]. This self-inactivation is a common feature of several actin-membrane linkers, including talin and vinculin, and in retrospect explains why the *in vitro* actin binding of these proteins was so difficult to characterize compared to proteins such as cofilin or filamin, where the actin-binding sites appear to be constitutively exposed. Biochemical and cell localization studies show that ERM proteins colocalize with transmembrane proteins in activated cells and that *in vitro* this association is stimulated by PPIs. The PIP2-dependent linkage of ezrin to ICAMs [45,46] involves reordering of the FERM domain, which contains an acidic loop distinct from the IP3-binding site that may also participate in binding to the basic juxta-membrane regions present in adhesion receptors such as CD44 [42]. The importance of the PIP2-binding regions for cellular localization and function of ERM proteins has been increasingly well demonstrated in recent studies. Mutation of four basic residues found in the PIP2-binding site prevented localization of ezrin to actin-rich membrane structures [47].

Talin

Like ezrin, talin is also activated by PIP2 to increase membrane association. In this case one consequence of PIP2 binding is an increased affinity of the intact protein for the cytoplasmic domain of beta 1 integrins [48]. The relevance of this interaction in a cellular context is reinforced by evidence that PIP2 cosediments after immunoprecipitation with anti-talin antibodies, and the amount of PIP2 shows a strong transient increase after suspended cells are plated on fibronectin, reaching a maximum 15 minutes after engagement of integrins that is five times higher than the initial state or the levels 1 hour after plating. The finding that only PIP2, but not PIP or PI, shows this transient change rules out the possibility that the lipid in the immunoprecipitates results from nonspecific contaminating membranes but also raises the question of the state of the lipid in these lipid-protein complexes.

Relation of Actin Assembly to Phosphoinositide-containing Lipid Rafts

The finding that the specialized regions of the plasma membranes such as caveoli or lipid rafts are potentially enriched in PPI [49] and that several cytoskeletal proteins do not bind PIP2 unless it is present in bilayers at approximately 10 mol percent [50] suggests that clustering of inositol lipids in specialized regions of lipid monolayers is an important aspect of their ability to modify specific cellular processes. Experiments with liposomes show that in the presence of multivalent cations PIP2 organizes into domains [51] and domains in PIP2-containing monolayers bind and are reorganized by peptides based on the PIP2-binding site of gelsolin [52]. Evidence that areas of local PIP2 concentration form in cells and are associated with actin assembly has emerged from several recent studies. In fibroblasts, the PH domains of PLCδ1 fused with GFP localized to actin-rich membrane ruffles and the selective concentration of the PLCδ1-PH-GFP in highly dynamic regions of the plasma membrane, which are rich in F-actin, supports the hypothesis that local synthesis and lateral segregation of PI(4,5)P2 spatially restrict actin polymerization [53]. In macrophages, the PLCδ1-PH-GFP protein localizes transiently to the phagosomal cups along with PLCγ2, PI(4)P 5-kinase and actin [54,55]. The dissociation of a PLCδ1-PH-CFP fusion protein was accompanied by recruitment of a C1-PKCδ-C1-YFP fusion protein, which suggests that PLCγ2 mediates the conversion of PI(4,5)P2 to diacylglycerol upon sealing of the phagosomal cup.

An immunocytochemical study with PI(4,5)P2 antibodies in PC12 cells, COS-7 cells, and hippocampal neurons has visualized clusters of PIP2 that co-localize with plasmalemma-associated PKC substrates that affect actin cytoskeleton: GAP43, myristoylated alanine-rich C kinase substrate (MARCKS), and CAP23 (GMC proteins) [17]. These clusters are interpreted to be raft domains, which were dispersed by membrane cholesterol extraction by using cyclodextrin. Cells that overexpress MARCKS exhibited larger macroscopic PIP2 clusters, whereas expression of MARCKS lacking basic effector domain exhibited reduced PIP2 clusters. These results suggest that GMC proteins regulate the availability of PIP2 for interaction with actin-binding proteins. A focal pattern of labeling suggesting colocalization of actin with PIP2 at the membrane has also been recently observed in NIH3T3 fibroblast plasma membranes treated with a fluorescent gelsolin-derived, cell permeant phosphoinositide–binding peptide [13].

Different Mechanisms of PPI-Actin Binding Protein Regulation

There are at least three distinct mechanisms and several variations by which membranes containing PPIs can alter

actin-binding protein function. The simplest mechanism shown in Fig. 1A is that an actin-binding site coincides with a PIP2-binding site and therefore targeting of the protein to PPI-rich membranes dissociates actin competitively without necessarily changing the protein structure. However, even for small monomer-sequestering proteins such as cofilin/ADF/actophorin, careful mapping of the actin- and PIP2-binding sites shows that they are not precisely coincident, and that specific residues can be altered to perturb one but not the other activity [27,56]. The recent finding that PIP2 promotes oligomerization of cofilin and subsequent actin filament bundling [57] further complicates the model of simple competition. Profilin likewise appears to have an extensive surface that interacts with PIP2, and binding to the lipid promotes increased alpha-helix in the protein [58].

A different model for inhibition of actin-binding function shown in Fig. 1B is that the binding to PIP2 causes a rearrangement of actin-binding domains or a local unfolding of polypeptide within these domains to derange the surface required to bind actin. This model appears to account for effects on gelsolin and related proteins [50]. This type of allosteric regulation may occur either with or without the protein inserting into the hydrophobic domain of the membrane.

The third mode of binding (Fig. 1C) involves docking of the protein to the membrane in a manner that disrupts interactions between domains within monomers or homo-oligomers that mask binding sites for actin or membrane anchors. This model, which may apply to ERM proteins, talin, alpha actinin, N-WASP, and vinculin, would result in activation rather than inhibition of the protein function. In the model drawn, both sites are activated after PIP2 binding, but it is also plausible that one of the sites remains occupied by the lipid and is then available only after PIP2 hydrolysis or reorganization of the membrane. Such a mechanism would explain how PIP2 binding can work to activate vinculin *in vivo* whereas purified PIP2 may inhibit vinculin-actin binding *in vitro* [59] and would allow for sequential activation of two sites as PIP2 is turned over at the cell membrane.

Effects on Lipid Membrane Structure

Binding of protein to membranes containing PPIs can have a number of effects on the membrane depending on the charge of the protein docking site, the degree of penetration into the hydrophobic domain, and the number of lipids to which the protein binds. In contrast to the extensive documentation of changes in protein function or structure, changes in lipid structure are less often studied but are likely to be equally important in a cellular context. Although it is often assumed that protein docking to PPIs in a membrane mainly localizes the protein to the surface, there are many specific changes that can occur, such as the recently documented extended conformation [60], especially for a lipid such as PIP2, which is relatively unstable in a bilayer. One interesting possibility is that some forms of binding to PIP2 can destabilize bilayer packing to the extent that loss of membrane asymmetry occurs. Resent observations indicate that PIP2 is a positive regulator of Ca^{2+}-induced lipid scrambling due to PIP2-enriched domain formation [61]. The possibility to reorganize membranes by specific interaction with PIP2 has been documented by electron spin resonance measurements that show disordering of lipid bilayer vesicles by myristoylated ARF6 only when the vesicles contain PIP2 [62]. Phospholipid scrambling was also observed in platelets and lipid vesicles containing PIP2 treated with a gelsolin-derived peptide [12]. These observations suggest that the binding of cytoskeletal proteins to membrane phosphoinositides has a vast potential for regulation and reorganization of cells that is now beginning to be appreciated.

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CHAPTER 157

The Role of PI3 Kinase in Directional Sensing during Chemotaxis in *Dictyostelium*, a Model for Chemotaxis of Neutrophils and Macrophages

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Introduction

Chemotaxis, or directional movement of eukaryotic cells toward a small molecular attractant, is highly conserved evolutionarily and is regulated by a variety of ligands (including chemoattractants, chemokines, and growth factors) that activate G-protein-coupled and receptor tyrosine kinase effector pathways. Concentration differences of only a few percent over the length of a cell are sufficient to be recognized and converted into directional motility. Chemotaxis of *Dictyostelium* cells toward the extracellular chemoattractant cyclic AMP (cAMP) provides a model for the mechanisms underlying chemotaxis in a number of mammalian cell types, including neutrophils and macrophages. The ability to employ genetic, biochemical, and single-cell assays makes *Dictyostelium* an exceptional system for finding new genes involved in chemotaxis and understanding the interplay of known and novel gene products in the signal transduction processes underlying chemotactic responses. Furthermore, the simplicity of *Dictyostelium* chemotaxis combined with a vast body of experimental knowledge makes this organism an ideal system for testing predictions made by theoretical models of the chemotactic response.

Here, we discuss the signaling events that control the ability of a cell to sense the direction of a chemoattractant gradient. The phosphatidylinositol-3 kinase (PI3K) pathway plays a pivotal role in the conversion of such a shallow external gradient into the extreme cytoskeletal polarization necessary for directed cell movement of neutrophils, macrophages, and *Dictyostelium* cells.

Directional Movement

When cells are placed in a chemoattractant gradient, there is a rapid polymerization of actin on the side of the cell closest to the chemoattractant source resulting in the formation of a new leading edge [1–6]. Polymerization of F-actin leads to protrusion of the plasma membrane [7]. Assembly of conventional, nonmuscle myosin II and F-actin at the

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posterior of the cell enables actin-myosin contractility, which lifts off the posterior (also called a uropod) and promotes a rapid contraction of the posterior of the cell [3,6].

Localization of Cytoskeletal and Signaling Components

Even in the absence of an external gradient, many chemotaxis-competent cells exhibit a polarized distribution of cytoskeletal components, with the majority of F-actin found at the leading edge and myosin II and the remaining F-actin found at the cell's posterior. Only when cells are placed in a gradient is there a dynamic activation of localized F-actin polymerization and myosin assembly resulting in directional cell movement. When the chemoattractant gradient changes direction, the cells respond by dismantling the old actin/myosin cytoskeleton and forming a new leading edge and uropod, thereby realigning their axis with the external gradient [1,3,4,6]. In *Dictyostelium*, neutrophils, and macrophages, members of the Rac/Cdc42 and RhoA family of small GTPases regulate WASP proteins (e.g. WASP, Scar/WAVE), members of the PAK family of serine/threonine protein kinases, and myosin kinases are required for chemoattractant-mediated actin polymerization, myosin assembly, and directional movement (Fig. 2). For a more detailed discussion of this topic see Nobes *et al*., 1999 [8]. In contrast to cytoskeletal elements, upstream signal transduction components exhibit a uniform distribution in the absence of an external signal. In response to stimulation, some of these components undergo a dramatic redistribution and polarization, reflecting an underlying signal processing that can recognize and amplify a shallow external gradient enabling the chemotactic response. Recent experimental progress has identified some of these proteins and has shed light on some of the mechanistic aspects regulating their change in subcellular localization.

Analysis of the subcellular localization of known cell surface sensors such as G-protein-coupled chemoattractant receptors has demonstrated that these receptors in *Dictyostelium* and neutrophils are uniformly localized along the plasma membrane [1,4–6], even in the presence of an external signal. Although the concentration of $G\beta\gamma$ subunits is highest at the anterior of the cell, the gradient is extremely shallow, comparable to the gradient of the external signal, and cannot account for the steep intracellular gradient of other signaling components [9].

The Signaling Pathways Controlling Directional Movement

Activation of PI3K at the leading edge appears to play a predominant role in controlling directional movement in many amoeboid cell types such as neutrophils, macrophages, and *Dictyostelium* cells [1,4–6]. Class I members of the PI3K family phosphorylate $PI(4,5)P_2$ and PI(4)P at the 3′ position in the inositol ring to produce the membrane lipids $PI(3,4,5)P_3$ and $PI(3,4)P_2$ (Fig. 1). $PI(3,4)P_2$ is also derived through the dephosphorylation of $PI(3,4,5)P_3$ by the 5′ inositol lipid phosphatase SHIP [10].

Studies in which PI3K function is abrogated through gene knockouts, use of PI3K-specific inhibitors, or PI3K isoform-specific antibodies demonstrate that PI3K is required for proper chemotaxis in neutrophils, macrophages, and *Dictyostelium* cells (see [1] for references). *Dictyostelium pi3k1/2* null cells, in which two of the three genes encoding the Class I PI3Ks PI3K1 and PI3K2 have been disrupted by homologous recombination, exhibit strong chemotaxis defects [11–15]. *Dictyostelium* wild-type cells chemotaxing toward cAMP are highly polarized, preferentially form a single pseudopod at the leading edge, and produce few if any lateral pseudopodia. In contrast, *pi3k1/2* null cells or wild-type cells treated with the PI3K inhibitor LY294002 exhibit a significant loss of polarity, move more slowly than wild-type cells, and produce multiple pseudopodia simultaneously along the periphery of the cell, although the cells still move predominantly toward the chemoattractant source [14]. These results indicate that PI3K is an important component of chemotaxis regulating directionality and polarity; however, these findings also imply that there are pathways parallel to those regulated by PI3K1 and PI3K2 contributing to directional movement. The nature of this pathway is currently unknown.

These observations make the PI3K pathway a likely candidate to be involved in polarization amplification. This notion is further supported by the subcellular localization of PI3K pathway components. Initial evidence for the localized activation of PI3K has derived from the demonstration, first in *Dictyostelium* and then in neutrophils and fibroblasts, of the preferential localization of a subfamily of PH-domain-containing proteins that preferentially bind the PI3K products $PI(3,4,5)P_3$ and $PI(3,4)P_2$ [14,16–19]. GFP fusions of these proteins function as reporters for local accumulation of $PI(3,4,5)P_3$/$PI(3,4)P_2$ corresponding to localized activation of Class I PI3Ks. These proteins are cytosolic in unstimulated cells and rapidly and transiently move to the plasma membrane in response to cells being globally stimulated by a chemoattractant (cells being rapidly bathed in chemoattractant) so that all of the receptors around the cell are uniformly activated. When cells are placed in a chemoattractant gradient, these proteins preferentially localize to the leading edge, suggesting that PI3K is preferentially localized in this region of the cell (Fig. 3). The localization of these PH-domain-containing proteins, which in *Dictyostelium* peaks at ~6–8 seconds after global stimulation with a chemoattractant, is one of the most rapid responses that has been described [14,16,17]. In *Dictyostelium*, three PH-domain-containing proteins exhibit identical patterns of spatial localization in chemotaxing cells and in response to global stimulation by a chemoattractant. These include CRAC, a PH-domain-containing protein required for chemoattractant-mediated activation of adenylyl cyclase; the serine/threonine protein kinase Akt/PKB, which is a PI3K effector important in

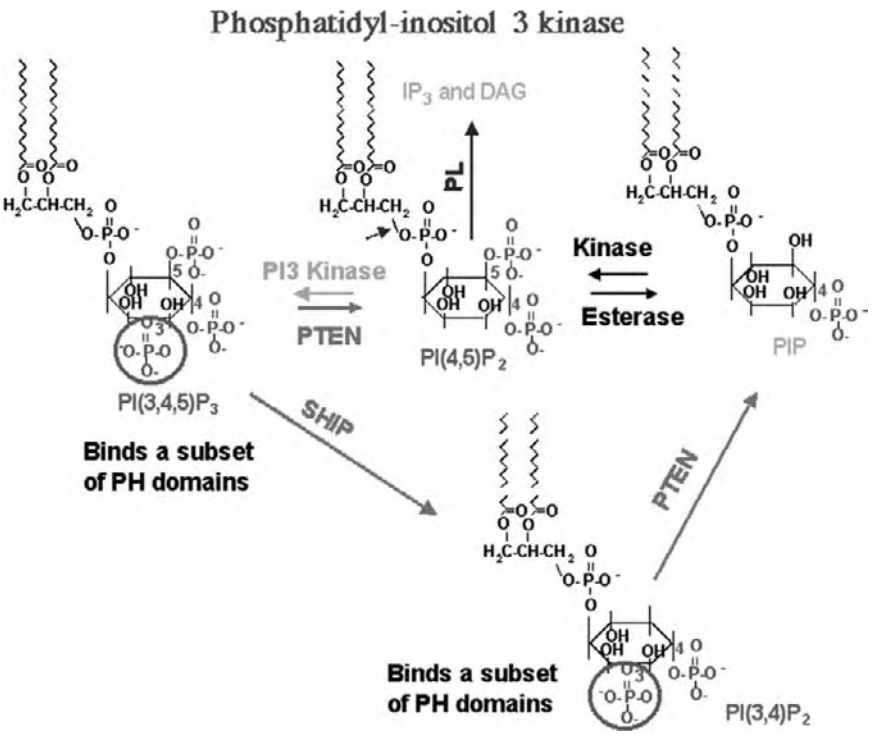


Figure 1 The phosphatidylinositol kinase/PTEN biochemical pathway. The figure illustrates the biochemical pathway for the production and degradation of $PI(3,4,5)P_3$ and $PI(3,4)P_2$, PI3K products that preferentially bind a subclass of PH-domain-containing proteins involved in chemotaxis. PI3K phosphorylates on the 3′ position of inositol, $PI(4,5)P_2$, also a substrate of phospholipase C (PLC), or PI(4)P. $PI(3,4,5)P_2$ can be degraded by the 5′ inositol phosphatase SHIP to produce $PI(3,4)P_2$. The tumor suppressor PTEN dephosphorylates $PI(3,4,5)P_3$ and $PI(3,4)P_2$ on the 3′ position of the inositol ring, thereby removing the binding sites for the PH domains on the plasma membrane.

regulating cell growth and cell survival; and PhdA, which is involved in the spatial-temporal control of F-actin polymerization at the leading edge. In neutrophils, the PH domain of Akt/PKB exhibits changes in its spatial distribution in response to chemoattractant stimulation similar to those of its counterpart in *Dictyostelium* cells [18].

Genetic and biochemical studies have demonstrated that these localizations occur in response to activation of PI3K. First, the localization is inhibited by the PI3K-specific inhibitor LY294002. Second, in *Dictyostelium* cells carrying disruptions of the genes encoding two of the Class I PI3Ks (PI3K1 and PI3K2), chemoattractant-mediated membrane localization is not observed. Finally, point mutations in the PH domain that abrogate the ability of the PH domain to bind PI3K lipid products prevent the PH domains from localizing to the leading edge in response to global chemoattractant stimulation (see [1] for references). These findings indicate that PH domain localization and thus PI3K activation respond to one of the primary downstream responses to chemoattractant stimulation. These findings have been reproduced in mammalian leukocytes [18].

The activation of this response at the leading edge is reflected by a very steep intracellular gradient of the PH domain localization at the plasma membrane from the front to the back of the cell. Since this occurs in a very shallow chemoattractant gradient (as low as a 2–5% difference between the front and back of the cell), there must be a mechanism by which an external, shallow gradient gives rise to a very steep intracellular gradient of the second messengers $PI(3,4,5)P_3$ and $PI(3,4)P_2$. Recent studies have demonstrated that PI3K in *Dictyostelium* preferentially localizes to the leading edge (Fig. 3). GFP fusions of PI3K1 or PI3K2 localize to the leading edge when cells are placed in a chemoattractant gradient and are transiently localized to the plasma membrane in response to global stimulation [20]. This provides for the localized activation of PI3K at the leading edge and thus the localized production of $PI(3,4,5)P_3$/$PI(3,4)P_2$. What localizes PI3K to the leading edge is presently unknown. However, as already mentioned, PI3K's localization is not due to a corresponding localization of either the chemoattractant receptor or the coupled heterotrimeric G protein.

PI3K Effectors and their Roles in Controlling Chemotaxis

pi3k1/2 null cells exhibit strong chemoattractant defects. *Dictyostelium pi3k1/2* null cells chemotaxing toward cAMP exhibit a significant loss of polarity and move more slowly than wild-type cells. Whereas wild-type cells preferentially

form a single pseudopod at the leading edge and produce few if any lateral pseudopodia, *pi3k1/2* null cells produce multiple pseudopodia simultaneously along the periphery of the cell, although the cells still move predominantly toward the chemoattractant source. Results from neutrophils and macrophages in which the function of PI3kγ and PI3Kδ, respectively, are abrogated are consistent with the observations in *Dictyostelium* (see [1] for references).

Biochemical analysis of *Dictyostelium pi3k1/2* null cells has provided insight into the downstream effector pathways. *pi3k1/2* null cells exhibit a reduced chemoattractant-mediated F-actin assembly and a more severe defect in the spatial-temporal regulation of F-actin assembly. When cells are chemotaxing toward a micropipette emitting a chemoattractant and the location of the micropipette is changed, resulting in a change in direction, the kinetics of the directional change in *pi3k1/2* null cells are delayed compared to those of wild-type cells. *Dictyostelium* cells carrying a disruption of the gene encoding PhdA, a PH domain-containing protein with PI3K-dependent localization to the leading edge, exhibit actin phenotypes similar to those observed for *pi3k1/2* null cells, suggesting that this function of PI3K is mediated, at least in part, through PhdA [14].

Akt/PKB is activated in response to chemoattractants in both *Dictyostelium* and neutrophils and probably in other cell types as well, and this activation is lost in *Dictyostelium* and mammalian *pi3k* null cells (see [1] for references), indicating these pathways are probably conserved between *Dictyostelium* and mammalian cells. Ligand-regulated Akt/PKB activity is required for proper chemotaxis in *Dictyostelium* and has been linked to the migration of mammalian endothelial cells [17,21].

In *Dictyostelium*, a gene knockout of Akt/PKB exhibits a subset of the *pi3k* null defects, including a reduction in cell movement, directionality, and chemoattractant-mediated myosin II assembly, providing a link between the activation of PI3K at the front of the cell and the regulation of myosin assembly at the cell's posterior [17,22] (Fig. 2). Myosin assembly and disassembly in *Dictyostelium* is regulated by phosphorylation of myosin heavy chain kinase (MHCK). Phosphorylation of myosin II by MHCK leads to myosin II disassembly, whereas phosphatase treatment leads to assembly [23]. Genetic and biochemical evidence demonstrates that PAKa, a p21-activated serine/threonine kinase related to mammalian PAK1 and yeast Cla4 and Ste20, is essential for myosin II assembly during cytokinesis and chemotaxis; *paka* null cells exhibit phenotypes similar to those of cells lacking myosin II. Moreover, PAKa colocalizes with myosin at the posterior of chemotaxing cells and at the contractile ring during cytokinesis. Studies have demonstrated that PAKa is a direct substrate, both *in vivo* and *in vitro*, of Akt/PKB. Phosphorylation of PAKa by Akt/PKB leads to its activation [22]. PAKa then is thought to function by inhibiting MHCK, leading to myosin II assembly. It is interesting that one of the MHCKs, MHCK-A, preferentially localizes to the leading edge in chemotaxing cells, where it is presumably activated, causing disassembly of myosin and more efficient pseudopod extension [24].

The Tumor Suppressor PTEN Regulates the Chemoattractant PI3K Pathways

PTEN, the tumor suppressor that acts as a negative regulator of the PI3K cell growth and cell survival pathway by dephosphorylating $PI(3,4,5)P_3$ and $PI(3,4)P_2$ on the 3′ position [25], also functions as a negative regulator of chemotaxis. *Dictyostelium* cells overexpressing PTEN exhibit a reduced activation of Akt/PKB and chemotaxis defects consistent with a reduced level of PI3K pathway activity [20]. Hypomorphs, cells expressing a lower level of PTEN, exhibit higher levels of Akt/PKB activation. The strongest link between PTEN and chemotaxis derives from the analysis of *Dictyostelium* PTEN null cells [26]. These cells exhibit prolonged localization of PH-domain-containing proteins in response to chemoattractant stimulation. Moreover, when chemotaxing cells are examined, PH domain protein localization extends around the side of the cell, including some localization to the cell's posterior. These phenotypes are very similar to those obtained by expressing myr-tagged PI3K. These results demonstrate that PTEN restricts the domain of $PI(3,4,5)P_3$/$PI(3,4)P_2$ localization and regulates the function of the PI3K pathway. Further linkage of the PI3K pathway to chemotaxis was demonstrated by the finding that PTEN null cells exhibit an elevated and prolonged level of chemoattractant-mediated F-actin assembly, indicating a linkage between PI3K and F-actin assembly. This observation suggests that PI3K activation at the leading edge may be one of the mechanisms that drives the expected activation of Rho exchange factors and F-actin polymerization at these sites. The results are consistent with the phenotypes of *pi3k* null cells and those of PhdA, a PI3K effector.

The subcellular localization of PTEN is also consistent with a role as a negative regulator of the PI3K pathway during chemotaxis: PTEN is uniformly localized around the plasma membrane of unstimulated cells and rapidly delocalizes from the plasma membrane in response to chemoattractant stimulation [20] (Fig. 3). In polarized, chemotaxing cells, PTEN is preferentially excluded from the leading edge, while it remains along the sides of the cell. This finding suggests that PTEN localization, like that of PI3K, is dynamic and is complementary to that of PI3K. Presumably, PTEN exclusion from the leading edge allows an amplification of the PI3K activity at this site of the cell, whereas its localization on the plasma membrane on the sides of the cell helps restrict PI3K activity and sharpen the boundary of PIP_3/PIP_2 localization.

Conclusions

Results in *Dictyostelium* provide a model of the mechanism controlling directional movement (Fig. 2). The localization of PI3K to the leading edge results in the production of the second messengers $PI(3,4,5)P_3$ and $PI(3,4)P_2$, causing a localization of PH-domain-containing proteins and regulation of downstream effector pathways. The delocalization of

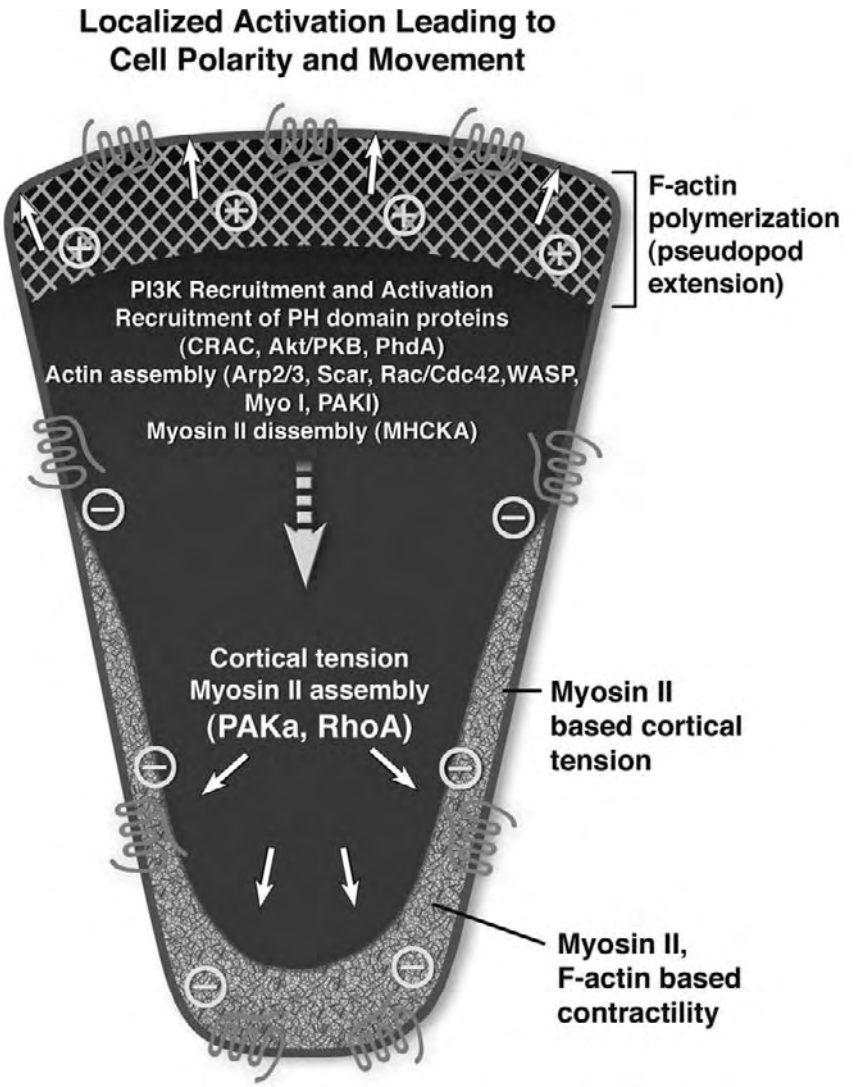


Figure 2 Spatial regulation of signaling components and the actin/myosin cytoskeleton. The image shows a polarized, chemotaxing amoeboid cell. Preferential activation in a series of signaling pathways at the leading edge results in F-actin polymerization and extension of the pseudopod. The pathways activated include phosphatidylinositol-3 kinase (PI3K), leading to the recruitment and activation of PH-domain-containing proteins. Other components that are localized to the leading edge include those regulating actin assembly (the Arp2/3 complex, the Wiskott-Aldrich Syndrome protein WASP and its relative Scar/WAVE, and the small GTPases Rac and Cdc42), and myosin I, which may regulate the translocation of the WASP/Arp2/3 complex along the F-actin filaments. Mammalian PAK1 is also vital in regulating pseudopod extension. Myosin assembly and contractility at the posterior of the cell is required for uropod contraction. In *Dictyostelium*, these processes are mediated by myosin heavy chain kinase A (MHCKA), which localized to the front of the cell, and PAKa found at the posterior of the cell. In mammalian cells, it is regulated by the small GTPase RhoA and downstream pathways. See text for additional details.

PTEN from the leading edge while PTEN remains along the sides of the cell helps localize and restrict the PI3K pathway. Downstream effector pathways include F-actin polymerization at the leading edge and myosin assembly at the cell's posterior. Other studies have implicated this pathway in controlling cell polarization, which is necessary for effective chemotaxis. Parts of this pathway have also been described in neutrophils and macrophages, including the essential role of PI3K in directional sensing and the localization of PH-domain-containing proteins at the leading edge. Because of the increased recognition of the importance of chemotaxis in a variety of cellular processes, including cell polarization, metastasis, and embryonic cell movement, understanding these mechanisms is paramount to providing mechanistic insights into basic biological processes and many aspects of human disease.

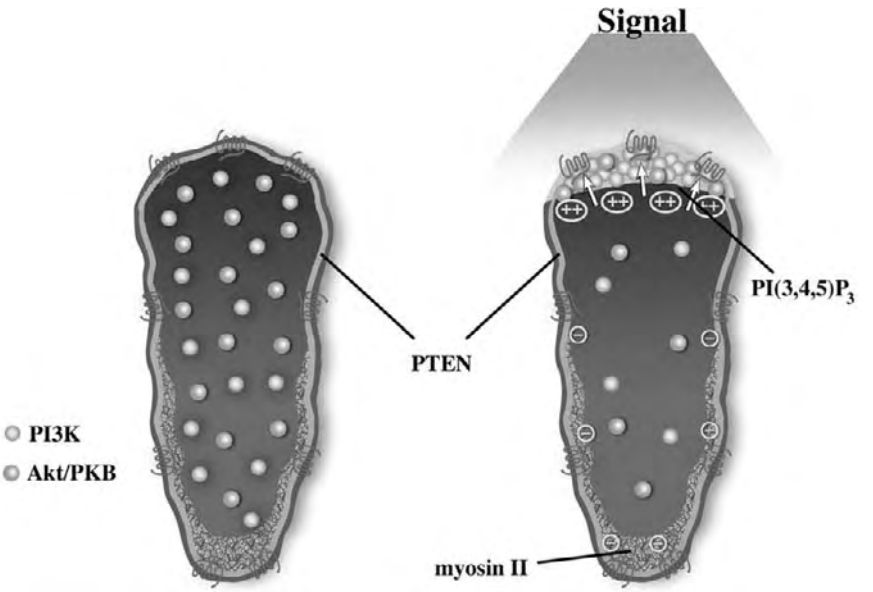


Figure 3 Differential localization of PI3K and PTEN in a chemotaxing cell. The figure on the right shows that in a resting cell, PI3K and PH-domain-containing proteins Akt/PKB, CRAC, and PhdA are uniformly distributed in the cytosol, whereas the tumor suppressor PTEN is localized uniformly at the edge of the cell. When cells are placed in a chemoattractant gradient, as illustrated in the panel on the right, PI3K preferentially localizes to the leading edge, causing the production of $PI(3,4,5)P_3$ and the localization of the PH-domain-containing proteins. PTEN is preferentially lost from the leading edge. This loss is thought to help sharpen the gradient by preferentially limiting the site of $PI(3,4,5)P_3$ membrane localization.

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CHAPTER 158

Phosphatidylinositol Transfer Proteins

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Introduction

The phosphatidylinositol transfer protein (PITP) family is defined by its ability to bind one molecule of either phosphatidylinositol (PtdIns) or phosphatidylcholine (PtdCho) and facilitate lipid transfer between separate membrane compartments [1]. PITPs have now emerged as critical regulators of phosphoinositide metabolism in specific cellular compartments where they participate in signal transduction and membrane traffic [2]. PITP was originally purified as a soluble 35 kDa protein, which is now known to contain a single structural domain [3]. The PITP domain has now been found in the larger RdgB proteins, originally identified in *Drosophila* as *r*etinal *d*egeneration (Class *B*) mutants. Today, the mammalian PITP family includes five proteins divided into three subgroups, all containing a PITP domain: the classical PITPs, α and β (35 kDa), two larger related proteins M-rdgBα1 and M-rdgBα2 (160 kDa), and the soluble M-rdgBβ protein (38 kDa) [4]. In addition to mammals, proteins with a PITP domain are found in *Caenorhabditis elegans* (worms), *Drosophila melanogaster* (flies) and *Dictyostelium discoideum* (soil amoebae), but not yeast or plants. The larger rdgB proteins are not found in *Dictyostelium*, however. The yeast Sec14p and its related family members form a separate group of PtdIns transfer proteins that, although they share lipid binding properties and transfer function with mammalian PITPs, have no sequence or structural similarity [3,5].

The Classical PITPs: α and β

PITPα and PITPβ are expressed ubiquitously in all tissues, are abundant proteins, and share 77% identity and 94% similarity in amino acid sequence. In addition to PtdIns and PtdCho transfer, PITPβ can also transfer sphingomyelin [6]. Transfer occurs down a concentration gradient without input of energy *in vitro*. Thus PITPs solubilize specific lipids from membranes and can facilitate their movement through the aqueous phase (Fig. 1). Although PITPs are defined by their ability to bind either one molecule of PtdIns or PtdCho, the affinity of PITPα for PtdIns is 16-fold greater compared to PtdCho. This reflects the lower levels of PtdIns compared to PtdCho in cells, and typically 30 to 40% of the PITPα and β proteins are PC-bound compared to 60 to 70% that are loaded with PtdIns.

PITPα and PITPβ are localized in different compartments. PITPα is present in the cytosol and the nucleus whereas PITPβ is localized at the Golgi and in the cytosol. In mammalian cells, the function of PITP was first identified in biochemical studies involving reconstitution of phospholipase C-signaling and exocytosis in cytosol-depleted cells [7,8]. Phospholipase C hydrolyses phosphatidylinositol(4,5)bisphosphate (PIP_2) to generate the second messengers, diacylglycerol and inositol(1,4,5)trisphosphate. Activation of G-protein-coupled receptors or receptor tyrosine kinases is responsible for increasing phospholipase C activity, and PITPα was identified as an essential component in ensuring PIP_2 supply for the enzyme [9,10]. Exocytosis could be similarly recovered in

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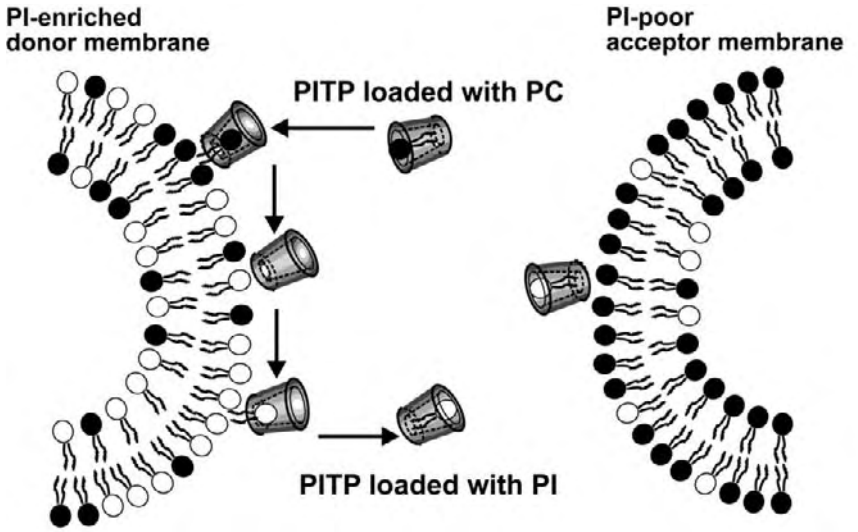


Figure 1 PITPs bind and transfer PtdIns and PtdCho between membrane compartments. Phosphatidylinositol transfer proteins (PITPs) were first purified based on their ability to transfer PtdIns between two membrane compartments *in vitro*.

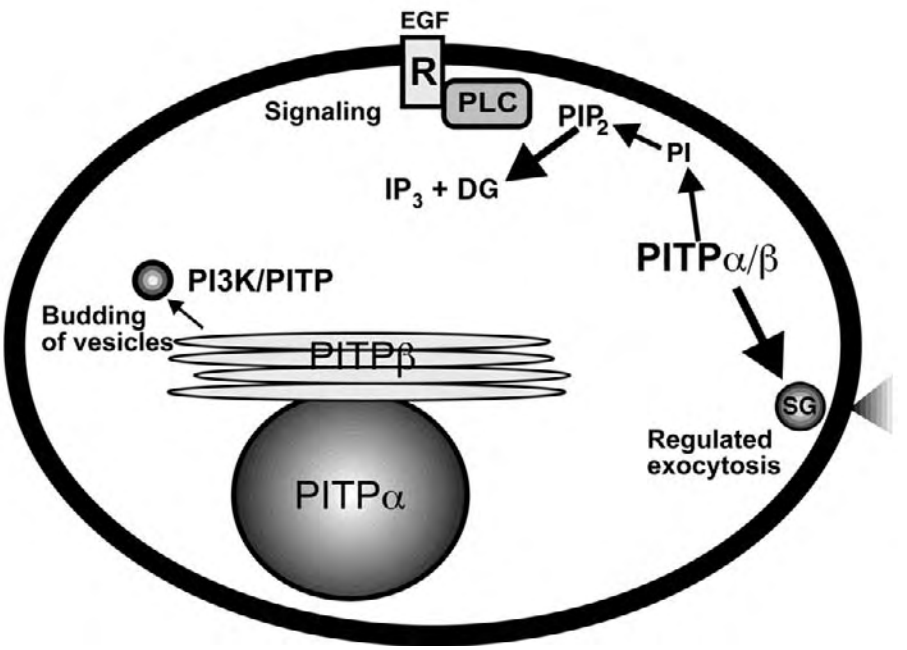


Figure 2 Functions and location of PITPα and PITPβ. PITPα is primarily localized in the cytosol and the nucleus. PITPα is required for supplying the substrate, PtdIns for PIP_2 synthesis utilized by phospholipase C and for maintaining a pool of PIP_2 for exocytosis. PITPβ is primarily localized at the Golgi and cytosol and is involved in the budding of vesicles by making available a pool of phosphoinositides at the Golgi. The function of PITPα in the nucleus is probably in making substrate available for phosphorylation.

permeabilized cells where PITPα and a PIP 5-kinase worked in synergy to make PIP_2 [11,12]. Finally, biogenesis of vesicles from Golgi was also dependent on cytosolic proteins, and PITP was thus purified [13,16]. In all these studies, both PITPα and PITPβ were equally capable of restoring function. Several of these functions are summarized in Fig. 2.

Studies aimed at elucidating the mechanism of action of PITP in each of these seemingly disparate functions have yielded a singular theme. The activity of PITP stems from its ability to transfer PtdIns from its site of synthesis (ER) to sites of cellular activity and to stimulate the local synthesis of phosphorylated forms of PtdIns, including PtdIns(4)P, PtdIns(4,5)P_2, PtdIns(3)P, and PtdIns(3,4,5)P_3 [14,15]. It is speculated that PITP could present PtdIns to the lipid kinases within a signaling complex. This concept is supported by observations that PITPα does associate with the EGF receptor phopsholipase Cγ and PtdIns 4-kinase following stimulation with EGF [9].

A reduction in the expression levels of PITPα, as seen in the *vibrator* mutation in mice leads, to neurodegeneration [17] in the presence of normal concentrations of PITPβ, suggesting that although these proteins share transfer activity and can substitute for each other in reconstitution assays [8,13,18], they do have distinct functions *in vivo*. PITPβ is an essential protein, since mice carrying mutations in PITPβ die early in embryogenesis, and PITPβ may be essential for stem cell viability [19]. PITPβ was originally cloned from a rat brain cDNA library by its ability to rescue *Sec14* mutants in yeast, *S. cerevisiae*. Mutations in *Sec14* lead to a defect in the formation of secretory vesicles destined for the plasma membrane. Despite the absence of sequence or structural homology, PITPβ was able to rescue the temperature-sensitive *Sec14* mutants. In mammalian cells, PITPβ also localizes to the Golgi, and considerable data have accumulated that suggest that PITPβ may be involved in vesicle budding in this compartment by maintaining a pool of phosphorylated PtdIns [16].

The amino acid sequence of the PITP domain is highly conserved in all isoforms, and no characteristic short sequence motifs have been identified. From the crystal structure of PITPα bound to PtdCho, the PITP domain comprises an amino-terminal lipid-binding region that contains an eight-stranded, concave, mostly anti-parallel ß-sheet and two helices, the carboxy-terminal helical region, and the intervening regulatory loop region [3]. Upon stimulation with receptor-directed agonists or PMA, PITPα is phosphorylated at Ser164, which resides in the regulatory loop region. This is an important regulatory control as mutation of the serine residue to glutamate (which mimics phosphorylation) inhibits transfer function as well as the ability to provide substrate for phospholipase C signaling.

The mechanism of how PITP can abstract a lipid from a bilayer and facilitate exchange can be conjectured from the extensive biochemical and structural analysis of PITPα. For PITPα to perform its task, a change in affinity for membranes has to occur for it to associate with membranes to exchange its bound lipid, but this change in affinity has to be reversed so that the protein can move rapidly away from the membrane. Deletions of the C-terminus induce a more relaxed conformation and enhances its affinity for membranes without affecting its lipid binding properties. Thus movement of the carboxy-terminal helical region very likely governs change in membrane affinity and also exposes the lipid tails toward the membrane. The lipid is now able to move out of its cavity, and lipid exchange can then occur. Following exchange the protein has to return to its compact structure to be released from the membrane.

One of the major roles of PITPα is to provide PtdIns for PLC signaling. PLC signaling is thought to occur in inositol-lipid enriched membranes rafts, since destruction of rafts inhibits PLC activation [20,21]. It may be speculated that the localized depletion of the inositol lipids in membrane rafts could result in changes of membrane bilayer curvature. Since the activity of PITPα is sensitive to membrane curvature, this would mean that the transfer activity is regulated by changes in the local membrane environment. This conclusion is supported by the observation that upon stimulation with EGF, PITPα is part of a signaling complex, which includes the EGF receptor, Type II PI-4-kinase, and phospholipase Cγ [9]. Thus in cells, PITPα does not randomly transfer lipids but does so at specific sites of active consumption of phosphoinositides.

RdgB Family of PITP Proteins

As already mentioned, the RdgB acronym is derived from a *r*etinal *deg*eneration mutant phenotype (type **B**) in *Drosophila* where this family of PITP proteins were first identified. The *D-rdgB* mutation causes abnormal photoreceptor responses and light-enhanced retinal degeneration, and genetic evidence indicates that the D-rdgB product acts within the light-triggered phosphoinositide cascade responsible for phototransduction. The *D-rdgB* gene encodes a 160 kDa protein that has an N-terminal PITP domain, an acidic Ca^{2+}-binding domain, and an extended hydrophobic region; in mammals, there are of two homologues, M-RdgBα1 and M-RdgBα2. In addition, a smaller protein of 38 kDa (RdgBβ) was identified by homology to rdgBα and this isoform is also found in both flies and mammals [4]. Transgenic expression of murine rdgBα1 or 2 rescues *rdgB* null *Drosophila.* However, deletion of RdgBα2 in mice has no obvious phenotype and phototransduction and photoreceptor survival is unaffected, whereas deletion of RdgBα1 is embryonically lethal [22].

M-RdgB1 specifically associates with phosphatidylinositol 4-kinase (α-isoform) and can increase the kinase activity [23]. These data are consistent with our proposal that PITP proteins mediate spatially restricted synthesis of phosphorylated inositol lipids. In conclusion, proteins with a PITP domain all appear to function in many aspects of biology by virtue of its ability to regulate phosphoinositides synthesis. Phosphoinositides play important roles not only for providing substrate for signaling pathways but also as ligands for proteins containing specific domains, including PH domains, PX domains, ENTH domains.

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CHAPTER 159

Inositol Polyphosphate Regulation of Nuclear Function

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Introduction

As several chapters in this Handbook attest, inositol signaling pathways have emerged as a multifaceted ensemble of cellular switches that regulate a number of processes well beyond calcium release, including membrane trafficking, channel activity, and nuclear function. Over 30 inositol messengers are found in eukaryotic cells that may be generally grouped into two classes: (1) inositol lipids or phosphoinositides (PIPs) and (2) water-soluble inositol polyphosphates (IPs). Insights into the roles of these messengers have come through the characterization of numerous gene products that control the metabolism of PIPs and IPs, over eighty in humans and twenty-six in budding yeast. This review will discuss in brief a small subset of the overall inositol signaling pathway, namely higher IPs, generally defined as having four or more phosphates. Two important concepts have emerged: (1) the higher IPs discussed here are derived from phospholipase C-dependent activation, thus IP_3 is both a messenger and a precursor to others, and (2) the higher IPs have been linked to the regulation of several nuclear processes. Emphasis will be placed on the gene products that synthesize IP_4, IP_5, IP_6 and diphosphoryl IPs and the processes they have been found to regulate. Several of these kinases appear to localize within the nucleus, and their activities are necessary for proper gene expression, mRNA export, and DNA metabolism. The breadth of nuclear processes regulated and the evolutionary conservation of the genes involved in their synthesis have sparked renewed interest in higher IPs as important intracellular messengers.

Inositol Signaling and the Molecular Revolution

The molecular revolution has left an indelible mark on inositol signaling pathways, fueling an expansion of our thinking [1–8]. As the roles of inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglyerol were forged as intracellular messengers, many researchers questioned whether other inositol lipids and inositol polyphosphates, some 30 in all, had important roles in cell signaling. The cloning and characterization of kinases, phosphatases, lipases, and effectors has made it clear that the functions of inositol phosphate derivatives are numerous. Over the past decades, dozens of gene products have been characterized as players that act in concert to generate a combinatorial ensemble of distinct chemical messengers with instructions for the cell. Nature may have utilized *myo*-inositol as a signaling scaffold because of its elegant chemistry—a six-carbon asymmetric cyclitol that is readily modified by combinatorial phosphorylation—and because it may be formed through two metabolic steps from glucose 6-phosphate. Ancestral relationships have been identified at the sequence and structural level among proteins involved in inositol signaling and those involved in nucleotide, protein, and carbohydrate metabolism, thereby providing clues as to how signaling machinery evolved. Examples of genetic economy are found among several promiscuous IP kinases and

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phosphatases harboring multiple specificities. Remarkably, a dual-functional gene product has been identified, conserved from yeast to man, which has two distinct autonomously folded inositol lipid phosphatase domains that together are capable of dephosphorylating all known PIPs. Three inositol lipid phosphatases—OCRL-1, MTM, and PTEN/MMAC—have been identified in which a loss of function results in human disease. Together these findings have generated much new excitement within the signaling community, and as we look to the future there is every expectation that we are in for many more surprises.

Links of Inositol Signaling to Nuclear Function

A recurring theme in intracellular signaling is the spatial restriction of pathways to selective compartments. In the past 15 years, discrete nuclear specific pathways of inositol metabolism have been identified that may provide a provocative mechanism by which extracellular stimuli may ultimately elicit nuclear responses. Initially it was demonstrated that phosphoinositides are present in nuclear membranes and that activities required for their synthesis and breakdown are within nuclear fractions [9–11]. The functional importance of such pathways were then suggested through studies of insulin-like growth factor I (IGF-I), which stimulates nuclear but not cytoplasmic phosphoinositide metabolism [12,13]. Studies by Crabtree and coworkers [14] have found that PIP_2 regulates chromatin-remodeling complexes. Many other studies have been recently reviewed by Divecha and coworkers [13], and hint that inositols influence nuclear processes such as DNA synthesis, cell cycle, nuclear calcium, chromatin structure, gene expression, and messenger RNA export.

Genetic and biochemical studies of a phospholipase C-dependent pathway in the budding yeast have provided compelling functional evidence for regulation of three distinct nuclear processes by higher IPs. The budding yeast genome contains a single phosphoinositide-specific phospholipase C gene (*PLC1*) whose activation induces a kinase pathway that sequentially converts $I(1,4,5)P_3$ to higher IPs, including $I(1,4,5,6)P_4$, $I(1,3,4,5,6)P_5$, IP_6, and PP-IPs (see Fig. 1) [15]. Examination of IP metabolism in a variety of yeast strains reveal that activation of Plc1 results in the production of IP_3, which is then sequentially phosphorylated by two kinases, Ipk2 and Ipk1, to IP_6 [15–17]. A third kinase, Kcs1, has been identified as a diphosphoryl inositol synthase, which generates PP-IP branches from IP_5 and IP_6 substrates [18,19].

Individual mutations in *plc1*, *ipk2,* or *ipk1* result in defects in the production of IP_6 as well as defects in efficient mRNA export [15]. In contrast, mutation in *kcs1* and hence PP-IP production does not appear to alter mRNA export [19]. Furthermore, induction of the pathway through overexpression of Plc1 results in suppression of defects in a *gle1-1* mRNA export mutant [15]. These data suggest that phospholipase C and kinase-dependent higher IP production, possibly IP_6 or some yet identified product of the Ipk1 2-kinase, regulates mRNA export. The cloning of Ipk1 orthologs from plants and

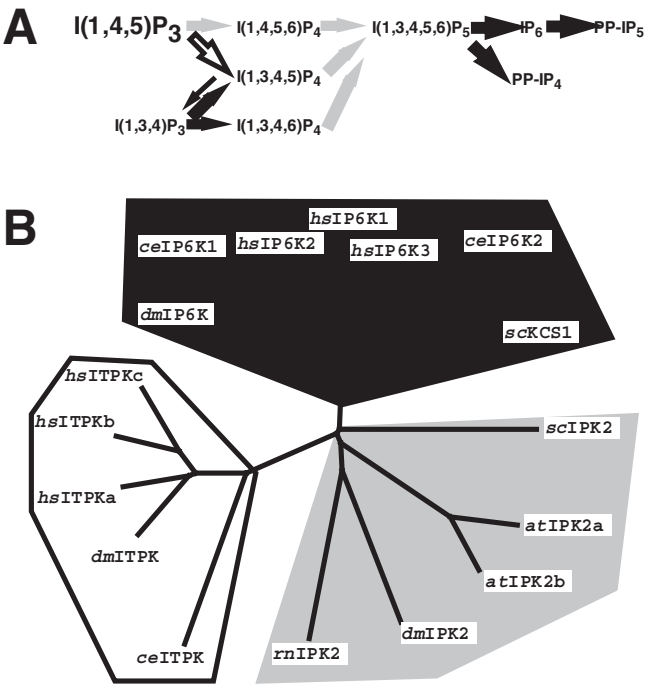


Figure 1 Higher inositol polyphosphate synthesis pathways. (A) An abridged description of IP_3 metabolism—most phosphatase activities and several higher IP intermediates have been omitted for clarity. (B) Dendogram showing three branches of the IPK family, including IPK2, ITPK, and IP6K kinases. Species are abbreviated: *hs, Homo sapiens; rn, Rattus norvegicus; dm, Drosophila melanogaster; at, Arabidopsis thaliana; sc Saccharomyces cerevisiae; ce, Caenorhabditis elegans.* Color legend: red—Ipk2 family members having 6-/3-/5-kinase activities; green—$I(1,4,5)P_3$ 3-kinase (ITPK); blue—diphosphoryl inositol synthetase, which generate PP-IPs (IP6K); black—IP_5 2-kinase; magenta—$I(1,3,4)P_3$ 5-/6-kinase; and cyan—IP_3 5-phosphatase.

mammals, and a recent report that reduction of higher IPs in mammalian cells also affects mRNA export suggests this pathway is conserved throughout eukaryotes (J. Stevenson-Paulik, R. A. Frye, and J. D. York, unpublished; [20,21]).

Second, a role for IP_4 and/or IP_5 in the regulation of gene expression has come from studies of a yeast IP_3/IP_4 kinase, Ipk2, which found it is identical to Arg82 [16,17]. Messenguy and co-workers [22,23] have studied Arg82 as a regulator of gene expression through the ArgR-Mcm1 transcription complex. Ipk2 is a dual-specificity 6-/3-kinase that sequentially converts IP_3 to IP_5, is localized within the nucleus, and is required to assemble protein complexes on DNA-promoter elements. Both Plc1 activity and Ipk2-mediated IP_4/IP_5 production are required for ArgR-Mcm1 transcriptional activation. Our results indicate that Ipk2 influences transcriptional responses through a two-step mechanism. First, Ipk2 protein but not IP synthesis is needed to enable formation of ArgR-Mcm1 complexes on DNA promoter elements. Second, production of IP_4 and possibly IP_5 through both phospholipase C and Ipk2 kinase activity is required to properly execute transcriptional control. While Messenguy and colleagues [24] have recently suggested that higher IPs are not required for ArgR-Mcm1 transcription, we find in using both genome-array

analysis and transcriptional reporter assays that both Plc1 and Ipk2 kinase activities are required for appropriate gene expression [A. R. Odom and J. D. York, unpublished]. Because Ipk1 is not required for complex formation or transcription control [16], we conclude that these two IP kinases generate distinct nuclear messengers.

It is also important to mention earlier studies of Henry and Coworkers that found that changes in cellular levels of *myo*-inositol regulate the transcription of *INO1*, whose gene product converts glucose 6-phosphate to D-inositol 3-phosphate (reviewed in [25]). This enables cells to initiate *de novo* synthesis of inositol under conditions in which it is unavailable in the growth medium. This transcriptional regulation is accomplished through defined *cis*-acting DNA elements and *trans*-acting factors. An important future area of study will be to determine how the cell detects changes in inositol.

A third role for phospholipase C pathway in nuclear function has come from studies of the Kcs1, a disphosphoryl synthase that generates PP-IPs from IP_5 and IP_6 substrates (referred to as an IP_6 kinase by Snyder and coworkers). *KCS1* was originally identified on a genetic screen as one of two genes that when mutated overcome a hyper-recombination phenotype found in certain mutant alleles of protein kinase C [26]. Snyder and coworkers [27] have demonstrated that Kcs1 has IP_6 kinase activity and find that a point mutation in the kinase domain results in rescue of hyper-recombination, indicating that PP-IPs play a role in DNA metabolism. Of note, Shears and co-workers [19,28] have suggested a role for PP-IPs in binding components involved in membrane trafficking and have recently reported that *kcs1* mutant yeast strains have aberrant vacuole morphology. Thus, it is possible that PP-IPs have distinct compartment specific functions.

An additional role for higher IPs in DNA metabolism is suggested by the work of West and coworkers [29]. This group finds that IP_6 is a regulator of non-homologous end-joining (NHEJ), a DNA repair pathway mediated through the DNA-dependent protein kinase (DNA-PK). Through an elegant biochemical purification of a cellular regulator of NHEJ, IP_6 was identified [29]. Subsequently it has been shown that inding of IP_6 occurs via the *KU* heterodimer, the noncatalytic subunit of DNA-PK [30,31]. While both *KU* heterodimer and NHEJ pathways are found in yeast, the yeast *KU* heterodimer does not bind IP_6, and unpublished studies of Llorente and Symington (referred to in [31]) have indicated that loss of IP_6 production does not impair NHEJ in yeast. It will be important to show that changes in IP_6 levels, or one of its metabolites such as PP-IP_5, within the cell regulate NHEJ *in vivo*.

The Inositol Polyphosphate Kinase (IPK) Family

The sequence motif "PxxxDxKxG" is conserved among a growing family of inositol polyphosphate kinases, which in general we have called IPKs, depicted by the dendogram in Fig. 1B. This motif was originally described as common to IP_3 3-kinases (reviewed in [32]), and subsequently it was shown by several research groups to be a hallmark of IPK family members discussed in the preceding section, which include IP_3 3-kinases, IP_3/IP_4 dual-specificity 6-/3-kinases, and diphosphoryl IP synthase. This motif is not found in IP kinases that phosphorylate the axial second position of the inositol ring [15], nor in I(1,3,4)P_3 5/6 kinases [33], suggesting these kinases evolved from different ancestors. Thus there appear to be three branches on the tree, each of which encodes kinases that regulate distinct processes within the cell.

The Ipk2 and diphosphoryl inositol synthase (IP6K) branches are conserved from yeast to man, but the IP_3 3-kinase (ITPK) branch is not. Two groups have found that certain Ipk2 proteins exhibit diphosphoryl synthase activity [34,35]. These data indicate that the Ipk2 branch may be the oldest and raises a question related to the origins of soluble inositol polyphosphate signaling and higher IP function. What is remarkable, all eukaryotes have pathways in which the activation of phospholipase C results in cleavage of PI(4,5)P_2 to produce inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. However, the commonly viewed cellular function of these two messengers in releasing intracellular calcium and stimulation of protein kinase C has not yet been described in lower eukaryotes. Budding yeast do not appear to have IP_3-mediated calcium release pathways (and no evidence of the IP_3 receptor in their genome), nor does diacylglycerol appear to activate yeast Pkc1. I will leave you with a final question: does this indicate that the primordial role of phospholipase C induced production of IP_3 is to serve as fuel for production of higher IPs and regulation of nuclear processes?

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CHAPTER 160

Ins(1,3,4,5,6)P_5: A Signal Transduction Hub

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Introduction

All nucleated cells contain approximately 15 to 50 μM Ins(1,3,4,5,6)P_5 [1]. In this review, I will illustrate how Ins(1,3,4,5,6)P_5 serves a number of signaling roles, both by itself, and also as a precursor pool for other physiologically active inositol polyphosphates (Fig. 1).

For example, Ins(1,3,4,5,6)P_5 dephosphorylation by a receptor-regulated 1-phosphatase [2] generates Ins(3,4,5,6)P_4, which inhibits CaMKII-dependent activation of a family of Cl^- channels in the plasma membrane [3–5]. This carefully controlled regulation of ion channel conductance, through a dynamic balance between competing stimulatory and inhibitory signals, permits a high degree of signal amplification. Enhancement of the signaling process is aided by the precipitous dose-response curve that describes the highly cooperative manner with which Ins(3,4,5,6)P_4 inhibits Cl^- channels [3,4,6]. Specificity is another of the hallmarks of an efficient cellular signal; this is certainly the case here. Cl^- channels are unaffected by Ins(1,3,4)P_3, Ins(3,4,5)P_3, Ins(3,4,6)P_3, Ins(4,5,6)P_3, Ins(3,5,6)P_3, Ins(1,3,4,6)P_4, Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4, and Ins(1,3,4,5,6)P_5 [2–4,7]. The efficacy of Ins(3,4,5,6)P_4 (IC_{50} = 3–7 μM) reflects a physiologically relevant concentration range (1–10 μM; [8]).

Ca^{2+} also directly activates some Cl^- channels, independently of CaMKII; this effect of Ca^{2+} is also blocked by Ins(3,4,5,6)P_4 in certain situations [7] although not in others [5]. Both CaMKII- and Ca^{2+}-regulated Cl^- channels regulate salt and fluid secretion [8,9], cell volume homeostasis [10], and electrical excitability in neurones and smooth muscle [11]. Recently [12] we showed that at least one member of a molecularly distinct Cl^- channel family, ClC, is also inhibited by Ins(3,4,5,6)P_4. These channels are located in secretory vesicles, endosomes, and lysosomes, where they act as a charge shunt that facilitates functionally indispensable vesicle acidification by ATP-driven H^+ pumps [13]. Inhibition of insulin granule acidification by Ins(3,4,5,6)P_4 attenuates Ca^{2+}-dependent insulin secretion [12]. We anticipate that further cellular functions for Ins(3,4,5,6)P_4 will emerge from its effect upon vesicle acidification. Thus, receptor-activated Ins(1,3,4,5,6)P_5 dephosphorylation regulates a versatile range of physiological activities that depend upon Cl^- channel activity.

The Ins(1,3,4,5,6)P_5 1-phosphatase is of particular interest because it is reversible *in vivo* [2]; in fact, this enzyme was originally identified as an Ins(3,4,5,6)P_4 1-kinase [14]. Furthermore, the 1-phosphate group that is removed from Ins(1,3,4,5,6)P_5 can be directly transferred to the 6-OH of Ins(1,3,4)P_3 [2]. By accepting this phosphate group, Ins(1,3,4)P_3 enhances the Ins(1,3,4,5,6)P_5 1-phosphatase activity [2]. This is how PLC-initiated increases in levels of Ins(1,3,4)P_3 elevate Ins(3,4,5,6)P_4 levels [15]. Thus, the 1-kinase and 1-phosphatase activities of a single enzyme switches Ins(3,4,5,6)P_4 signaling on and off. These opposing reactions offer an alternative to general doctrine that intracellular signals are regulated by integrating multiple, distinct phosphatases and kinases [16].

A different role for Ins(1,3,4,5,6)P_5 concerns its interaction with PTEN, a tumor suppressor [17]. PTEN has classically been recognized as a PtdIns(3,4,5)P_3 3-phosphatase that downregulates the lipid's enhancement of cell proliferation and Akt-dependent cell survival [17]. My laboratory

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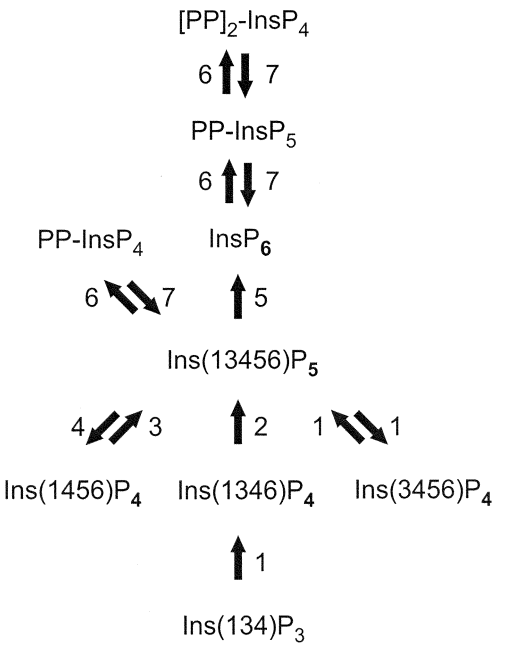


Figure 1 Enzymes that synthesize and metabolize Ins(1,3,4,5,6)P_5. (1) The multifunctional Ins(1,3,4)P_3 6-kinase/Ins(3,4,5,6)P_4 1-kinase/ Ins(1,3,4,5,6)P_5 1-phosphatase [2]. (2) Ins(1,3,4,6)P_4 5-kinase [1]. (3) Ins(1,4,5,6)P_4 3-kinase [1,21,23]. (4) PTEN [18] and MIPP [26]. (5) Ins(1,3,4,5,6)P_5 2-kinase [27]. (6) Diphosphoinositol polyphosphate synthase (a.k.a. "InsP_6 kinase") [30,31]. (7) Diphosphoinositol polyphosphate phosphatase [33].

recently discovered that PTEN is also a high-affinity Ins(1,3,4,5,6)P_5 3-phosphatase [18]. Competition from soluble Ins(1,3,4,5,6)P_5 will temper the ability of PTEN to bind and dephosphorylate PtdIns(3,4,5)P_3 and further restrict PTEN's already weak protein phosphatase activity [19]. Ins(1,3,4,5,6)P_5 may therefore be viewed as "clamping" PTEN activity, the significance being that overall regulation of a signaling system is much tighter, and permits greater amplification, if it has to be de-inhibited (i.e. when PTEN escapes Ins(1,3,4,5,6)P_5) as well as activated (i.e. when PTEN locates PtdIns(3,4,5)P_3). Ins(1,3,4,5,6)P_5 that is dephosphorylated by PTEN will be replenished by Ins(1,4,5,6)P_4 3-kinase activity; we discovered this kinase over 10 years ago, and we also demonstrated the dynamic nature of Ins(1,3,4,5,6)P_5 3-phosphatase/Ins(1,4,5,6)P_4 3-kinase metabolic cycling *in vivo* [1,20]. Perhaps this cycling is simply the metabolic "price" for regulation of PTEN. Alternately, this cycle may itself have specific functions, for example in the nucleus, since that is where most mammalian Ins(1,4,5,6)P_4 3-kinase is located [21], together with some PTEN [22]. Furthermore, in yeast there is evidence that Ins(1,4,5,6)P_4 synthesis regulates transcription [23]. Salmonella's SopB and SopE virulence factors activate rapid Ins(1,3,4,5,6)P_5 hydrolysis to Ins(1,4,5,6)P_4 as an obligatory part of the process by which the bacteria activate host cell Rac/Rho GTPases, which promotes cellular invasion [24,25]. There is a mammalian enzyme (MIPP; multiple inositol polyphosphate phosphatase) that further dephosphorylates Ins(1,4,5,6)P_4 to Ins(1,4,5)P_3 [26]. Thus, Ins(1,3,4,5,6)P_5 potentially provides a PLC-independent source of Ins(1,4,5)P_3, although to date, only *Dictyostelium* MIPP is proven to generate Ins(1,4,5)P_3 in this manner [26].

Ins(1,3,4,5,6)P_5 is also phosphorylated by a 2-kinase [27], yielding InsP_6, to which a number of diverse functions have been attributed, but unfortunately, *in vitro* experiments with InsP_6 provide many opportunities for nonphysiological artifacts, so the significance of many of these studies has been criticized [28]. This is why genetic manipulations of InsP_6 levels inside cells are more likely to uncover useful information. Finally, there are enzymes that convert Ins(1,3,4,5,6)P_5 to a diphosphorylated derivative (PP-InsP_4) [29]. This kinase family generally receives more attention for phosphorylating InsP_6 [30], but Ins(1,3,4,5,6)P_5 is also a substrate [31]. Indeed, metabolic cycling between Ins(1,3,4,5,6)P_5 and PP-InsP_4 is at least as extensive in intact cells as is the cycling between InsP_6 and its diphosphorylated derivatives (PP-InsP_5 and [PP]$_2$-InsP_4 [29]). All of the diphosphorylated inositol phosphates are considered "high-energy" phosphate donors that apparently regulate vesicle trafficking and possibly other energy-demanding processes [31,32].

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CHAPTER 161

Phospholipase D

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Introduction

Phospholipase D (PLD) activity is found throughout the biological world, and enzymes have been characterized from a broad spectrum of organisms. Phosphatidic acid is a key molecule in the metabolic pathways for phospholipid synthesis and degradation. One pathway for the production of this lipid is hydrolysis of glycerophospholipids by PLD enzymes, which produce phosphatidic acid (PA) and the associated base or headgroup.

Numerous hormones, growth factors, neurotransmitters, and other cellular stimuli regulate the generation of PA by PLD in mammalian cells. This has led to an emerging role for the mammalian enzymes and PA in signal transduction. Phosphatidic acid is hypothesized to act as a second messenger through direct interaction with a variety of targets, which are discussed elsewhere [1,2]. In addition, PA is a precursor for formation of diacylglycerol (DAG) and lysophosphatidic acid (LPA). DAG can act as a second messenger to regulate the activity of protein kinase C (PKC) isozymes; LPA can function as an autocoid or paracrine through interaction with receptors in the edg family.

This brief overview summarizes the properties and functions of PLD enzymes with a focus on the mammalian proteins. More detailed information can be found in several reviews that provide excellent depth [1] and earlier perspectives [2–4], as well as description of the enzymes in plants [5,6] and yeast [7].

Structural Domains and Requirements for Activity

Two mammalian PLD isozymes have been identified and characterized at the molecular and biochemical levels. A schematic representation of the domain structure of these and several PLD enzymes from other organisms is presented in Fig. 1A. The phospholipase D enzymes belong to a larger family of proteins that includes several endonucleases, cardiolipin synthases, and phosphatidylserine synthases. These enzymes are characterized by the presence of HKD motifs (consensus sequence, HxKxxxxD). Structures have been determined for two members of this superfamily, the PLD from *Streptomyces* sp. Strain PMF [8] (MMDB, 15995; PDB, 1FO1) and the dimer of the endonuclease, Nuc, from *Salmonella typhimurium* [9] (MMDM, 11347; PDB, 1BYR). The structures clearly show that two HKD motifs come together to form a single functional catalytic site with the two histidines as likely nucleophiles for catalysis. Whereas most of the enzymes in this superfamily contain both HKD motifs in a single polypeptide, Nuc contains only a single motif and dimerizes to form its active site (Fig. 1B). Mutations of conserved residues in the HKD motifs have indicated their requirement for catalytic activity in the mammalian enzymes. The C-terminus is a second region required for activity by the mammalian enzymes. Evidence from Liu and colleagues [10] indicates that C-terminal residues and especially the C-terminal α-carboxyl group were required for activity. The actual role of these residues in the catalytic reaction is unknown.

Figure 1A identifies other potential regulatory features that distinguish PLD enzymes. The classical plant PLDs, represented by the enzyme from maize, contain an N-terminal C2 domain, which provides for binding of Ca^{2+} and phospholipids [6]. In contrast, the mammalian enzymes contain putative PX and PH domains that may be important for interaction with regulatory molecules. The presence of the latter domains extends to PLDs from many organisms, including *D. melanogaster*, *C. elegans,* and yeast. Recently, two PLD

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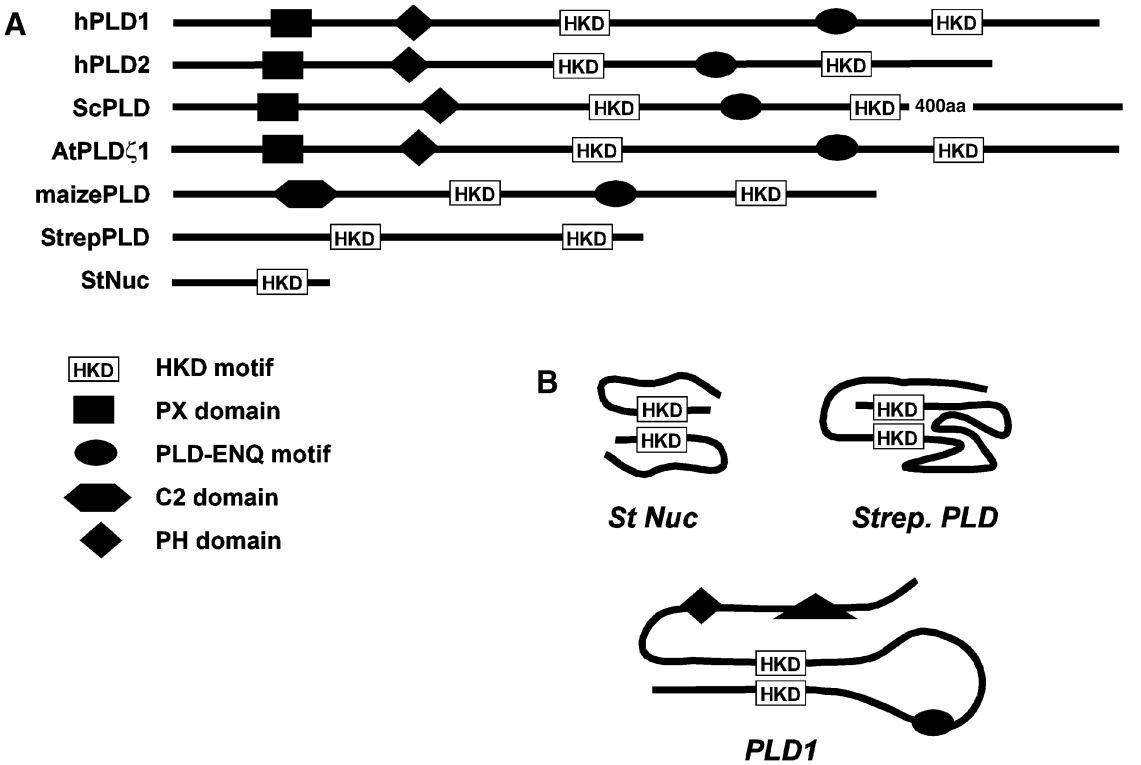


Figure 1 A. Schematic representation of several members of the PLD superfamily: hPLD1, human PLD1 (GI:4505873); hPLD2, human PLD2 (GI:20070141); ScPLD, SPO14 gene from *Saccharomyces cerevisiae* (GI:1174406); AtPLDζ1, isozyme from *Arabidopsis thaliana* (GI:20139230); maizePLD, PLDα1 from *Zea mays* (GI:2499708); StrepPLD, enzyme from *Streptomyces septatus* (GI:15823702); StNuc, nuclease from *Salmonella typhimurium* (GI:6435643). B. Dual HKD motifs cooperate to form a single active site. In the case of Nuc, which only contains one HKD motif, the protein dimerizes to form an active catalytic site.

enzymes that closely resemble the mammalian structural paradigm were identified in *Arabidopsis thaliana* (see AtPLDζ1, Fig. 1). This departure from the classical plant PLDs suggests a much broader scope for regulation of PLD activity in plants [11].

Catalysis: Mechanism and Measurement

It has been appreciated for some time that the enzymatic cleavage carried out by PLD is a two-step process (Fig. 2, see [12] for details). The preferred substrate for the mammalian enzymes is phosphatidylcholine (PC) [13]. The initial reaction involves the formation of a phosphatidylated enzyme with the concomitant release of choline. This attachment is presumably to one of the histidines of the HKD motifs. Under physiological conditions, water is used to release phosphatidic acid and regenerate the active enzyme. A common substrate for assessment of PLD activity *in vitro* is [^{3}H-choline]-PC; the reaction is easily followed by measurement of released choline. Optimal assay procedures, which use phospholipid vesicles containing the labeled PC, have been described in detail [14].

Primary alcohols are much better acceptors for the phosphatidic acid than water. In solutions containing 1% ethanol, the primary product of PLD is phosphatidylethanol rather than PA. This property, referred to as transphosphatidylation, is unique to PLD enzymes and is exploited to measure PLD activity *in vivo*. The pool of PC in cultured cells can be preferentially labeled with [^{3}H]-myristate or labeled lysophosphatidylcholine. The exposure of cells to ethanol or lower concentrations of n-butanol results in the formation of the labeled phosphatidyl alcohol, which can be uniquely distinguished after separation by thin later chromatography or other resolving techniques. An extensive discussion of this and other methods to measure PLD activity is available [13].

Modification of Mammalian PLDs

Two types of modification have been observed in the mammalian enzymes. Two cysteines (Cys240 and Cys241) in the PH domain of PLD1 can be acylated [15]. Elimination of this acylation in PLD1 [15] and PLD2 [16] results in altered cellular location and modest reduction in membrane association. However, a definitive role of this modification in physiological regulation remains to be elucidated.

The PLD isozymes can be phosphorylated by PKCα, *in vitro*, and on Ser/Thr and Tyr, *in vivo*. While these modifications are putative mechanisms for modulation of PLD

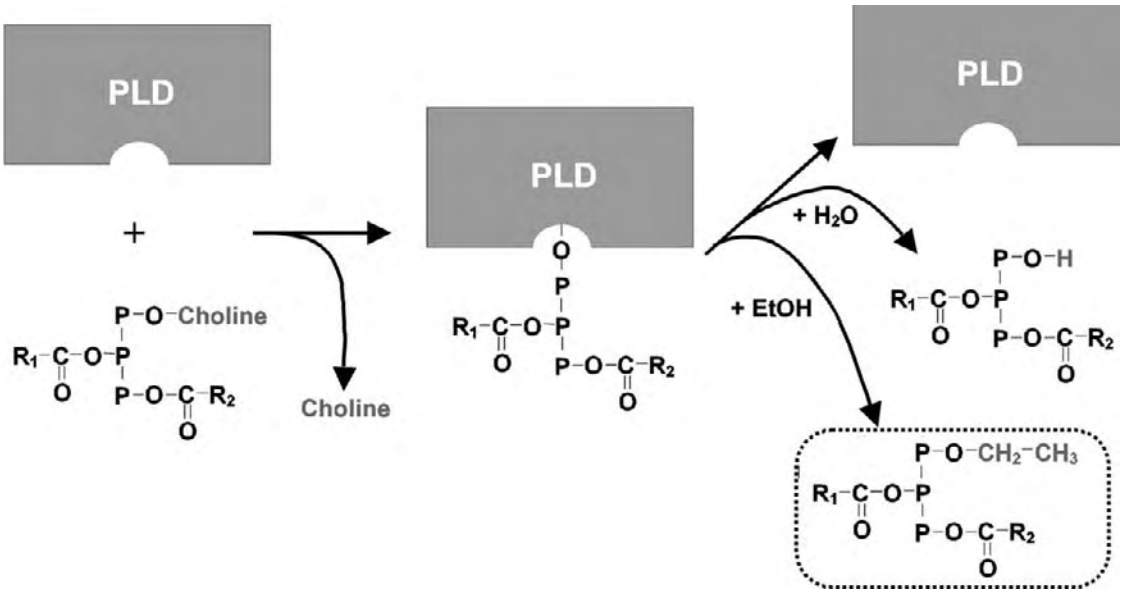


Figure 2 Catalytic mechanism for PLD. Hydrolysis of PC involves two steps. The first cleavage releases choline while forming an intermediate phosphatidylated enzyme. Subsequent hydrolysis with water yields the normal product, phosphatidic acid. In the presence of low concentrations of primary alcohols, the reaction can be shunted to form the phosphatidyl alcohol.

activity by G-protein-coupled receptors and growth factors, the evidence for stimulation of PLD activity by direct phosphorylation is not clear [1].

Regulatory Inputs for Mammalian PLD

Phospholipase D activity in mammalian cells can be stimulated by numerous hormones that act through G-protein-coupled receptors, growth factor receptors, and other stimuli (see [1,2] for surveys). Potential mechanisms for regulation include at least four direct activators that have been identified and characterized, *in vitro*; these are two families of monomeric GTPases (Arf and Rho), protein kinase C (PKC), and phosphatidylinositol 4,5-bisphosphate (PIP_2) (Fig. 3). The lipid PIP_2 was originally identified as a key component of substrate vesicles used to assay the mammalian enzyme [17,18]. Subsequently, this lipid was shown to be an efficacious activator of both PLD1 and PLD2, SPO14 from yeast, and most recently two isoforms of PLD from *Arabidopsis* [11]. While many reviews and papers have stated that this lipid is a cofactor or essential for phospholipase activity, the mammalian PLD1 is clearly active and regulated by Arf in the absence of PIP_2 [14]. Thus, this lipid should be considered a bonafide regulator of the enzyme. Phosphatidylinositol 3,4,5-trisphosphate has also been shown to activate PLD, but the low abundance of this lipid in cells suggest this is unlikely to be a physiological mechanism.

Members of the Arf family of monomeric GTPases were the first identified protein regulators of PLD activity [17,19]. Although they are potent activators of PLD1, Arfs provide only slight modulation of wild type PLD2 [20]; this becomes more efficacious if the N-terminus of PLD2 is truncated [21]. The physiological relevance of this is not known. A second family of monomeric GTPases, the Rho proteins, can also directly activate PLD1 but not PLD2. All subgroups of the Rho family (Rho, Rac, and Cdc42) have proven effective in this function. For both Arf and Rho proteins, regulation of PLD activity occurs through the activated form of the GTPase and thus downstream of the regulation of these proteins.

A more traditional pathway for regulation of PLD is via the classical forms of PKC. It had been noted for some time that direct stimulators of PKC (e.g. phorbol myristate acetate, PMA) increased the activity of PLD in cells. The unusual feature of this regulation, when assessed *in vitro*, is that direct stimulation of PLD by activated PKCα does not require phosphorylation, but rather may occur through the regulatory domain [22]. There is evidence, however, that phosphorylation mechanisms may be required *in vivo* [1].

An intriguing property of these activators is their synergistic action when combined *in vitro* [22,23]. Such action is especially noted among the protein activators, as well as in conjunction with PIP_2. This finding suggests that the most efficacious stimulation of PLD activity *in vivo* may require the action of multiple regulatory pathways.

Regulatory Pathways

Evidence has been presented to implicate these stimulatory molecules in the regulation of PLD activity in cells. Extensive discussions are found elsewhere [1,2]. One clear mechanism for G-protein-coupled receptors is through the stimulation of phospholipase Cβ and activation of protein kinase C (Fig. 3). Similarly, growth factors can stimulate PKC via PLCγ.

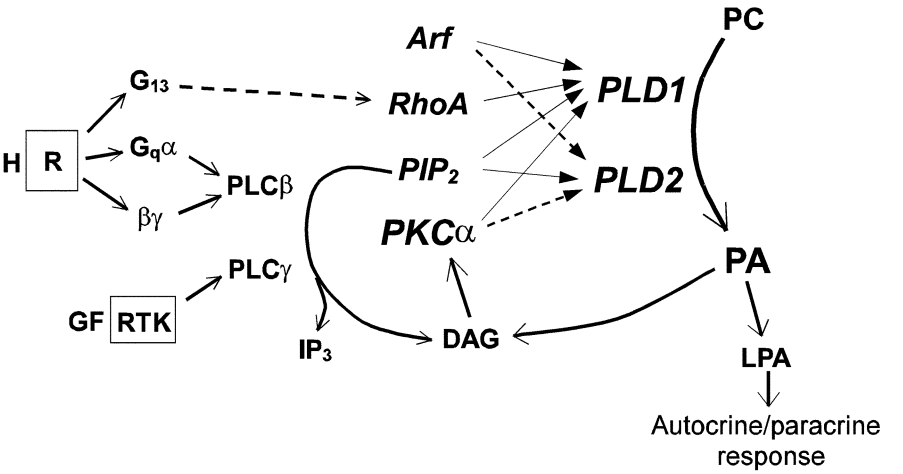


Figure 3 Potential pathways for regulation of mammalian PLD isozymes. See text for discussion of interactions and pathways.

Use of inhibitors of PKC and other strategies to reduce PKC activity in cells is generally effective for attenuation of PLD activity due to direct stimulation of PKC with PMA. In contrast, hormone responses are often retained. The use of PKC to stimulate PLD is intriguing because it potentially leads to an autocatalytic cycle in which the product of PLD activity could lead to further activation of PKC via conversion of PA to DAG. The ineffectiveness of PKC inhibitors in many systems (including some hormones that stimulate PLC activity) indicates that this regulation is more complex and that other mechanisms for hormonal stimulation are operative.

Evidence for the role of Rho proteins in regulation of PLD activity derives largely from the use of C3 toxin from *C. botulinum* and expression of dominant negative forms of the GTPases (summarized in [1]). The interpretation of these data is complicated by the potential regulation of PIP_2 synthesis by Rho proteins and the potential for nonspecific effects of disruption of cytoskeletal architecture through attenuation of these GTPases. The Rho proteins offer an attractive mechanism for regulation of PLD by receptors coupled to the G_{12} and G_{13} proteins. At this time, evidence for this regulation by endogenous receptors and G proteins is lacking.

Physiological regulation of PLD by PIP_2 is still an open question. Various studies, especially in permeabilized cells, that correlate PLD activity with manipulations to vary PIP_2 are suggestive [1,2]. However, the multiple actions of PIP_2 in cells suggests that global change in the concentration of PIP_2 is an unlikely regulatory mechanism. Recent studies that show increased activity of PLD2 when coexpressed with Type1α PIPkinase and the potential association between this kinase and the PLDs [24] support hypotheses that regulation could occur through localized and coordinated changes in the lipid.

The emerging picture predicts that several pathways regulate PLD activity *in vivo*. The primary pathways used in any one system will probably depend on the cell type, the stimulus used, and the local and temporal environment, such as the state of other pathways being utilized in the cells.

Physiological Function of PA

The number of stimuli that regulate PLD activity strongly indicate the importance of PA in signal tranduction. The known activators of PLD have given rise to hypotheses that include roles in basic hormone signaling (PKC), vesicle trafficking (Arf), cytoskeletal rearrangements (Rho), and exocytosis (Arf and Rho) [1,2]. Yet the various roles proposed are largely unproven.

Evidence for prolonged production of DAG via PLD activity is clear, and downstream regulation is probable. This pathway offers a mechanism for regulation by DAG that is independent of Ca^{2+} and selective for a spectrum of PKC isozymes different from those activated by the action of phospholipase C. The potential role of PA in facilitation of membrane rearrangements required for vesicle budding and fusion is attractive. Thus, production of PA, and subsequent products, DAG or LPA, could profoundly affect curvature and stability of the membranes involved. However, the evidence for these events is controversial. Finally, numerous proteins have been shown to interact with or be affected directly by PA *in vitro* [1,2]. These represent potential targets for regulation by the lipid, but definitive demonstration of these putative pathways has been elusive.

Localization of PLD

Phospholipase D activity is found primarily in particulate fractions of mammalian cells and tissues. Subcellular distribution of PLD isozymes within the cell is still in question and has been inferred largely from studies with exogenous expression of tagged proteins [1,2]. In several studies, overexpession of PLD1 resulted in localization to perinuclear regions,

frequently with a punctate appearance. In contrast, overexpressed PLD2 was found in the plasma membrane and potential endosomal vesicles near the surface of cells. One report, which examined endogenous PLD1, found the enzyme enriched in the Golgi apparatus, but diffuse staining also indicated a more diverse distribution [25].

Future Directions

Which PLD isozyme is responsible for activity observed in response to hormones? PLD1 was initially favored because of its reponsiveness *in vitro* to regulatory molecules in the known hormone pathways (PKC and Rho in particular). Yet the presumed main site for regulation of PLD activity by hormones is the plasma membrane, and by this criterion, localization studies suggest PLD2 as the potential target. Studies with overexpressed enzymes demonstrate that PLD2 is responsive to activation by PKC in the cellular environment and can enhance stimulations observed with some hormonal stimuli. In contrast, overexpressed PLD1 can be unresponsive to agonists that stimulate putative activators of the enzyme. The discordance between regulation observed *in vitro* and *in vivo* indicates there is still much to be learned about the molecular mechanisms of PLD regulation.

In addition to understanding the responsiveness of individual isozymes, basic questions about the putative roles of PA and its metabolites abound. Fundamental to this are clear determinations of the specific regulation of putative effector proteins and the potential role for these lipids as mediators of membrane restructuring. As for the enzyme itself, little is known about its three-dimensional structure or the molecular mechanisms by which multiple activators can synergistically stimulate its activity. A clear understanding of these mechanisms *in vitro* should facilitate investigation of hormonal pathways *in vivo*.

Acknowledgments

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CHAPTER 162

Diacylglycerol Kinases

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Introduction

Many signaling cascades are initiated by phospholipase C (PLC) isozymes. One product of this reaction is diacylglycerol (DAG), a prolific second messenger that activates proteins involved in a variety signaling cascades. The protein kinase Cs (PKCs) are the best-characterized DAG-activated proteins, but diacylglycerol also activates other proteins [1], including RasGRP and two guanine nucleotide exchange factors (GEFs), CalDAG GEFs I and III. The chimaerins, which are GTPase-activating proteins (GAPs) for Rac, and the *Unc-13* gene product from *Caenorhabditis elegans* also bind to DAG. Because it can associate with a diverse set of proteins, DAG potentially activates numerous signaling cascades. Thus, its accumulation needs to be strictly regulated. Diacylglycerol kinases (DGKs), which phosphorylate DAG, are widely considered to be responsible for terminating diacylglycerol signaling [2,3]. But the product of the DGK reaction, phosphatidic acid (PA), also can be a signal: it can activate phosphatidylinositol 4-phosphate 5-kinases and PKCζ, participates in recruiting Raf1 to the plasma membrane, and is involved in vesicle trafficking. Because they manipulate both DAG and PA signaling, the DGKs can regulate numerous signaling events.

The DGK Family

DGKs have been identified in most organisms that have been studied, and it appears that they have gained specialization in more complex species. For example, bacteria express only one DGK, which is an integral membrane protein capable of phosphorylating DAG and other lipids such as ceramide. This DGK does not appear to have structural elements that allow regulation of its activity, indicating that the limiting factor is access to its substrates. With the exception of yeast, in which no DGKs have been identified, higher organisms appear to have several DGKs that can be grouped by common structural elements into five subfamilies. The mammalian DGKs are the best characterized, and nine of them have been identified [2,3]. All of these DGKs have two common structural features: a catalytic domain and at least two C1 domains, which are thought to bind diacylglycerol (Fig. 1). Other structural domains, which form the basis of the five subtypes, appear to have regulatory roles. For example, type I DGKs, α, β, and γ, have calcium-binding EF hand motifs that make these enzymes more active in the presence of calcium. Type II DGKs, δ and η, have pleckstrin homology (PH) domains near their amino termini. DGKδ also has a sterile alpha motif (SAM) at its carboxy terminus that may allow protein-protein interactions. The only type III DGK, ε, does not have identifiable structural motifs outside its C1 and catalytic domains. It is interesing that this is the only DGK that displays specificity toward acyl chains of DAG—it dramatically prefers DAGs with an arachidonoyl group at the *sn*-2 position. Type IV DGKs, ζ and ι, have a motif enriched in basic amino acids that acts as a nuclear localization signal and is a substrate for conventional PKCs. This motif is homologous to the phosphorylation site domain of the myristoylated alanine rich C kinase substrate (MARCKS) protein. Type IV DGKs also have four ankyrin repeats at their carboxy termini that may be sites of protein-protein interactions. The only type V DGK, θ, is distinguished by three C1 domains, a PH domain, and a Ras-association (RA) domain. To date, no binding partners for the PH and RA domains have been identified. Based on their structural diversity, the mammalian DGKs likely have specific roles dictated by their unique structural motifs.

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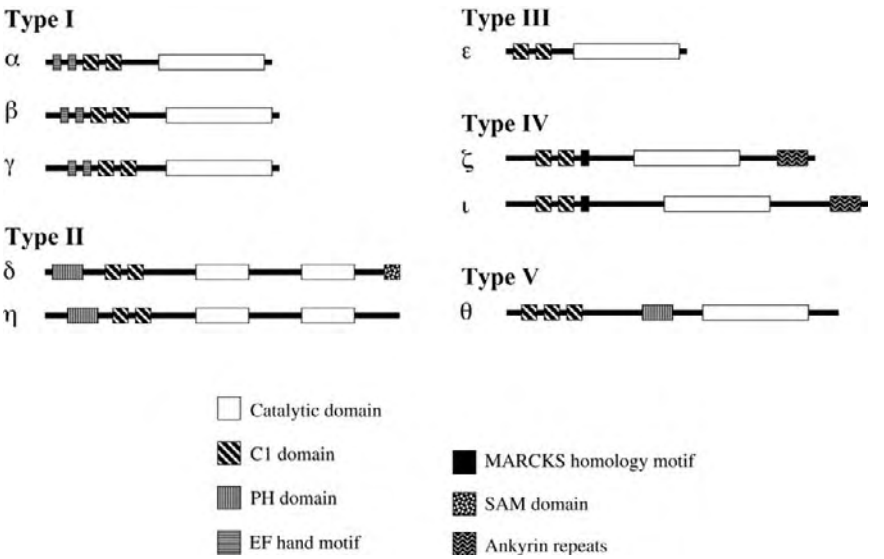


Figure 1 The nine members of the mammalian diacylglycerol kinase family are grouped by sequence homology into five subtypes. Shown are protein motifs common to several DGKs.

Regulation of DGKs

Activation of DGKs is complex, requiring translocation to a membrane compartment as well as binding to appropriate cofactors. Additional regulation of their activity occurs by posttranslational modifications. This complexity allows tissue- or cell-specific regulation depending on the availability of cofactors and the type of stimulus that the cell receives. Tissue-specific alternative splicing of DGKs β and ζ—and probably other isotypes—adds additional opportunities for regulation.

DGKα demonstrates the complex regulation of DGKs. In T lymphocytes, it translocates to at least two membrane compartments depending upon the agonist used to activate the cells. For example, stimulation of T cells with IL-2 causes DGKα to translocate from the cytosol to a perinuclear region [4]. But activation of the antigen receptor causes DGKα to translocate to the plasma membrane [5]. At the membrane, the activity of DGKα can be modified by the availability of several co-factors. Calcium is known to bind to the EF hand structures and stimulates DAG kinase activity *in vitro*, and lipids modify its activity: phosphatidylserine and sphingosine activate DGKα *in vitro* and probably *in vivo*. Finally, DGKα can be phosphorylated by several protein kinases, including PKC isoforms and Src. Although the consequences of these phosphorylations are not clear, evidence suggests that phosphorylation by Src enhances DAG kinase activity [6]. Thus, several events can modify the activity of DGKα, and combinations of them likely allow titration of its activity depending upon the cellular context.

Like DGKα, other DGK isotypes appear to be regulated by access to DAG through membrane translocation and by the availability of lipid or protein co-factors. Members of each DGK subfamily are likely to be regulated similarly, although there probably are subtle differences between subfamily members due to tissue-specific expression patterns, unique binding partners, alternative splicing, and subcellular localization. Type II DGKs, for example, have a PH domain, and this motif in DGKδ binds to phosphatidylinositols. Sakane *et al.* using an *in vitro* system, could not detect activation of DGKδ by phosphatidylinositols [2], suggesting that binding to these lipids instead provides a localization cue. The activity of types III and IV DGKs can be modified by lipids: DGKε is inhibited by phosphatidylinositols and by phosphatidylserine, while type IV DGKs are activated by phosphatidylserine. Type IV DGKs are also strongly regulated by subcellular translocation. They are imported into the nucleus, which requires their MARCKS homology domain, a nuclear localization signal that is regulated by PKC phosphorylation [7]. There is also evidence that the syntrophin family of scaffolding proteins regulates nuclear import of DGKζ by associating with its carboxy-terminal PDZ binding domain to sequester DGKζ in the cytoplasm [8]. And we have observed that DGKζ has a strong nuclear export signal (M. K. Topham, unpublished). Thus, nuclear accumulation of type IV DGKs is exquisitely regulated, suggesting an important nuclear function for these isozymes. Finally, DGKθ, a type V DGK, can be regulated through its association with active RhoA [3]. Binding to RhoA completely inhibits its DAG kinase activity and is the only example of regulation of a DGK through a protein-protein interaction.

Paradigms of DGK Function

Although there is substantial information regarding their regulation, little is known of the biologic functions of the individual DGKs. But recently, a few paradigms have emerged.

Spatial Regulation of DAG Signaling

Evidence suggests that DGKs selectively associate with and regulate DAG-activated proteins. Van der Bend *et al*. [2] initially tested this concept by either initiating spatially restricted DAG synthesis through receptor activation or by causing nonspecific, global DAG generation with exogenous PLC. They observed DAG kinase activity—measured by generation of PA—following receptor activation, but not after treating the cells with exogenous PLC. Their data demonstrate that DGKs are active only in spatially restricted compartments following physiologic generation of DAG. Recently, more specific examples of spatially restricted DAG kinase function have emerged. We found that DGKζ associated with RasGRP, a guanine nucleotide-exchange factor for Ras [9]. Their association was enhanced in the presence of phorbol esters, which are slowly metabolized DAG analogues. Since RasGRP requires DAG to function, we hypothesized that DGKζ associated with it to spatially metabolize DAG and consequently to regulate the function of RasGRP. Indeed, we found that kinase-dead DGKζ did not affect the function of RasGRP. Demonstrating the specificity of this regulation, we found that five other DAG kinases did not significantly inhibit RasGRP activity. Thus, our data demonstrate that in some cases DAG kinase activity is spatially restricted and serves to specifically regulate DAG-activated proteins.

Nurrish *et al.* presented another example of compartmentalized DGK function [3]. They isolated a *Caenorhabditis elegans* strain resistant to serotonin-induced inhibition of locomotion. The mutated gene responsible for the effect, *dgk-1*, is homologous to DGKθ. Their data suggested a model in which serotonin signaling activated DGK-1 to reduce local accumulation of DAG. This resulted in inhibition of UNC-13, a protein activated by DAG that may mediate acetylcholine release. Thus, their results represent another example of compartmentalized DGK function that modulates the activity of a DAG-activated protein.

Regulation of Signaling Through Fatty Acid Specificity

Inositol phospholipids, including PIP_2, a precursor of DAG, are enriched in arachidonate at the *sn*-2 position. Some DAG targets, including PKCs, are specifically activated by diacylglycerol-containing unsaturated fatty acids, such as arachidonate. So to maintain the integrity of some DAG-activated signaling cascades, it is important that phosphatidylinositols maintain a proper fatty acid composition. Because DGKε selectively phosphorylates arachidonoyl-DAG, the first step in resynthesis of phosphatidylinositols, DGKε may be responsible for their enrichment with arachidonate. Inositol lipid signaling is an important component of neuronal transmission. In a collaborative effort we examined seizure susceptibility in mice with targeted deletion of DGKε [10] and found that the null mice were resistant to seizures induced by electroconvulsive shock. Examination of brain lipids revealed that compared to wild-type mice, DGKε-deficient mice had reduced levels of arachidonate in both PIP_2 and DAG. This lipid profile demonstrated a critical role for DGKε in maintaining a proper balance of arachidonate-enriched inositol phospholipids. Thus, through its selectivity for arachidonoyl-DAG, DGKε regulates lipid signaling events and, consequently, seizure susceptibility.

Nuclear DGKs

There is a nuclear phosphatidylinositol cycle regulated separately from its plasma membrane/cytosolic counterpart [7]. DAG is present in nuclear preparations and appears to fluctuate with the cell cycle, but the specific pattern of its accumulation is not clear because of the many different methods used to isolate nuclei. DAG kinases have also been observed in nuclei and appear to have a prominent role there. Some DGKs, like DGKs α, ζ, and ι, translocate to the nucleus, while others, like DGKθ, are constitutively located there [3]. These DGKs are confined to specific compartments within the nucleus. For example, both DGKθ and DGKζ appear in a speckled pattern within the nucleus, while DGKα associates with the nuclear envelope [3]. This compartmentalization suggests that DGK isotypes have specific roles in the nucleus. Movement of proteins in the nucleus largely occurs by random diffusion, so overexpression of one DGK isotype may interfere with the function of another DGK. This fact, combined with the lack of specific DGK inhibitors, has made it difficult to study the nuclear function of the different DGK isotypes. But they are likely to affect nuclear signaling either by terminating DAG signals or by generating PA. For example, in T lymphocytes, the PA produced by nuclear DGKα appears to be necessary for IL-2-mediated progression to S phase of the cell cycle [4]. Conversely, nuclear DGKζ inhibits exit from G1 phase of the cell cycle by metabolizing DAG [7]. These data indicate both the complexity and importance of lipid signaling and DGK function in the nucleus.

Visual Signal Transduction

A clear role for DGK function has been demonstrated in *Drosophila*. A mutant strain, *rdgA*, undergoes rapid retinal degeneration after birth. The defect is due to a deficiency in retinal DGK activity because of a point mutation that inactivates dDGK2, a DAG kinase very similar to mammalian type IV DGKs. Although there are differences in photoreceptor signaling between *Drosophila* and vertebrates, these observations indicate that DGKs may be functionally important in the vertebrate retina. Three mammalian DGK isoforms, γ, ε, and ι, have been definitively localized to the retina, but their functions there have not yet been identified [2]. However, there is evidence of light-dependent activation of PIP_2 hydrolysis and generation of PA in vertebrate retina, indicating a role there for DGK activity.

Conclusions

Diacylglycerol kinases are expressed in all multicellular organisms that have been studied. Their structural diversity and complexity indicate that they are functionally important in a variety of cellular signaling events. Since they can affect both DAG and PA signals, DGK activity plays a central role in many lipid signaling pathways.

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CHAPTER 163

Sphingosine-1-Phosphate Receptors

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Introduction

The endothelial differentiation gene (EDG) family of G-protein coupled receptors (GPCRs) comprises high-affinity receptors for the lysophospholipids, lysophosphatidic acid (LPA), and sphingosine-1-phosphate (S1P) [1,2]. The homologous EDG receptors are clearly divided into two classes: three that bind LPA (EDG-2/LPA_1, EDG-4/LPA_2, EDG-7/LPA_3) and five that bind S1P (EDG-1/$S1P_1$, EDG-3/$S1P_3$, EDG-5/$S1P_2$, EDG-6/$S1P_4$, EDG-8/$S1P_5$). Molecular modeling and targeted mutagenesis have shown that S1PRs and LPARs use very similar motifs for binding of ligands, with one amino acid primarily determining the difference in specificity [3]. As several excellent reviews have recently appeared on LPARs and our studies have concentrated on dissecting molecular signaling pathways regulated by S1P [4,5], we have focused in this chapter on lipid signaling to and through S1PRs.

S1P is formed by sphingosine kinase (SphK), of which there are two known mammalian isoforms (for review, see [6]). SphKs are evolutionarily conserved and catalyze the ATP-dependent phosphorylation of the primary hydroxyl of sphingosine, the common backbone of mammalian sphingolipids. S1P is an interesting molecule that is an intercellular messenger and an intracellular second messenger [7]. This greatly complicates interpretation of results when adding exogenous S1P to cells: Is the response observed due to cell surface receptors, effects on intracellular targets, or both? A preponderance of studies have indicated that many of the biological effects of S1P are mediated by specific S1PRs and the lack of confirmed intracellular targets appears to bolster these claims. However, others have suggested that certain results are better explained by receptor-independent intracellular effects of S1P. First, the well-known S1PRs typically have K_ds in the 2–30 nM range [2,8], whereas effects of S1P on growth and suppression of apoptosis usually require micromolar concentrations [9]. In addition, dihydrosphingosine-1-phosphate (dhS1P), which has the same structure as S1P but only lacks the 4,5-*trans* double bond, binds to and activates all of the S1PRs. However, dhS1P does not mimic the effects of S1P on growth and survival [10], thus suggesting that these effects are likely to be mediated by intracellular actions of S1P.

The S1PRs

S1P was identified as the natural high affinity ligand of $S1P_1$ [2], which was shown to be highly specific, only binding S1P and dhS1P [11,12]. $S1P_1$ is coupled to $G_{\alpha i}$ and $G_{\alpha o}$ [13] but not $G_{\alpha s}$, $G_{\alpha q}$, or $G_{\alpha 12/13}$ [14]. Thus, pertussis toxin, which inhibits $G_{\alpha i/o}$ proteins, is a useful tool for dissecting signaling through $S1P_1$. *$s1p_1$* deleted mice died *in utero* between E12.5 and E14.5 due to massive hemorrhaging [15]. Although vasculogenesis and angiogenesis are normal in the *$s1p_1$-/-* mice, vascular smooth muscle cells failed to completely surround and seal the vasculature, thereby leading to hemorrhage. On a cellular level, the defect was linked to an inability of $S1P_1$ null fibroblasts to migrate toward S1P, likely due to dysfunctional Rac activation, and indicated the important role of S1P/$S1P_1$ signaling in motility.

$S1P_2$ is unique in being the only one of the S1PRs with a significantly poorer affinity for dhS1P than S1P [16]. $S1P_2$ has a wide tissue distribution [17] and a K_d for S1P of 20–30 nM [11]. In addition to $G_{\alpha i/o}$, $S1P_2$ couples to $G_{\alpha q}$ and $G_{\alpha 12/13}$ [14]. $S1P_2$ has been linked to increases in cAMP levels and thus may couple weakly to $G_{\alpha s}$ in some cell types depending on the pattern of expression of both GPCRs and

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G proteins [18]. $S1P_2$ regulates diverse signaling pathways, including calcium mobilization, stimulation of NF-κB, and inhibition of Rac-dependent cell migration in certain cell types [19,20]

$S1P_2$ has also been knocked out in mice [21] and in contrast to *s1p$_1$-/-* mice, these mice have no obvious anatomical or physiological phenotypes. It is interesting that mammalian $S1P_2$ is highly homologous to the zebrafish gene *miles apart* [22]. Two inactivating mutations in *miles apart* prevent the normal migration of heart primordia, thus resulting in abnormal cardiac development. The heart precursor cells from the *miles apart* mutants migrated normally when transplanted into wild-type embryos, but wild-type cells failed to migrate in mutant embryos, a result that suggests that the zebrafish $S1P_2$ homologue is required for generating a migration-permissive environment.

$S1P_3$ was shown to be activated by S1P [23] with a K_d of 20–30 nM [11,12] and to couple to $G_{\alpha i/o}$, $G_{\alpha q}$, and $G_{\alpha 12/13}$, but not $G_{\alpha s}$ [14]. $S1P_3$-null mice have been generated and also have no obvious phenotype [24]. $S1P_3$ has been linked to many signaling pathways, including calcium mobilization, stimulation of NF-κB, and NO production [25,26].

The two remaining S1PRs have a more narrow tissue distribution. $S1P_4$ is expressed almost exclusively in lymphoid and hematopoietic cells, as well as in the lung [27]. $S1P_4$ has a K_d for S1P of 12–63 nM, as determined by different groups [28,29], and couples to $G_{\alpha i/o}$. The final S1PR, $S1P_5$, previously named EDG-8 and *nrg-1*, is expressed predominantly in the central nervous system and to a lesser extent in lymphoid tissue [8,30]. $S1P_5$ couples to $G_{\alpha i/o}$ and $G_{\alpha 12/13}$, but not to $G_{\alpha s}$ or $G_{\alpha q}$ [31] and has a K_d for S1P of 2–6 nM [8,31].

S1P Signaling via S1PRs

Intriguing questions concerning lysolipid messengers are what regulates the levels of these amphipathic molecules and how do they get to their target cells? Platelets are known to store S1P and release it upon stimulation (reviewed in [32]). HUVECs and C6 glioma cells release S1P to the extracellular milieu [33,34]. Moreover, even when S1P release from cells is below detectable limits, co-culturing cells expressing $S1P_1$ with cells producing S1P due to overexpression of SphK induced activation of $S1P_1$ on adjacent as well as distant cells, thus indicating either that vanishingly small amounts of S1P are released or that it can be transferred from one cell to another by cell–cell interactions, or both [35]. Thus, S1P can act in an autocrine and/or paracrine manner. In support of this concept, the chemoattractant PDGF recruits SphK to the plasma membrane, where S1PRs are located, and especially to structures known as lamellipodia [36]. Given the importance of lamellipodia and S1PRs in chemotaxis, this finding suggests that S1P is produced and released from the cell in a spatially restricted manner, providing cells with a sense of direction. In addition, a recent report claims that type 1 SphK is secreted from cells in a catalytically active form and may catalyze the formation of SIP at or near the plasma membrane [33]. Further studies are necessary to confirm a role for extracellular SphK.

Transactivation of S1PRs

An intriguing aspect of the *s1p$_1$-/-* phenotype is that it appears to be nearly identical to that of the PDGF-BB and PDGFR-β knockouts [15], as these embryos also die because of a vascular smooth muscle cell migration defect. Because PDGF stimulates SphK and increases S1P [10], it therefore appeared possible that $S1P_1$ and PDGF signaling pathways are linked. Indeed, embryonic fibroblasts from *s1p$_1$-/-* mice, in contrast to wild-type cells, failed to migrate toward both S1P and PDGF [35]. Moreover, enforced expression of $S1P_1$ in HEK 293 cells, which express low basal levels of $S1P_1$, increased their ability to migrate toward PDGF, and antisense ablation of $S1P_1$ significantly inhibited migration toward PDGF [36]. A specific inhibitor of SphK also blocked PDGF-induced motility. Taken together, these results suggest a transactivation pathway linking PDGF though SphK to the autocrine and/or paracrine release of S1P that then stimulates $S1P_1$ to regulate motility. Furthermore, it was independently shown that $S1P_1$ potentiated the response to PDGF in HEK 293 cells overexpressing PDGFR [37]. However, in this case, these effects appeared to be independent of SphK, and it was suggested that PDGFR and $S1P_1$ were tethered in a complex that was activated independently of S1P.

Downstream Signaling from S1PRs

Because the S1PRs are coupled to heterotrimeric G proteins, the types of signals transduced are many and varied, depending on the specific isoforms of G_α and $G_{\beta\gamma}$ that are present. Thus, signals linked to a S1PR in one cell type may not be linked in the same manner in a second cell type. For example, transfection with $S1P_1$ increases S1P-induced calcium mobilization in CHO cells [38] but not in COS-7 cells [39]. Determining which specific S1PR is involved in a particular response is difficult because most cells express multiple S1PRs. To date, S1PR specific agonists or antagonists have not been developed. Thus, to elucidate the role of a particular S1PR, either transfection of receptor negative or knockout cells or antisense approaches have been used. Given the diversity of GPCR signaling, it is not surprising that results from these experiments demonstrate that S1PRs control the major lipid-mediated signaling pathways, as discussed below.

Phospholipase C. Many of the responses linked to S1PR signaling involve increases in intracellular calcium. Generation of the second messenger inositol trisphosphate (IP_3) by activation of phospholipase C (PLC) is the major pathway leading to intracellular calcium increases. CHO cells transfected with $S1P_1$, $S1P_2$, $S1P_3$, or $S1P_4$, but not vector controls, had increased IP_3 production and calcium release in an

S1P-dependent manner [28]. In contrast, in Jurkat T cells, $S1P_2$ and $S1P_3$, but not $S1P_1$, elicited IP_3-mediated calcium responses [25]. On a more physiological level, in HUVECs, which express $S1P_1$, and to a lesser extent $S1P_3$, S1P stimulated nitric oxide (NO) production by calcium-dependent epithelial nitric oxide synthase (eNOS) [40]. NO production was blocked by the PLC inhibitor U73122, the calcium chelator BAPTA-AM, and antisense oligonucleotides to $S1P_1$ or $S1P_3$, thus demonstrating a role for both S1PRs in activation of PLC. Furthermore, fibroblasts from $S1P_3$-null mice, but not littermate controls, failed to activate PLC upon S1P addition [24].

Phospholipase D. Another important lipid second messenger is phosphatidic acid (PA), which is generated by activation of phospholipase D (PLD). Overexpression of $S1P_1$ in HEK 293 or NIH 3T3 cells did not result in activation of PLD [9]. However, in C2C12 skeletal muscle cells, S1P stimulated PLD via either $S1P_1$, $S1P_2$, or $S1P_3$ in a pertussis toxin–sensitive manner [41]. Transfection of either $S1P_1$ or $S1P_2$ in C6 glioma cells conferred S1P-dependent PLD stimulation and PA formation [42]. $S1P_3$ also induced production of PA, specifically through activation of PLD2 in CHO cells [43].

Phosphatidylinositol-3-kinase. Activation of phosphatidylinositol-3-kinase (PI3K) promotes cell survival, cytoskeletal remodeling, and vesicular trafficking [44]. PI3K also promotes activation of the protein kinase Akt in two ways: translocation of Akt to the membrane by binding phosphatidylinositol-3,4-bisphosphate and activation of phosphoinositide-dependent kinases, which phosphorylate and activate Akt (reviewed in [45]). Though the S1PR(s) involved were not identified, S1P induced chemotaxis and angiogenesis of endothelial cells both *in vivo* and *in vitro* in a PI3K- and Akt-dependent manner [46,47]. $S1P_1$ transiently transfected in COS-7 cells led to activation of Akt, which was inhibited by the PI3K inhibitor wortmannin [48]. Further work from this group implicated $G_{\beta\gamma}$ stimulation of the PI3Kβ isoforms in S1P-dependent signaling to PI3K [49]. On a more physiological level, ventricular cardiomyocyte hypertrophy induced by S1P was inhibited by both wortmannin and by $S1P_1$ antibody [50]. $S1P_1$, $S1P_2$, and $S1P_3$ transfected into CHO cells each activated PI3K in response to S1P [43,51]. It is interesting that in this system, $S1P_1$ and $S1P_3$ promoted S1P-induced chemotaxis, while $S1P_2$ inhibited it.

Sphingosine Kinase. S1P has been demonstrated to release calcium from non-IP_3 releasable microsomal stores, though the intracellular receptor(s) are unknown [52–54]. Meyer zu Heringdorf and colleagues demonstrated that HEK 293 cells endogenously expressing $S1P_1$, $S1P_2$, and $S1P_3$ mobilized calcium in response to S1P [55]. However, in these cells, PLC was not activated and there was no measurable production of IP_3. What is especially interesting, they found that S1P stimulated SphK and S1P production, and the increase in S1P levels, as well as calcium release, was reduced by inhibitors of SphK. S1P production was also completely blocked by pertussis toxin, indicating the involvement of G_i-linked GPCRs in the process. Thus, the remarkable observation was made that extracellular S1P regulates intracellular S1P formation [55].

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CHAPTER 164

SPC/LPC Receptors

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Introduction

Among LPLs, the biological effects and signaling mechanisms of lysophosphatidic acid (LPA), sphingosine-1-phosphate (S1P), and their receptors (LPA_{1-3} and $S1P_{1-5}$) have been studied most extensively [1–4]. The signaling mechanisms of their corresponding choline derivatives, lysophosphatidylcholine (LPC) and sphingosylphosphorylcholine (SPC), however, have been examined to a much lower extent, although their extracellular existence and evidence of their signaling properties have long been recognized.

Addition of a positively charged choline group to the negatively charged phosphate group provides LPC and SPC with zwitterionic and detergent-like properties. In fact, LPC is cell lytic at concentrations >30 μM when bovine serum albumin (BSA) is absent [5]. Moreover, the specific receptors for LPC and SPC were not previously identified. Thus, controversy has arisen as to whether LPC, and possibly SPC, act as specific signaling molecules or molecules modulating cellular functions nonspecifically, and whether their actions are receptor-mediated. This situation has been changed recently with the identification of three G-protein-coupled receptors (GPCRs)—OGR1, GPR4, and G2A—as receptors for LPC and SPC [6–8]. These discoveries provide an intriguing and novel opportunity to study the pathophysiological and functional roles of SPC, LPC, and their receptors.

Physiological and Pathological Functions of LPC and SPC

The potential physiological and pathological functions of LPC and SPC have been recently reviewed [9,10]. While LPA, S1P, LPC, and SPC may share similar, overlapping, or opposing effects in some cellular systems, each of these lipids may also have its own unique functions. For example, all four of these LPLs have been shown to play some role in wound healing and some inflammatory processes [11–15]. LPA and its receptors are involved in nervous system development, and S1P has been implicated in cardiovascular development [12,16,17]. LPC and SPC are implicated more specifically in diseases involving immunological and inflammatory processes, such as atherosclerosis and systemic lupus erythematosus [18–21].

The metabolic pathways involved in synthesis and release of LPC and SPC are closely related to those of LPA and potentially S1P. A lysophospholipase-D (lysoPLD) activity, which directly converts LPC to LPA, has been reported previously [22,23]. We have recently observed that when sterile, cell-free ovarian cancer ascites samples, but not non-malignant ascites, were incubated at 37°C, LPA levels were increased over time (Fig. 1A). The LPA production was completely abolished when EDTA or EGTA was added to the ascites, indicating that the LPA production in ovarian cancer ascites was probably due to a soluble enzymatic activity that requires bivalent metal ions and calcium. It is interesting that during the same time course, LPC levels were decreased (Fig. 1B), suggesting that a lysoPLD-like activity may be responsible for LPA production in ovarian cancer ascites. Furthermore, SPC is a substrate for bacterial PLD (unpublished observations), and thus SPC may be converted to S1P in mammalian cells *in vivo*, although such an endogenous activity has not been identified. These data support the notion that the physiological roles of LPLs may be closely related and intertwined and therefore may play a more complex role *in vivo* than what is observed *in vivo* when a single LPL is tested.

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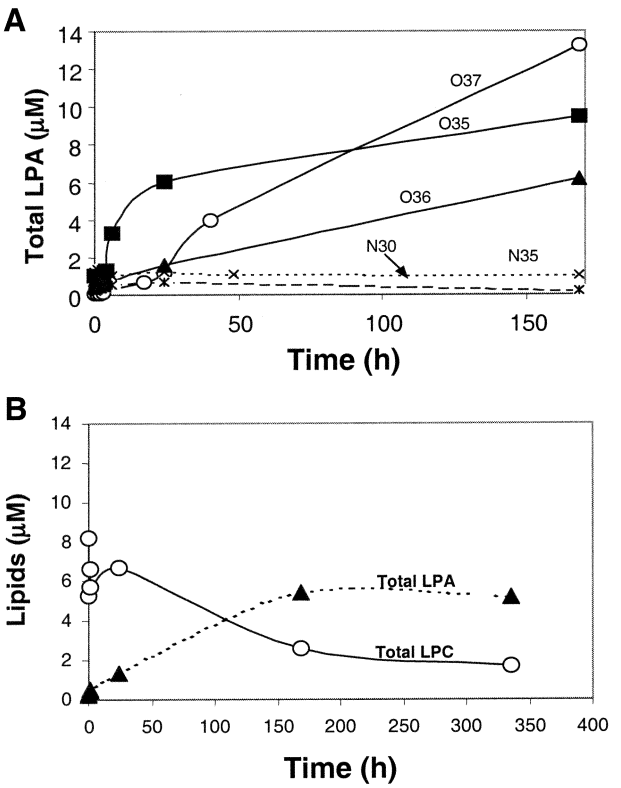


Figure 1 LPA production and LPC degradation in ascites samples. The ascites samples were incubated at 37°C for different durations as indicated, and then quantitatively analyzed for LPA and LPC content by ESI-MS [34,35]. (A) LPA production in ascites from patients with ovarian cancer (O35, O36, and O37) and nonmalignant diseases (N30 and N35). (B) LPC reduction and LPA production in ascites from a patient with ovarian cancer.

Identification of Receptors for SPC and LPC

The identification of receptors for SPC and LPC first began with the cloning of OGR1 from the HEY ovarian cancer cell line by using a PCR-based cloning strategy with primers based on the sequences of receptors for platelet-activating factor (PAF) and thrombin [24]. OGR1 shares approximately 30% sequence homology with the PAF receptor, a finding that indicated that the ligand for OGR1 may be also a lipid molecule and may also contain a choline group. Functional analyses were performed, which provided evidence for OGR1 as the first high-affinity receptor identified for SPC [6]. Similar studies were performed to determine whether SPC and/or LPC are ligands for GPR4 and G2A [7,8]. OGR1, GPR4, and G2A have no or very low affinity to LPA, S1P, PAF, lyso-PAF, lysophosphatidylinositol (LPI), PAF, sphingomyelin, ceramide, psychosine, glucosyl-β1,1′-sphingosine (Glu-Sph), galactosyl-β1,1′-ceramide (Gal-Cer), and lactosyl-β1, 1′-ceramide (Lac-Cer) [6–8]. TDAG8, a fourth related receptor that is 36% homologous to OGR1, has been recently identified as a receptor for a glycosphingolipid, psychosine (galactosyl-β1,1′-sphingosine) [25]. Due to the relative high homologies of these receptors, the potentials exist for TDAG8 to also be a LPC/SPC receptor and/or for OGR1, GPR4, and G2A to be receptors for psychosine.

Although LPA/S1P and SPC/LPC subfamily receptors are GPCRs for structurally related lysolipids, the two receptor subfamilies share little sequence homology and may prefer different G-protein coupling in certain signaling pathways. Evidence supports that three major G protein families (G_i, G_q, and $G_{12/13}$) are coupled to these receptors. Compared to the majority of GPCRs, which employ G_q as a mediator for calcium mobilization, SPC- and LPC-induced calcium release from intercellular stores are mediated through G_i in MCF10A cells, although other cell lines remained to be tested. Furthermore, while ERK activation via GPCRs is mainly mediated through G_i, SPC-induced PI3K and ERK activation via OGR1 appear to be mediated by a PTX-insensitive G protein ([6] and unpublished observations). Nonetheless, these differences are not restricted to LPC/SPC receptors. G_i-mediated calcium release and G_q-mediated PI3K and ERK activation have been reported previously [26–29].

The K_d values for LPC/SPC ligand binding are about one to two orders of magnitude higher than those for LPA/S1P receptors. Likewise, the serum and plasma concentrations of LPC are usually one to two orders of magnitude higher than those of LPA. Thus, the K_d values of their receptors may reflect a physiological adaptation to their concentrations. At the normal physiological concentrations of LPC (5–180 μM), if all of the LPC were in an active form, then its receptors would be saturated, downregulated, and/or desensitized. However, *in vivo*, the functionally available concentration of LPC may be affected by such conditions as percentage of LPC bound to albumin or lipoprotein and compartmentalization (i.e., tissue, cellular, and subcellular distribution) of LPC [30–32]. The concentrations of both S1P and SPC in physiological fluids are in the nM to sub-μM range. The lower affinity of SPC for its receptors may suggest (1) a physiological adaptation for a lower response to SPC; (2) the presence of a different, higher-affinity receptor(s) for SPC, which cannot be ruled out; and (3) these receptors (LPC/SPC subfamily receptors) may have different endogenous ligand(s). These issues remain to be further investigated.

Perspectives

Until now, very limited information has been accumulated regarding the pathophysiological functions of SPC, LPC, and their receptors. G2A-null mice develop a late-onset autoimmune disease [33]. Some of the effects of G2A may be compensated by other LPC receptors. Generation of OGR1- and GPR4-null mice is in progress and will provide important information about the physiological functions of these receptors. Comparative studies between LPA/S1P and LPC/SPC may generate interesting data to advance our understanding of these lipids, since: (1) LPA and S1P are prototypes of bioactive extracellular lipid signaling molecules; (2) LPC and SPC share similar, yet distinct signaling pathways as those induced by LPA and S1P; (3) the metabolic pathways LPA/S1P and LPC/SPC; are linked between and (4) all of these LPLs are present in serum and plasma. LPA, SPC, and

LPC levels are elevated under pathological conditions. It can be foreseen that the identification of their receptors will facilitate our understanding of the roles these LPLs play in physiological and pathological processes.

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CHAPTER 165

The Role of Ceramide in Cell Regulation

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During the past several years there has been a dramatic increase in information on the role of ceramide in many cellular events. Many excellent and thorough reviews have been written on ceramide; therefore, the purpose of this chapter is to offer the reader a starting point in the body of literature focused on the role of ceramide in cell regulation.

Ceramide-Mediated Cell Regulation

Ceramide is involved in several cellular processes, including apoptosis, cell senescence, differentiation, and cell stress. Many cytokines and stress agents, such as tumor necrosis factor (TNF) heat, and chemotherapy agents induce the production of ceramide, and several studies suggest critical roles for ceramide in regulating specific cell responses to these agents (Fig. 1). For example, augmentation of ceramide levels can lead to apoptosis in many cancer cells, whereas inhibition of ceramide formation can attenuate apoptosis in many, but not all, cell types and in response to several agonists. In other cell types, such as endothelial cells and fibroblasts, ceramide is more closely related to cell cycle arrest, differentiation, and senescence rather than apoptosis [1].

Biochemical Pathways of Ceramide Generation

De Novo Biosynthesis

The sphingolipid class of cell membrane lipids includes the sphingoid bases, ceramides (sphingoid bases with N-linked acyl groups), and complex sphingolipids based on ceramide and containing polar head groups. The committed step in sphingolipid biosynthesis is the condensation of palmitate with serine, which is catalyzed by serine palmitoyltransferase (SPT), a pyridoxal 5′-phosphate-dependent enzyme composed of two subunits and requiring (at least in yeast) another small subunit for maximal activity [2]. SPT generates 3-ketosphinganine, which is then reduced to dihydrosphingosine (DHS). The N-linked addition of a fatty acid to DHS, catalyzed by dihydroceramide synthase, yields dihydroceramide, which is subsequently desaturated to form ceramide in mammalian cells, or hydroxylated to form phytoceramide in yeast. From ceramide a variety of complex sphingolipids are derived. For example, complex glycosphingolipids including cerebrosides are formed by the addition of sugar groups to the ceramide backbone, and gangliosides are formed by the further addition of sialic acid. Sphingomyelin (SM) is generated by the transfer of a phosphocholine headgroup from phosphatidylcholine to ceramide. Each of these sphingolipid classes has distinct structural and/or functional roles [3].

Importantly, Fas, TNF, B-cell receptor stimulation, angiotensin II, palmitate loading, several chemotherapeutic agents (such as etoposide, CPT-11, and daunorubicin), phorbol esters, and UV radiation have been shown to activate the *de novo* pathway, leading to ceramide accumulation. Inhibition of this pathway with either myriocin, an SPT inhibitor, or with Fumonisin B_1, an inhibitor of dihydroceramide synthase, blocks formation of ceramide and attenuates the apoptotic response to these agents, thus implicating this pathway in the regulation of apoptosis [2]. Also, overexpression of glucosyl ceramide synthase (GCS), which clears

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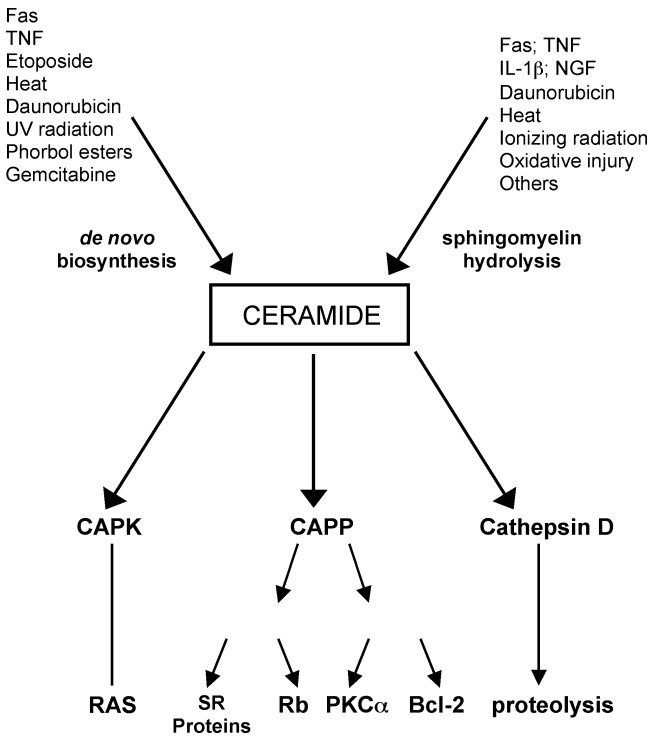


Figure 1 Several mechanisms of ceramide mediation of cell responses to extracellular signals.

ceramide (possibly with preference to *de novo* generated ceramide), attenuates apoptotic responses.

The Sphingomyelin Cycle

Ceramide can also be formed from hydrolysis of complex sphingolipids by stepwise removal of headgroups. Thus, sphingomyelinases (SMases) catalyze hydrolysis of SM, generating ceramide and phosphorylcholine. SM-derived ceramide can then act as a lipid mediator, undergo cleavage into sphingoid bases, or be reincorporated into SM, the latter completing the SM cycle. There are several different sphingomyelinase isoforms, displaying different pH optima, subcellular localization, and cofactor requirements [4].

SMase-derived ceramide has been shown to be an important component of the cell response to factors such as TNF, FAS, IL-1β, Ara-C, heat, and ionizing radiation. Cellular activities of ceramide derived from SM hydrolysis depend on the subcellular colocalization of the SMase as well as the SM pool, and studies suggest that the topology of ceramide production by SMase is a key factor in determining ceramide's ultimate cellular effects [4]. For example, recent results suggest that SM hydrolysis in the mitochondrion is sufficient to promote apoptosis [5], whereas ceramide at the membrane inhibits protein kinase C (PKC) translocation [6] and augments Fas action [7].

Moreover, as subsequent resynthesis of sphingomyelin from SMase-generated ceramide consumes phosphatidylcholine, yielding diacylglycerol (DAG), the dual action of SMase and SM synthase plays a role in the regulation of both ceramide and DAG, another important lipid mediator, primarily through activation of PKC, whose effects are often antagonistic to those of ceramide [4].

Ceramide Targets

Several important biochemical signaling pathways are regulated by ceramide, including pathways of stress and apoptosis. There are several key enzymes regulated by ceramide *in vitro*; thus serving as direct targets of ceramide, mediating at least some of its effects on these pathways.

CAPK

A ceramide-activated kinase activity which phosphorylates Raf has been shown to be the same as the kinase suppressor of Ras (KSR). KSR is activated by cytokine-induced ceramide production, coupling agents such as TNFα to apoptosis through regulation of MAP kinase pathways [8]. Other protein kinases for which there is evidence for direct ceramide-activation include PKCζ [9] and Raf [10].

CAPP

The discovery that ceramide specifically increased phosphatase activity in crude cell extracts resulted in the purification of the activity and subsequent identification of protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1) as ceramide-activated protein phosphatases (CAPP) [11]. Indeed, ceramide causes dephosphorylation of several key phosphoproteins with important roles in cell regulation. For example, inhibitors of ceramide biosynthesis blocked the TNF-induced, PP2A-dependent, dephosphorylation of PKCα, thus providing strong evidence for the involvement of *de novo* synthesized ceramide in PKCα regulation. Ceramide generation has also been shown to couple TNFα to the dephosphorylation of c-jun via PP2A. Furthermore, ceramide activation of PP2A caused dephosphorylation of bcl-2 and blocked its anti-apoptotic effects. Akt, a kinase involved in insulin signaling, mitogenesis, and apoptosis, has been shown to be dephosphorylated and inhibited in response to ceramide. Also, ceramide induces the PP1-mediated dephosphorylation of the retinoblastoma gene product (Rb), a key regulator of the cell cycle. Additionally, data indicate that the FAS-mediated dephosphorylation of SR proteins, which play important roles in mRNA splicing, is mediated by ceramide activation of PP1.

Cathepsin D

Cathepsin D is a lysosomal protease that has recently been shown to be activated by ceramide *in vitro*. Association of ceramide with the pre-pro cathepsin D causes autocatalytic cleavage to produce the 32-kDa active protease [12], which is thought to mediate several apoptotic events. This is particularly interesting as it presents a novel mechanism of ceramide-mediated responses independent of protein

phosphorylation, and it highlights the compartment-specific functions of ceramide.

Mechanisms of Ceramide-Protein Interaction

Mechanisms of ceramide activation of these proteins are far from fully understood, however, some proteins that interact with ceramide have cysteine-rich domains which have been hypothesized to accommodate protein-ceramide interaction [13], but this has not been demonstrated. On the other hand, many ceramide-interacting proteins, including PP1 and PP2A, lack such domains. Therefore more research is needed to define ceramide-binding motifs.

Physical Properties of Ceramide

Because sphingolipid-enriched lipid microdomains such as rafts and caveolae exhibit distinct biophysical properties, it is possible that some of the described effects of ceramide are mediated via organization of such structures and/or assembly of signaling complexes [14]. Furthermore, ceramide is located in the membrane fractions of cells, and, based on its biochemical and biophysical properties, ceramide should not diffuse freely throughout the cell [15].

Conclusions

The field of ceramide signaling has rapidly expanded as studies reveal diverse roles for ceramide in cell biology and mechanisms of its regulation and action. Priorities for further study include determining the downstream actions of ceramide on protein targets that mediate specific cellular responses as well as determining the upstream events leading to ceramide production. Other key areas in need of further investigation include the subcellular localization of the substrates, enzymes, and protein targets of these sphingolipid signaling cascades.

Acknowledgments

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CHAPTER 166

Phospholipase A_2 Signaling and Arachidonic Acid Release

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Introduction

Phospholipase A_2 (PLA_2) has attracted considerable interest in view of its role in lipid signaling and its involvement in a variety of inflammatory conditions. PLA_2 cleaves the sn-2 ester bond of cellular phospholipids, producing a free fatty acid and a lysophospholipid, both of which are implicated in lipid signaling. The free fatty acid produced is frequently arachidonic acid (AA, 5,8,11,14-eicosatetraenoic acid), the biosynthetic precursor of the eicosanoid family of potent inflammatory mediators that includes the prostaglandins, thromboxane, leukotrienes, and lipoxins. The other product of PLA_2 action on phospholipids is a lysophospholipid, which, depending on its molecular composition, may be converted into platelet-activating factor, another potent inflammatory mediator.

Phospholipase A_2 (PLA_2) consists of a superfamily of enzymes that catalyze the hydrolysis of the sn-2 ester bond in phospholipids, generating a free fatty acid and a lysophospholipid. This reaction is of the utmost importance in the context of cellular signaling, since it constitutes the main pathway by which arachidonic acid (AA) is liberated from phospholipids. Free AA is the precursor of a large family of compounds known as the eicosanoids, which includes the cyclooxygenase-derived prostaglandins and the lipoxygenase-derived leukotrienes [1]. If the other product of PLA_2 action on phospholipids is a choline-containing lysophospholipid possessing an alkyl linkage in the sn-1 position, then an acetyltransferase can act upon it to produce platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-3-phosphocholine).

The importance of the eicosanoids and platelet-activating factor as key mediators of inflammation as well as other pathophysiological conditions is now universally accepted [1]. Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) are well established as cyclooxygenase inhibitors, and are widely used in clinical practice. Similarly, the pharmaceutical industry has been actively pursuing lipoxygenase inhibitors and receptor antagonists for both leukotrienes and PAF. Note that since prostaglandins, leukotrienes, and PAF all derive from the action of a PLA_2, direct inhibition of such an enzyme would have the potential of blocking all three of the pathways at once, which could be of therapeutic advantage in certain settings. This is why the pharmaceutical industry has been actively pursuing the design of drugs with potential anti-PLA_2 effects, and some compounds are now in advanced clinical trials. Furthermore, $cPLA_2$ knockouts show distinct advantages in certain diseases [2,3].

The above approach is hampered, however, by the fact that cells in general contain multiple PLA_2 enzymes. For example, in humans, no less than 15 different proteins have been identified to possess PLA_2 activity [4]. Thus a first step in a rational PLA_2 drug design strategy is to define the different PLA_2 classes present in cells, as well as their putative roles in eicosanoid and PAF synthesis.

PLA_2 Groups

PLA_2s have been systematically classified according to their nucleotide sequence [4]. The latest update to this classification,

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published in October 2000, included eleven groups, most of them with several subgroups [4], but new PLA_2 enzymes have been described since, leading to a twelfth group [5–7]. Only PLA_2s whose nucleotide sequence has been determined should be included in the classification. However, this obvious criterion is the cause of some confusion, since many reports have appeared that erroneously link certain enzyme activities and functions to particular PLA_2 groups without it having been verified that such an association actually exists.

A parallel classification of the PLA_2s on the basis of biochemical properties is also frequently used, and it has value in describing PLA_2 activities for which sequence data are unavailable. This classification contemplates three main PLA_2 classes based on whether the enzyme is secreted ($sPLA_2$), cytosolic Ca^{2+}-dependent ($cPLA_2$), or cytosolic Ca^{2+}-independent ($iPLA_2$). One must be aware of the fact that this classification is not devoid of problems either, e.g. the Group IVC PLA_2 is generally referred to as $cPLA_2$-γ, despite its being a Ca^{2+}-independent enzyme. In addition, the PAF acetylhydrolase PLA_2s (Groups VII and VIII) also distribute among these categories.

Generally, the $sPLA_2$s (Groups I, II, III, V, IX, X, XI, XII) require millimolar levels of Ca^{2+} for activity, have low molecular masses, and lack specificity for arachidonate-containing phospholipids. The $cPLA_2$s (Group IV, comprising three subgroups) have higher molecular masses, require Ca^{2+} for translocation to membranes but not for activity, and are selective for arachidonate-containing phospholipids. Finally, the $iPLA_2$s (Group VI, and also Group IVC; see above) have high molecular masses but are not selective for arachidonate-containing phospholipids [4].

Cellular Function

A key determinant of the role of PLA_2s in a given cellular function is the mechanism of PLA_2 regulation/activation during such a process. A myriad of agents that exert effects on cells via receptor-dependent or independent pathways elicit a series of signals that ultimately lead to increased PLA_2 activity. Elucidation of these signals has been the subject of much effort for the last ten years [8]. The situation is further complicated by the evidence that most cells contain several PLA_2 forms and that all of them may eventually participate in the signaling process. Figure 1 shows the PLA_2 signal transduction mechanism developed over the years in our laboratory for the $P388D_1$ macrophage-like cells.

The scheme shown in Fig. 1 has been generally confirmed by many other laboratories and thus can be regarded as the currently accepted paradigm of PLA_2 signaling in immunoinflammatory cells. The cells may respond to two different kinds of signals that generate either a delayed response (bacterial lipopolysaccharide, LPS) or an immediate one (PAF). LPS acts primarily by inducing the cells to synthesize new proteins involved in the process. However, PAF acts on preexisting proteins. In either case, the foremost event is the translocation and activation of the $cPLA_2$ in an intracellular compartment. The mechanism of activation of this enzyme has been the subject of many studies, and generally involves the concerted action of the mitogen-activated protein kinase (MAPK) cascade and transient elevations of the intracellular Ca^{2+} concentration [9]. There are a few exceptions however, in which $cPLA_2$ activation has been described as being activated in a Ca^{2+}-independent manner [10] and/or

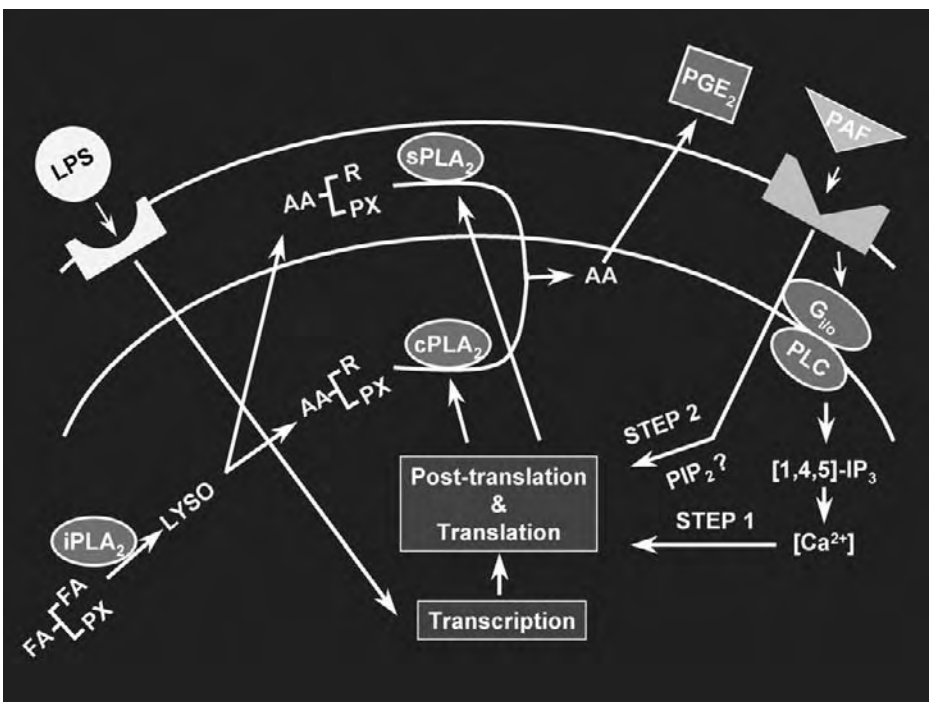


Figure 1 Signal transduction mechanism in $P388D_1$ macrophages. Adapted from [13].

phosphorylated by kinases not of the mitogen-activated protein kinase family [11].

Activation of the $cPLA_2$ is followed by activation of a $sPLA_2$, which, depending on cellular type, may belong to Groups IIA, V, or perhaps other groups. Depending on the stimulation conditions, the $cPLA_2$ modulation of $sPLA_2$ cellular activity may occur at a gene regulatory level (delayed responses) [12] or at the level of regulation of enzyme activity itself (immediate responses) [13]. In the latter case, a variety of cellular mechanisms may account for this activation, from $cPLA_2$-induced rearrangement of membrane phospholipids that enables further $sPLA_2$ attack to more sophisticated biochemical mechanisms such as inactivation of endogenous $sPLA_2$ inhibitors or Ca^{2+} fluxes. While it is clear that the $cPLA_2$ acts on perinuclear membranes, the precise site of action of the $sPLA_2$ has been the subject of numerous recent studies. The enzyme appears to be released to the extracellular medium, from which it re-associates with the outer cellular surface, where it hydrolyzes phospholipids. However, recent studies have suggested that the enzyme is re-internalized deep into the cell, probably via the caveolin system to the vicinity of nuclear membranes [14]. Whether the enzyme is still active in the cellular interior or this represents a signal termination mechanism is unclear at present [15]. This is currently an area of active study.

Free arachidonate generated by both $cPLA_2$ and $sPLA_2$ should be readily converted to prostaglandins and other eicosanoids by the cyclooxygenases and/or lipoxygenases. These eicosanoids are subsequently secreted to the extracellular medium, where they can act in both autocrine and paracrine manners.

Summary

The model depicted in Fig. 1 contemplates a scenario where the concerted action of two distinct PLA_2s leads to a full AA release response. The $cPLA_2$ appears to initiate the response and plays primarily a regulatory role, whereas the $sPLA_2$ acts in a second "wave" to amplify the response by providing the bulk of the AA liberated. Needless to say, in those cells that do not express a $sPLA_2$, the $cPLA_2$ would be the only one responsible for the release.

Cells usually also contain measurable levels of $iPLA_2$. This enzyme is frequently suggested to play a role in AA mobilization and eicosanoid production [16]. However, such an involvement remains controversial because practically all the evidence favoring this view has been inferred from studies utilizing bromoenol lactone, a compound that manifests high selectivity for the $iPLA_2$ *in vitro* but fails to do so *in vivo*. For example, a recent report has shown that the $cPLA_2$ counts among the cellular targets of bromoenol lactone in cells [17]. The $iPLA_2$ may also be involved indirectly in AA mobilization by modulating fatty acid reacylation reactions. The lysophospholipids produced by the $iPLA_2$ may be used to re-incorporate part of the fatty acids (including AA) that have previously been released by its Ca^{2+}-dependent counterparts. Thus, by regulating AA reacylation reactions, the $iPLA_2$ may participate in the formation of the cellular AA pools. Thus, all three types of PLA_2 ($sPLA_2$, $cPLA_2$, $iPLA_2$) appear to serve important but distinct functions in cells.

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CHAPTER 167

Prostaglandin Mediators

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Introduction

Arachidonic acid (AA), a 20-carbon unsaturated fatty acid containing four double bonds (Δ5,8,11,14:C20:4), circulates in plasma in both free and esterified forms and is a natural constituent of the phospholipid domain of cell membranes. AA is mobilized for release by phospholipases (PLs) A_2, particularly type IV cytosolic (c) PLA_2, [1] following its calcium-dependent translocation to the nuclear membrane and the endoplasmic reticulum (Fig. 1). Three major groups of enzymes, prostaglandin G/H synthase (PGHS), lipoxygenase, or cytochrome p450, then catalyze the formation of the prostaglandins (PGs) and thromboxane A_2 (TxA_2), the leukotrienes, or the epoxyeicosatrienoic acids, respectively. Collectively, these products are known as eicosanoids. A parallel family of free radical catalyzed isomers, the isoeicosanoids, are formed by direct peroxidation of AA *in situ* in cell membranes [2]. This chapter will focus on the PGs and TxA_2, collectively termed the prostanoids.

The Cyclooxygenase Pathway

Prostanoids are formed by the action of PGHS, or cyclooxygenase (COX), on AA to form bisenoic products containing two double bonds, denoted by a subscript 2, (e.g. PGE_2 [3]). COX-1 or COX-2 dimers [4], homotypically inserted into the ER membrane, contain both cyclooxygenase and hydroperoxidase activities [3]. AA is sequentially transformed into the unstable cyclic endoperoxides, PGG_2 and PGH2, for delivery to downstream isomerases and synthases to generate TxA_2 and D, E, F, and I series PGs (Fig. 1). It is presently not understood either how AA is delivered specifically to COX or how PGH_2 is presented to downstream enzymes. Two COX genes have been identified: COX-1 is expressed constitutively in most cells while COX-2 is upregulated by cytokines, shear stress, and tumor promoters [3]. These observations suggest housekeeping functions, such as gastric epithelial cytoprotection and hemostasis, for COX-1-derived prostanoids, although it appears that both enzymes contribute to the generation of autoregulatory prostanoids. Conversely, the inducible COX-2 is considered the dominant source of prostanoid formation in inflammation and cancer, although both isozymes can contribute to prostanoid formation in syndromes of human inflammation, including atherosclerosis [5] and rheumatoid arthritis [6].

COX-1 and COX-2 are closely related in their amino acid sequence [7] and crystal structure [8]. Although both isozymes demonstrate similar subcellular distribution [9], preference for downstream enzymes is sometimes evident in heterologous expression systems and apparently *in vivo*. COX-1 preferentially couples with TxS, PGFS [10], and the cytosolic (c) PGES isozymes [11]. COX-2 prefers PGIS [10] and the microsomal (m) PGES isozymes, which are induced by cytokines and tumor promoters [12]. Two forms of PGDS [13,14] and PGFS [15,16] have been identified, underscoring the diversity of the isomerases and synthases.

COX Deletion Deletion of COX-2 results in multiple defects of implantation and reproduction, leading to breeding difficulties [17]. Offspring have variably revealed cardiac fibrosis, renal defects and impaired inflammatory responses; however, the extent to which these phenotypes are modulated by genetic background is presently unclear. Impaired inflammatory responses [18] and delayed parturition [19] secondary to COX-1 deletion have been reported. Deletion of the COX-2 but not the COX-1 gene increases the frequency of patent ductus arteriosus (PDA) in newborn pups [20]. Coincidental deletion of COX-1 increases the frequency of the COX-2 knockout PDA phenotype [20]. It is the absence of PGE_2 that underlies this phenotype; deletion of 15 PGE_2 deydrogenase, the major inactivating enzyme of PGE_2, produces sustained high levels of PGE_2 throughout the perinatal period and results in ductal closure [21]. Both COX-1 and

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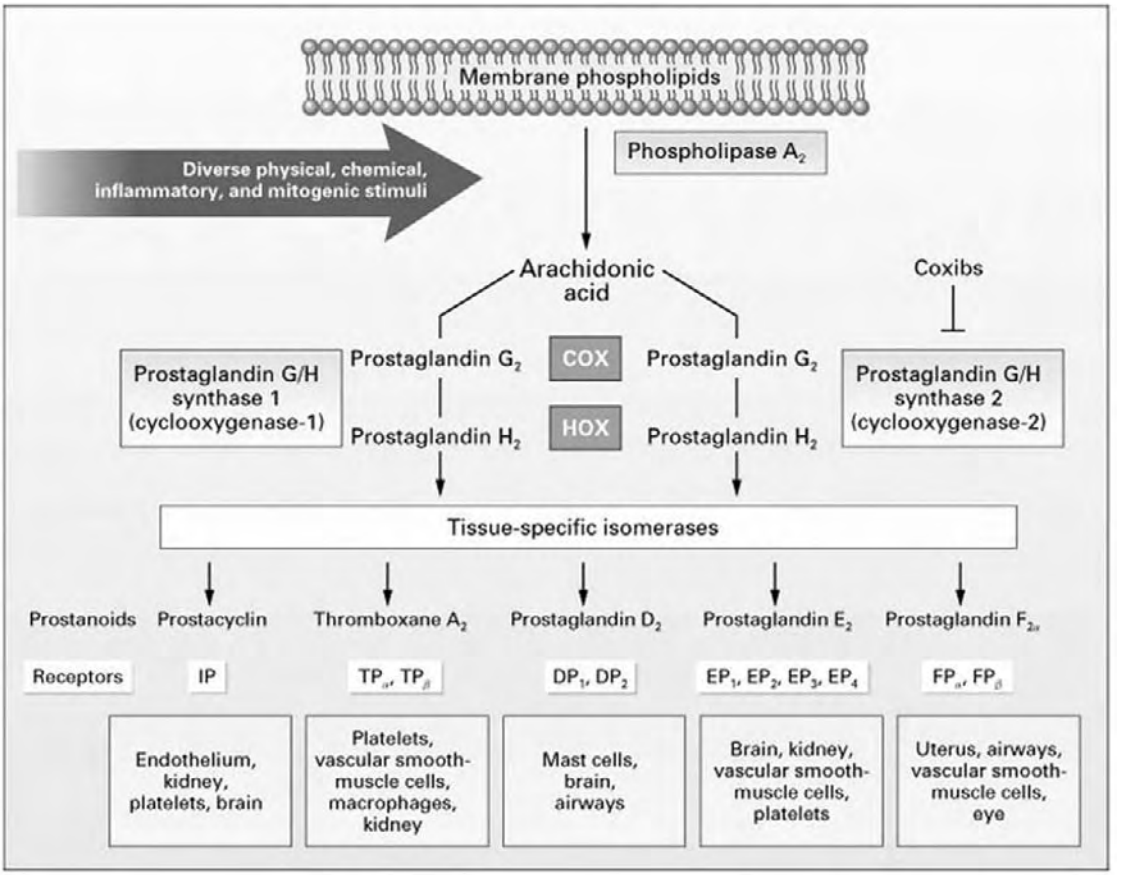


Figure 1 Production and actions of prostanoids: Arachidonic acid, a 20-carbon fatty acid containing four double bonds, is liberated from the *sn*2 position in membrane phospholipids by PLA_2. COX converts arachidonic acid to the unstable intermediate prostaglandin H2, which is converted by tissue-specific isomerases to multiple prostanoids. These bioactive lipids activate specific cell-membrane receptors of the superfamily of GPCRs.

COX-2 are subject to developmental regulation, and interference with their developmental expression may condition adult phenotypes [22]. COX-1 and COX-2 are expressed in a spatially and temporaly segregated manner during thymic development, where they influence T-cell maturation [23].

Prostanoid Receptors

Due to their short half lives (seconds to minutes), prostanoids act as autacoids, rather than circulating hormones, by activating membrane receptors at, or close to, the site of their formation. Specific G-protein-coupled receptors (GPCRs) have been cloned for all the prostanoids [24]. A single gene product has been identified for prostacyclin (the IP), $PGF_{2\alpha}$ (the FP), and TxA_2 (the TP), while four distinct PGE_2 receptors (the EP_{1-4}) and two PGD_2 (DP_1 and DP_2) have been cloned. The prostanoid receptors appear to derive from an ancestral EP receptor and share high homology [24]. Phylogenetic comparison of this family reveals three subclusters: first the EP_2, EP_4, IP, and DP_1, the relaxant receptors, which increase cAMP generation; second EP_1, FP, and TP, the contractile receptors, which increase intracellular calcium levels; and third the EP_3, which elevates intracellular calcium and decreases cAMP [24,25]. The DP_2, a member fMLP receptor superfamily [26,27], is the exception to this characterization. Differential mRNA splicing gives rise to additional isoforms of the TP (α and β) [28], FP (A and B) [29], and EP_3 (A–D) [30]. The prostanoid receptors have been reviewed thoroughly elsewhere [24,31].

Thromboxane A_2 (TxA_2)

TxA_2, the major product of platelet COX-1, is a potent vasoconstrictor [32], mitogen [33] and platelet activator [34]. Despite the diversity of platelet agonists, inhibition of platelet TxA_2 formation apparently accounts for cardioprotection from aspirin [35], reflecting the importance of TxA_2 as an amplification signal for more potent agonists, such as thrombin and ADP [34]. TxA_2, also a major product of macrophage COX-2, contributes to atherogenesis in mouse models [36]. Analogous to its role in vascular proliferation (see below), TxA_2 may also mediate cellular hypertrophy [37].

Two forms of the platelet TP have been segregated pharmacologically, one mediating shape change, the other aggregation [38]. However, the cloned human TP splice variants (splice variants of the mouse TP are not apparent),

do not account for this distinction, and TPα is apparently the sole isoform expressed in platelets [39,40]. Recognized differences between the splice variants are limited to G-protein activation in heterologous expression systems [41,42] and agonist-induced desensitization and sequestration [43,44]. Given the identification of distinct low homology GPCRs mediating ADP-induced platelet shape change and aggregation [45], it seems likely that at least one more distinct TP remains to be identified. In this regard, evidence suggests that $iPF_{2\alpha}$-III, an isoprostane, acts *in vivo* at the TP [46] but does not bind to either isoform *in vitro* [47], further suggesting the existence of another TP. Distinct receptor sites can be generated through GPCR heterodimerization [48]. It is interesting that TPα and TPβ appear to dimerize, and their coexpression augments $iPF_{2\alpha}$-III signaling compared to either receptor alone [49]. The extent to which associations between TPα with TPβ, and/or other prostanoid receptors, might contribute to the family of prostanoid receptors remains to be examined. The cloned TPs couple via G_q, G_{11}, $G_{12/13}$, and G_h (which is also tissue transglutaminase II) to activate PLC-dependent inositol phosphate generation and elevate intracellular calcium [24,31]. Activation of the TP isoforms may also activate or inhibit adenylyl cyclase, via G_s (TPα) or G_i(TPβ), respectively, and signal via G_q and related proteins to MAP kinase signaling pathways.

TP mRNAs are expressed widely in lung, liver, kidney, heart, uterus, and vascular cells with TPα usually the predominant isoform [50]. Despite reports of abundant TP expression in thymus, the role of TxA_2 in lymphocyte development and function is presently unclear. A naturally occurring mutation in the first intracellular loop of the TP is associated with a mild bleeding disorder and platelet resistance to TP agonists [40], while a polymorphism in the TP has been linked to bronchodilator resistance in asthma [51].

TP Deletion, Overexpression Deletion of the TP reveals a mild haemostatic defect and resistance to AA-induced platelet activation [52], reflecting the role of TxA_2 in vascular biology. TP null mice have reduced proliferative responses to vascular injury, while the opposite is true of mice engineered to overexpress TPβ in the vasculature [53]. TPβ overexpressors also develop a syndrome reminiscent of intrauterine growth retardation, probably secondary to placental ischemia [54].

Prostacyclin (PGI_2)

A major product of COX-2 in healthy individuals [55], PGI_2 is a potent vasodilator, inhibitor of platelet aggregation by all recognized agonists [56], and an inhibitor of cell proliferation *in vitro* [57]. PGI_2 biosynthesis is increased in syndromes of platelet activation [58,59], perhaps as a homeostatic response to accelerated platelet-vascular interactions. Chronic PGI_2 treatment can reduce pulmonary vascular resistance in patients with primary pulmonary hypertension [60]. Delivery of the gene for PGIS *in vivo* diminishes vascular smooth muscle cell proliferation and migration in response to injury [61]. PGIS polymorphs have been associated with essential hypertension [62] and myocardial infarction [63], while PGI_2 attenuates angiotensin II-induced renal vasoconstriction and systemic hypertension [64]. PGI_2 also attenuates the response to thrombotic stimuli in dogs [65] and specifically limits the effects on TxA_2 on platelets and the vessel wall in mice [53].

The sole identified IP couples to activation of adenylyl cyclase via G_s, although it can also activate phospholipase C via G_q. IP mRNA is abundantly expressed in kidney, where PGI_2 may regulate renal blood flow, renin release, and glomerular filtration rate and in lung, where PGI_2 can modulate vascular tone [24,31]. The IP is also expressed in the spinal column, where PGI_2 plays a role in pain perception, and in the liver, where its role is unknown. Expression within the cardiovascular system is most abundant in the aorta, consistent with the major biological role of PGI_2 in platelet and macrovascular homeostasis. IP expression in the heart, together with reports that COX-2-dependent PGI_2 formation limits oxidant-induced injury in cardiomyocytes [66], suggests a possible protective role for PGI_2 in cardiac tissue. A major difference between humans and rodents is the marked expression of IP in the thymus [67], although the functional relevance of this observation is not clear.

It is likely that at least one other IP remains to be identified. Pharmacologically distinct IP sites in the brain and kidney are not attributable to the cloned IP [68,69]. In addition, PGI_2 may activate the peroxisome proliferator activated receptors (PPARs). However, while both PGI_2 and iloprost activated PPARα and PPARδ *in vitro*, another PGI_2 analog, cicaprost, did not [70], and it is as yet unclear whether PGI_2 activation of PPARs occurs *in vivo*. The loss of PGI_2-mediated PPARδ activation was thought to underlie the implantation defect in COX-2-deficient mice, although no implantation defect was evident in PPARδ-deficient mice [71]. Two IP polymorphs, with some alterations in ligand binding and signaling in overexpression systems, have been identified [72].

IP Deletion Although results in IP-deficient mice have implicated PGI_2 in the mediation of pain and inflammation [73], these consequences seem conditioned by genetic background. Platelets of IP-null mice are resistant to disaggregation by IP agonists [73] and the thrombotic and proliferative response to vascular injury is enhanced [53], as is hypoxia-induced pulmonary hypertension and remodeling [74]. Despite its expression in murine thymus, disordered T-cell function secondary to IP deletion has not been reported, and the IP appears to play a minor role, if any, in murine T cell maturation [23]. Deletion of the IP undermines the atheroprotective effect of female gender in LDL receptor deficient mice [75], a possible consequence of estrogen-mediated upregulation of PGIS [76] and/or its protection from free radical attack. Interestingly, unlike their normotensive IP knock out counterparts, mice deficient in PGIS are hypertensive [77]. However, while this supports the possibility of a second IP, formation of both PGE_2 and TxA_2 are increased in the PGIS knockouts, perhaps due to rediversion of PGH_2.

Prostaglandin D_2 (PGD_2)

PGD_2, the major COX product formed by mast cells, is released during allergic responses, including asthma and systemic mastocytosis [78,79]. Infusion of PGD_2 in humans results in flushing, nasal stuffiness, and hypotension [80]. In mice, overexpression of lipocain-like PGDS increases response to bronchial challenge with ovalbumin [81]. The hematopoietic PGDS is expressed abnormally in patients with coronary disease [82] and a polymorphic variant has been linked to human asthma [83]. PGD_2, an abundant COX product in brain, is considered an important regulator of sleep-wake cycles [84]. Deletion of PGDS abolishes allodynia (touch-evoked pain) in mice [85], demonstrating a role for PGD_2 in pain perception.

The DP_1 is coupled positively to adenylyl cyclase through G_s [24,31], which directs PDG_2-induced inhibition of platelet aggregation, bronchodilation, and vasodilation. Among the prostanoid receptors the DP_1 is the least abundant, with minor expression reported in mouse ileum, lung, stomach, and uterus and expression in the CNS limited specifically to the leptomeninges. Recently, a chemoattractant receptor–homologous molecule (CRTH2), expressed on T-helper (H) type-2 cells [27], was classified as the DP_2. This receptor is distinct from other prostanoid receptors, couples to increased intracellular Ca^{2+}, and directs PGD_2-induced chemotaxis and migration of TH2 cells. Both DPs integrate coordinately the effects of PGD_2 on eosinophils, modulating chemokinesis, degranulation, and apoptosis [86]. DP_2 and PGDS are coordinately expressed at the fetal/maternal interface in human deciduas, where they may participate in lymphocyte recruitment [87].

PGD_2 may be metabolized to PGJ_2 and its metabolite, 15-deoxy Δ (12,14) PGJ_2 [88], a possible natural ligand for PPARγ, regulating adipogenesis, inflammation, tumorigenesis, and immunity [89]. However, while PGJ_2 and its metabolite can activate the nuclear receptor *in vitro* [90], it is presently unclear whether sufficient concentrations are formed *in vivo*.

DP Deletion Deletion of the DP_1 sharply reduces ovalbumin-induced lymphocytes and eosinophils infiltration and airway hyperreactivity, reflecting PGD_2's apparent role in asthma [91]. Work with these mice demonstrates the action of PDG_2 on arachnoid trabecular cells in the basal forebrain to increase extracellular adenosine, which in turn facilitates induction of sleep [92]. DP_2 null mice have not yet been generated.

Prostaglandin E_2 (PGE_2)

PGE_2 regulates diverse biological processes, including cell growth, inflammation, reproduction, sodium homeostasis and blood pressure [93]. Its biological effects are complex and often opposing; vasodilation in the arterial and venous systems [94] but constriction of smooth muscle in the trachea, gastric fundus, and ileum [95]. Like COX-2, the inducible mPGES isoforms [96] may contribute to the increase in PGE_2 associated with inflammatory and pyretic responses. The COX-1-cPGES axis is considered the predominant source of homeostatic PGE_2 [11], although mPGES and COX-1 seen coupled in the mouse kidney [97]. However, COX-2-derived prostanoids may differentially regulate salt excretion and glomerular circulation in volume overload or depletion [98]. PGE_2, along with PGI_2, apparently derived from COX-2, maintains renal blood flow and salt excretion [99], effects that may be counterbalanced by COX-1-derived TxA_2 [64].

Both the EP_2 and the EP_4 activate adenylyl cyclase via G_s [24,31]. Differences in agonist-induced desensitization may be one reason for the presence of such similar receptors for PGE_2. The EP_1, via an unclassified G-protein, and the EP_{3D}, via G_q, are coupled to PLC activation [24,31]. A splice variant of the EP_1 in the rat may antagonize coupling of other EPs [100]. The EP_{3B}/EP_{3C} couple to G_s-mediated activation, and the EP_{3D}/EP_{3A} to G_i-mediated inhibition, of adenylyl cyclase.

The mRNAs for all four EPs are widely expressed; however, the limited distribution of EP_1 and EP_2, compared with EP_3 and EP_4, together with induction of EP_2 in response to inflammatory stimuli [101], suggests specialized functions for the different EPs [24,31]. The biological actions of PGE_2 may be conditioned by this differential receptor expression and/or PGE_2 levels. EP_4 directs platelet inhibition at low PGE_2 concentrations, while increased PGE_2 levels in, for example, inflammation, lead to EP_3-mediated platelet aggregation [102]. Indeed, high concentrations of PGE_2 condition platelet responses through EP_3- and IP-mediated regulation of intracellular cAMP [102]. Despite higher renal EP_4 expression [31], evidence supports EP_2-mediated renal vasodilation and salt handling [103], while an EP_1-directed increase salt excretion may contribute to PGE_2-dependent natriuresis [31]. PGE_2 may also directly stimulate renin and angiotensin II generation in the kidney [104], or directly constrict the renal vasculature [105] leading to hypertension. In the gastrointestinal tract, cytoprotective effects are mediated by EP_1 in stomach [106] but by EP_3 and EP_4 in the intestine [107]. EP_1 and EP_3 receptors appear responsible for myometrial contractility caused by PGE analogs, such as misoprost, used to induce labor [108], while selective EP_2-mediated inhibition of myometrial contractility [109] may be useful against preterm labor. EP_2- [110] and EP_3-mediated [111] interactions with growth factors may underlie the proliferative and angiogenic actions of PGE_2 in cancer. In the immune system, activation of the EP_2 inhibits T-cell proliferation, while both EP_2 and EP_4 receptors regulate antigen-presenting function *in vivo* [112]. Circulating levels of interleukin-1β induce coordinate COX-2-mPGES expression at the blood brain barrier, permitting activation of the central EPs [113]. Localized infusions of PGE_2 into the third ventricle induce wakefulness via the EP_1 and EP_2 receptors, while EP_4 activation in subarachnoid space induces sleep [114]. Pyrexial responses may mediated through the EP_3 [115], while the EP_1 and the EP_3 increase neuronal excitability and pain perception [115,116].

EP Deletion Knockout mouse models have been generated for all the EPs. EP_1-deficient mice have reduced nociceptive perception, while male, but not female, knockouts have reduced systolic blood pressure accompanied by elevated renin-angiotensin activity [116]. EP_2-deficient mice are normotensive at baseline but demonstrate increased salt- and pressor hormone-induced hypertension, although this is modified by genetic background [103]. The EP_2 knockouts also demonstrate a preimplantation defect, which may underlie some of the breeding difficulties seen in the COX-2 knockouts (see above). EP_3-deficient mice are resistant to pyrogen-induced fever [115]. However, despite its abundant expression in the kidney, there is no renal phenotype in EP_3 knockouts [117]. Deletion of the EP_4 results in PDA and neonatal death [118].

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)

$PGF_{2\alpha}$ actions include luteolysis [119] and smooth muscle contraction across a variety of tissues [120,121]. PGFS catalyzes the reduction of PGH_2 to $PGF_{2\alpha}$, PGD_2 to 9α 11β $PGF_{2\alpha}$ [122], and retinal to retinol [123]. It exists in at least two isoforms, identified initially in liver [16] and lung [122], and is also expressed in lymphocytes [122] and spinal chord [124]. $PGF_{2\alpha}$ induces cardiac myocyte hypertrophy and induction of myofibrillar genes, independent of muscle contraction [125], suggesting a role for this eicosanoid during development, in compensatory hypertrophy, and/or in recovery of the heart from injury.

Thus far, one GPCR for $PGF_{2\alpha}$, the FP, which couples via G_q activation of PLC, has been cloned [24,31]. Stimulation of FP also activates Rho kinase, leading to the formation of actin stress fibers, phosphorylation of p125 focal adhesion kinase, and cell rounding [126]. Similar to the EP_3 and TP, carboxy terminal splice variants, FP_A and FP_B [29], have been identified. These are indistinguishable in their ligand-binding properties and signaling but may differ in their constitutive activity [29] and rates of desensitization [127]. FP_A and FP_B also differ in coupling to the Tcf/β catenin-signaling pathway, which may underlie the prolonged cytoskeletal effects mediated thought FP_B [128]. The FP is expressed in kidney, heart, lung, and stomach; however, it is most abundant in the corpus luteum, where its expression varies during the estrus cycle, consistent with the role for $PGF_{2\alpha}$ in luteolysis. The FP is also expressed in the ciliary body of the eye, where FP agonists have clinical utility in the treatment of raised intraocular pressure in patients with glaucoma [129]. Although activation of the FP results in vaso- and broncho-constriction [121], cell proliferation [130], and cardiomyocyte hypertrophy [131], the role of this prostanoid in cardiopulmonary disease is poorly characterized. Similarly, activation of the FP blocks preadipocyte differentiation *in vitro* [132], but the role of the FP, if any, in obesity is poorly understood.

FP Deletion Mice deficient in the FP do not deliver at term, resulting from a failure to induce the oxytocin receptor and lack of the normal decline in elevated progesterone levels [133]. Ovariectomy restores responsiveness to oxytocin and permits successful parturition. COX-1-derived $PGF_{2\alpha}$ in these mice appears important for luteolysis, consistent with delayed parturition in COX-1-deficient mice [19]. Subsequent upregulation of COX-2 generates prostanoids, including $PGF_{2\alpha}$ and TxA_2, important in the final stages of parturition [134]. Mice lacking both COX-1 and oxytocin underwent normal parturition, demonstrating the critical interplay between $PGF_{2\alpha}$ and oxytocin in onset of labor [19].

Concluding Remarks

The cyclooxygenase pathway of arachidonic acid metabolism generates a family of evanescent mediators with wide and varied physiological and pathophysiological actions. Understanding the biological role of the prostanoids requires examination of the biosynthetic pathways that lead to their temporal and tissue-specific generation together with the array of signaling pathways activated by their multiple receptors.

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CHAPTER 168

Leukotriene Mediators

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Introduction

The leukotrienes (LT) constitute a group of bioactive lipids derived from the metabolism of polyunsaturated fatty acids, e.g., arachidonic acid [1]. In two consecutive reactions, arachidonic acid is transformed into an unstable epoxide compound, LTA_4. This intermediate is either hydrolyzed into the dihydroxy acid LTB_4 or conjugated with glutathione to form LTC_4. The latter compound together with its metabolites LTD_4 and LTE_4 are referred to as the cysteinyl-containing leukotrienes (cys-LTs).

Leukotrienes possess a wide range of biological activities elicited via specific, G-protein-coupled, cell surface receptors [2]. LTB_4 is a very potent chemoattractant for neutrophils and recruits inflammatory cells to the site of injury. This compound also induces chemokinesis and increases leukocyte adhesion to the endothelial cells of the vessel wall. The cys-LTs are potent constrictors of smooth muscles, particularly in the airways, leading to bronchoconstriction. In the microcirculation, the cys-LTs constrict arterioles and increase the permeability of the postcapillary venules, which results in extravasation of plasma. Due to their potent biological activities, leukotrienes are considered to be chemical mediators in a number of inflammatory and allergic disorders, e.g. rheumatoid arthritis, inflammatory bowel disease, and bronchial asthma [3].

Five-Lipoxygenase

Five-lipoxygenase (5-LO) catalyzes the first two steps in leukotriene biosynthesis [4] (Fig. 1). Free arachidonic acid is oxygenated into the hydroperoxide 5-HPETE, which is subsequently dehydrated to yield the unstable epoxide intermediate LTA_4. The enzyme, which predominantly is found in bone marrow derived cells, is stimulated by Ca^{2+} and ATP. Furthermore, it contains one atom of non-heme iron that is involved in catalysis [5]. Mutagenetic analysis has demonstrated that His-372, His-550, and the C-terminal isoleucin Ile-673 are iron ligands [6]. The gene encoding human 5-LO, as well as the promoter, has been characterized, and some important features are listed in Table I, together with data for the other key enzymes in the leukotriene cascade.

The only crystal structure of a mammalian lipoxygenase that has been determined is rabbit 15-LO [7]. This enzyme contains an N-terminal β-barrel domain, a structure also found in the C-terminal domain of lipases. The role of this domain for lipoxygenases is presently unclear but for 5-LO it has been shown to bind Ca^{2+}, which stimulates enzyme activity and presumably facilitates its association of 5-LO with membranes during catalysis (see following section) [8]. 5-LO is also a substrate for p38 kinase-dependent MAPKAP kinases *in vitro*, suggesting that phosphorylation may be one additional factor, which determines 5-LO translocation and enzyme activity [9].

Five-Lipoxygenase Activating Protein (FLAP) and Cellular Leukotriene Biosynthesis

Cellular 5-LO activity is dependent on a small membrane protein, five lipoxygenase activating protein (FLAP), which presumably presents or transfers arachidonic acid to 5-LO [10].

Early studies showed that upon cell stimulation leading to an increase in Ca^{2+}, 5-LO is activated and translocates to a membrane compartment [11]. Of particular interest was the discovery that FLAP is localized to the nuclear envelope of

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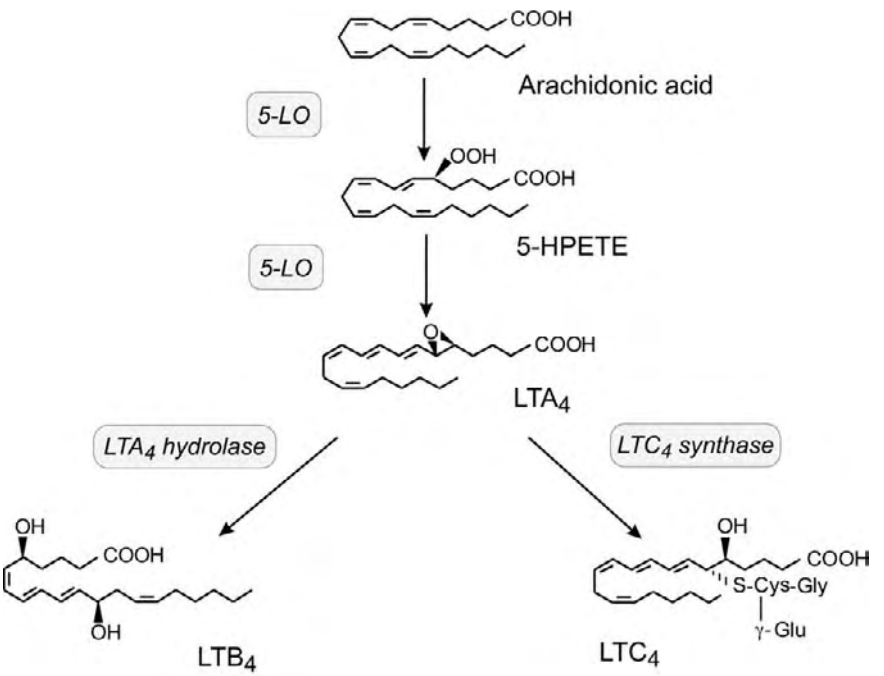


Figure 1 Enzymes and intermediates in the leukotriene cascade.

Table I Properties of Enzymes and Receptors in Leukotriene Biosynthesis and Action[a]

Protein	Protein size (no. of amino acids)[b]	Prosthetic group[c]	Gene size (kb)	Exon no.	Putative *cis*-elements of promoter regions	Chromosomal location	Gene deficient mice
5-Lipoxygenase	673	Fe	>82	14	Sp1, AP-2, NF-κB	10	+
FLAP	160	—	>31	5	TATA, AP-2, GRE	13	+
LTA_4 hydrolase	610	Zn	>35	19	XRE, AP-2	12	+
LTC_4 synthase	149	—	2.5	5	Sp1, AP-1, AP-2	5	+
BLT_1	351	—	5.5	3	Sp1, CpG site, NFκB AP-1	14	+
BLT_2[d]	357	—	ND[c]	1[c]		14	–
$CysLT_1$	336	—	ND	ND		X	+
$CysLT_2$	345	—	ND	ND		13	–

[a]Data refer to human proteins. ND, not determined.
[b]Initial methionine excluded.
[c]1 mol metal per mol protein.
[d]The ORF of BLT_2 is included in the promoter of the BLT_1 gene.

neutrophils and that 5-LO, upon cell activation, translocates to the same compartment [12] (Fig. 2). Further analysis revealed that 5-LO can also be present in the nucleus of *resting* cells associated with the nuclear euchromatin, a site from which it translocates to the nuclear envelope. In addition, 5-LO has been shown to associate with growth factor receptor-binding protein 2 (Grb2), an "adaptor" protein for tyrosine kinase-mediated cell signaling, through Src homology 3 (SH3) domain interactions [13]. It is interesting that inhibitors of tyrosine kinase activity, a determinant of SH3 interactions, also inhibited the catalytic activity of 5-LO and its translocation during cellular activation. In addition, an internal bipartite nuclear localization sequence, spanning Arg-638–Lys-655, has been shown to be necessary for the redistribution of 5-LO to the nuclear compartment [14,15]. Moreover, recent data indicate that also the N-terminal β-barrel domain in 5-LO plays a role in this process [16].

Not only 5-LO and FLAP are associated with the cell nucleus and nuclear membrane. Thus, LTC_4 synthase (see section entitled Leukotriene C_4 Synthase) resides in this compartment, and recent data suggest that the enzyme is located on the *outer* nuclear membrane and peripheral endoplasmic reticulum [17]. It is interesting that the soluble LTA_4 hydrolase (see section entitled Leukotriene A_4 Hydrolase) was also reported to reside in the nucleus of rat basophilic leukemia cells and rat alveolar macrophages [18].

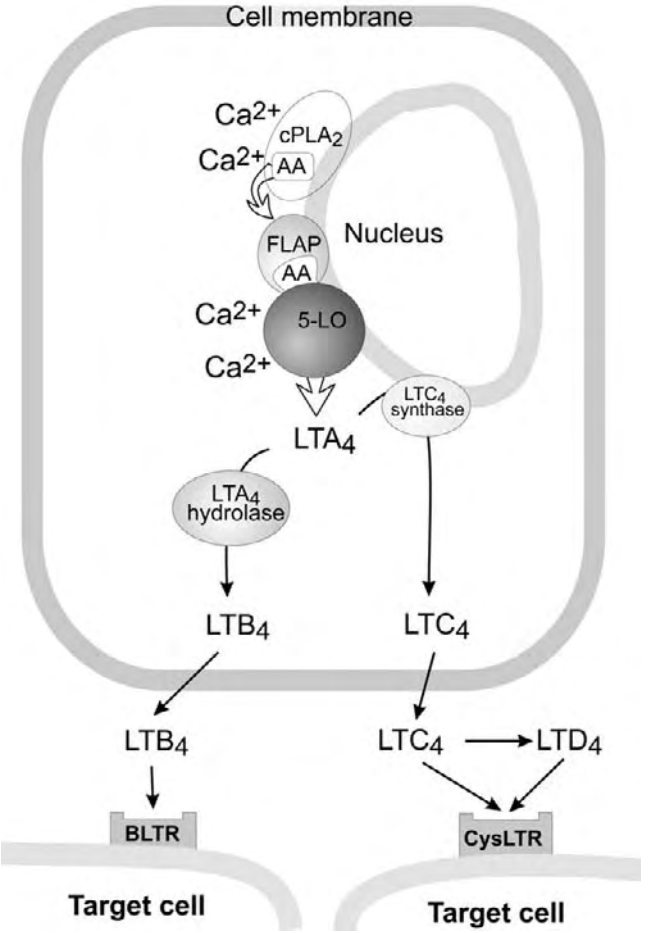


Figure 2 Leukotriene biosynthesis at the nuclear membrane of an activated leukocyte.

Together, these findings imply that leukotriene biosynthesis is carried out by a complex of enzymes assembled at the nuclear membrane (cf. Fig. 2). This conclusion in turn suggests that these enzymes and/or their products may have additional intracellular and intranuclear functions, perhaps related to signal transduction or gene regulation. In line with this notion, it has been reported that LTB_4 is a natural ligand to the nuclear orphan receptor PPARα, suggesting that LTB_4 may have intranuclear functions [19]. It was also reported that 5-LO can interact with several cellular proteins, including coactosine-like protein (CLP) and transforming growth factor type β-receptor-I-associated protein (TRAP-1) [20]. In addition, 5-LO interacts with a human homologue of the protein "Dicer," a member of the RNase III family of nucleases, which is implicated in the RNA interference mechanism of gene regulation [20,21].

Leukotriene A_4 Hydrolase

Leukotriene A_4 hydrolase catalyzes the final step in the biosynthesis of the proinflammatory compound LTB_4 (Fig. 1). In contrast to 5-LO, LTA_4 hydrolase is widely distributed and has been detected in almost all mammalian cells, organs, and tissues examined. The enzyme has been purified from several mammalian sources, and cDNAs encoding the human, mouse, rat, and guinea-pig enzymes have been cloned and sequenced [22].

Sequence comparison with certain zinc metalloenzymes revealed the presence of a zinc-binding motif (**H**EXX**H**-X_{18}-**E**) in LTA_4 hydrolase [23]. Accordingly, LTA_4 hydrolase was found to contain a catalytic zinc. The three proposed zinc-binding ligands, His-295, His-299, and Glu-318, were verified by mutagenetic analysis. Furthermore, the enzyme was found to exhibit a chloride-activated peptidase activity. Based on its zinc signature, sequence homology, and aminopeptidase activity, LTA_4 hydrolase has been classified as a member of the M1 family of the MA clan of metallopeptidases [24].

Identification of Catalytically Important Amino Acid Residues and Crystal Structure of LTA_4 Hydrolase

In addition to the zinc-binding ligands, several amino acid residues of catalytic importance have been identified by site-directed mutagenesis. Thus, mutagenetic replacements of Glu-296 in LTA_4 hydrolase abrogated only the peptidase activity, a finding that suggests a direct catalytic role for Glu-296 in the peptidase reaction, possibly as a general base [25]. Furthermore, sequence comparisons and mutational analysis have demonstrated that Tyr-383 plays an important role in the peptidase reaction of LTA_4 hydrolase, presumably as a proton donor [26].

Typically, LTA_4 hydrolase undergoes "suicide" inactivation with a concomitant covalent modification of the enzyme by its substrate LTA_4 [27]. Mutational analysis has demonstrated that Tyr-378 is a major structural determinant for suicide inactivation [28]. Mutated proteins, carrying a Gln or Phe residue in position 378, were neither inactivated nor covalently modified by LTA_4.

Recently, the X-ray crystal structure of LTA_4 hydrolase in complex with the competitive inhibitor bestatin was determined [29]. The protein molecule is folded into an *N*-terminal, a catalytic, and a C-terminal domain, packed in a flat triangular arrangement. Although the three domains pack closely and make contact with each other, a deep cleft is created between them. At the bottom of the interdomain cleft, the zinc site is located. As predicted from previous work, the metal is bound to the three amino acid ligands, His-295, His-299, and Glu-318. In the vicinity of the prosthetic zinc, the catalytic residues Glu-296 and Tyr-383 are located at positions that are commensurate with their proposed roles as general base and proton donor in the aminopeptidase reaction.

Close to the catalytic zinc, a glutamic acid residue (Glu-271), belonging to a conserved G*X*MEN motif in the M1 family of zinc peptidases, was identified [29]. By mutational analysis and crystallography it was shown that Glu-271 is necessary for *both* catalytic activities of LTA_4 hydrolase [30]. Presumably, the carboxylate of the glutamic acid residue participates in the opening of the epoxide moiety of LTA_4

and formation of a carbocation intermediate. In the peptidase reaction, the role of Glu-271 may be to serve as an N-terminal recognition site and to stabilize the transition state during turnover of peptide substrates.

The crystal structure, in combination with site-directed mutagenesis studies, also suggested that Asp-375 is a critical determinant for the introduction of the 12R-hydroxyl group of LTB_4 [31].

Leukotriene C_4 Synthase

Leukotriene C_4 synthase catalyzes the committed step in the biosynthesis of cys-LTs through conjugation of LTA_4 with glutathione. The enzyme is a membrane-bound homodimer with a subunit molecular mass of 18 kDa [32]. LTC_4 synthase has been cloned and sequenced [33,34]. Two consensus sequences for protein kinase C phosphorylation were found, and subsequent studies have shown that phosphorylation reduces the LTC_4 synthase activity [35]. Sequence comparisons of LTC_4 synthase and FLAP demonstrated a surprising 31% identity between the two proteins. In addition, recent work has identified two microsomal GSH transferases (MGST2 and MGST3) that possess LTC_4 synthase activity and exhibit a high degree of similarity to both LTC_4 synthase and FLAP [36,37].

Leukotriene Receptors

For LTB_4, two types of surface receptors are known (BLT_1 and BLT_2). The BLT_1-receptor has been cloned and characterized as a 43 kDa, G-protein-coupled receptor with seven transmembrane-spanning domains (7TM) [38]. The BLT_1 receptor is only expressed in inflammatory cells [39] and shows a high degree of specificity for LTB_4 with a K_d of 0.15–1 nM [38,40].

A second G-protein-coupled 7TM receptor for LTB_4, BLT_2, has recently been identified [40–42]. This receptor is homologous to the BLT_1 receptor but has a higher K_d value for LTB_4 (23 nM) [43]. In contrast to the BLT_1 receptor, BLT_2 is ubiquitously expressed in various tissues.

The cys-LTs are recognized by at least two receptor types ($CysLT_1$ and $CysLT_2$), both of which have been cloned and characterized as G-protein-coupled 7TM receptors [44–48]. The $CysLT_1$ receptor mRNA is found in, for example, spleen, peripheral blood leukocytes, lung tissue, smooth muscle cells, and tissue macrophages [45,47]. The preferred ligands for the $CysLT_1$ receptor are LTD_4 followed by LTC_4 and LTE_4 in decreasing order of potency.

The $CysLT_2$ receptor contains 345 amino acids with approximately 40% sequence identity to the $CysLT_1$ receptor [44,46,48]. This receptor binds LTC_4 and LTD_4 equally well, whereas LTE_4 shows low affinity to the receptor. Studies on the tissue distribution of the $CysLT_2$ receptor show high levels of mRNA in heart, brain, peripheral blood leukocytes, spleen, placenta, and lymph nodes, whereas only small amounts are found in the lung. The functional role(s) of the $CysLT_2$ receptor is presently unclear, but its wide tissue distribution suggests many possibilities, including regulation of brain and/or cardiac functions.

Gene Targeting of Enzymes and Receptors in the Leukotriene Cascade

The roles of the key enzymes and two of the receptors (BLT_1 and $CysLT_1$) in the leukotriene cascade have been studied by gene targeting. 5-LO-deficient mice are more resistant to lethal effects of PAF-induced shock and also show a marked reduction in the ear inflammatory response to exogenous arachidonic acid [49]. Furthermore, 5-LO null mice are more susceptible to infections with *Klebsiella pnemoniae* [50], exhibit a reduced airway reactivity in response to methacholine, and have lower levels of serum immunoglobulins [51].

FLAP deficient mice, like the 5-LO (–/–) mice, showed a blunted response to topical arachidonic acid, had increased resistance to PAF induced shock, and responded with less edema in zymosan-induced peritonitis [52]. Furthermore, the severity of collagen-induced arthritis was substantially reduced in FLAP (–/–) mice, thereby indicating a role for leukotrienes in this model of inflammation [53].

LTA_4 hydrolase (–/–) mice are resistant to the lethal effects of systemic shock induced by PAF, thus identifying LTB_4 as a key mediator of this reaction [54]. In zymosan A-induced peritonitis, LTB_4 modulates only the cellular component of the response, whereas the LTC_4 synthase (–/–) mice displayed a reduced plasma protein extravasation in this type of inflammation [55]. Furthermore, the LTC_4 synthase (–/–) mice were less prone to develop passive cutaneous anaphylaxis. Recently, the role of LTC_4 in plasma protein extravasation following zymosan A–induced peritonitis and IgE-mediated passive cutaneous anaphylaxis was confirmed in mice lacking the $CysLT_1$ receptor gene [56].

Finally, the role of the BLT_1 receptor has also been studied by targeted gene disruption [57,58]. The receptor was necessary to elicit the physiological effects of LTB_4 (e.g. chemotaxis, calcium mobilization, and adhesion to endothelium) and important for the recruitment of leukocytes in an *in vivo* model of peritonitis. As also observed in mice lacking 5-LO, FLAP, or LTA_4 hydrolase, BLT_1 (–/–) mice were protected from the lethal effects of PAF-induced anaphylaxis.

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CHAPTER 169

Lipoxins and Aspirin-Triggered 15-epi-Lipoxins: Mediators in Anti-inflammation and Resolution

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ASA	acetylsalicylic acid
ATL	aspirin-triggered lipoxin, 15*R*-LXA_4
COX-2	cyclooxygenase 2
EPA	eicosapentaenoic acid
HEPE	hydroxyeicosapentaenoic acid
HETE	hydroxyeicosatetraenoic acid
LT	leukotriene
LX	lipoxin
LXA_4	5*S*, 6*R*,15*S*-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid
15-epi-LXA_4	5*S*,6*R*,15*R*-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid
NSAID	non-steroidal anti-inflammatory drug
PUFA	polyunsaturated fatty acid
PMN	polymorphonuclear leukocytes

Lipoxin Signals in the Resolution of Inflammation

Biosynthesis

Cell-cell interactions and transcellular biosynthesis of mediators are now well recognized as important means of generating new signals [1]. In humans, lipoxin (LX) biosynthesis is an example of LO-LO interactions via transcellular circuits. Lipoxins are a separate class of mediators produced from arachidonic acid in that they contain a conjugated tetraene and trihydroxy structure, a feature that departs from the other structural classes of eicosanoids (see [2] and chapters therein) and that gives them distinct biological roles. The lipoxins are generated by two main routes (Fig. 1): the first involves initial lipoxygenation by 15-LO that inserts molecular oxygen in predominantly the *S* configuration at carbon 15 followed by 5-LO based transformation. This route is particularly relevant when polymorphonuclear leukocytes (PMN) interact with mucosal surfaces. A second route, which occurs predominantly as a major intravascular origin within blood vessels when, for example, platelet intracellular glutathione is depleted, involves the conversion of 5-LO-derived LTA_4 that is released from leukocytes and subsequently converted to lipoxins. On their own, human platelets do not generate lipoxins but become an important source of LX as they interact with leukocytes (reviewed in [3]).

Relation of Lipoxins to Diseases and Bioactions

In humans during disease processes, lipoxins are generated by airway, kidney, joints [see reviews [3,4] and references therein) and liver [5]. Their production within exudates is both temporally and spatially separated from the formation

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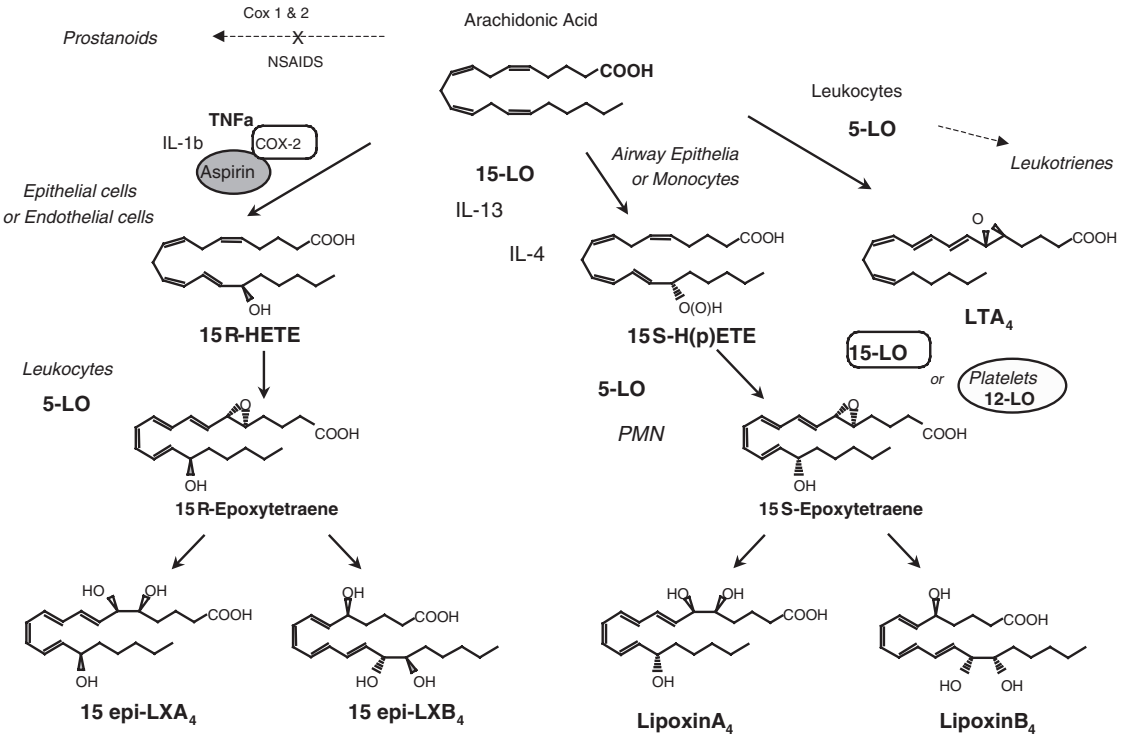


Figure 1 Biosynthesis of lipoxins and aspirin-triggered 15-epi-lipoxins. *Right*: 15-lipoxygenase initiated pathway and 5-lipoxygenase-12-lipoxygenase pathway. *Left*: ASA triggered pathway; irreversible acetylation of COX-2 by aspirin changes the enzyme's product from prostaglandin intermediate to precursors of ATL. The acetylated COX-2 remains catalytically active (see text).

of leukotrienes or prostaglandins [6], and the main actions of lipoxins stand apart from other known eicosanoids and lipid mediators (Table I). Acting in the nanomolar range, native lipoxins selectively regulate the motility of PMN, eosinophils, and monocytes in a stereospecific fashion, a result that raised our awareness to the possibility that lipoxins and related compounds could serve as endogenous "stop signals" of select leukocytes to help resolve local inflammation [3,4]. Much as lipoxins serve as endogenous suppressors of leukocyte-mediated tissue injury, their epimeric form generated with aspirin treatment (Fig. 1), namely aspirin-triggered lipoxins (ATL) 15-epi-LX (stereoisomers at carbon 15 position of native LX) and related compounds, may be the effectors of well-established anti-inflammatory therapies.

Lipoxins are rapidly generated within seconds to minutes, act locally, and are swiftly inactivated via enzymatic routes. Based on knowledge of LX routes of inactivation and the identity of a receptor for LXA_4 [7], metabolically stable LX and ATL analogs that resist rapid metabolic inactivation and are potent regulators of leukocyte traffic (*in vitro* and *in vivo*) were designed and synthesized by total organic synthesis [8]. Some of these more potent LX-mimetics are also topically active inhibitors of acute inflammation (Table I) and are potent inhibitors of TNF_α signals as well as IL-8 formation [9]. ATL and its active analogs compete with LXA_4 at its own receptor on leukocytes and act as agonists that stimulate and induce intracellular "stop signaling" that can have both rapid and gene transcriptional associated events [10]. These recent findings support the notion that ATL and native lipoxin prevent tissue damage by serving as endogenous anti-inflammatory molecules that also stimulate macrophage clearance of spent PMN and resolution of edema [11,12].

Table I Main Anti-Inflammatory and Resolving Actions of Lipoxins and Novel Aspirin-Triggered Lipoxins[a]

Compound/mediator	Response/action
LX and/or ATL[b]	• Regulate leukocyte traffic in acute inflammation & injury ("stop" PMN and eosinophils, "go" monocytes non-phlogistic activation) [3, 4]
	• Redirect chemokine-cytokine axis (gene expression, i.e. IL-8, IL-1) [10]
	• Reduce edema [11]
	• Stimulate clearance and phagocytosis of apoptotic PMN [12]
	• Turn down pain signals: downregulate PMN in neuropathic pain [25]
18*R*-EPA series (i.e. 18*R*,5,12-tri-HEPE) and 15-epi-LXA_5 series	• Inhibit PMN transmigration and block cytokine-stimulated inflammation *in vivo* [19]

[a]For further details, see text. For further details and original citations of bioactions in isolated cell systems and *in vivo* with disease models, please see [3].

[b]See abbreviations list in text.

Aspirin-Triggered Lipoxins and Other Polyunsaturated Fatty Acid–Derived Mediators

Despite nearly 100 years of wide use, the therapeutic impact of acetylsalicylic acid (ASA) is still evolving, and new beneficial effects are still being uncovered [13,14]. The irreversible acetylation of both cyclooxygenase 1 and 2 (COX-1 and COX-2) with subsequent inhibition of prostaglandin biosynthesis is well appreciated and explains some, but not all, of ASA's pharmacological actions [15], and until recently the mechanism for ASA's impact *in vivo* on PMN recruitment in inflammation remained largely unknown. In 1995, Clària and Serhan found that ASA treatment triggers formation of novel series of lipid mediators termed the aspirin-triggered lipoxins (ATL). Their formation relies on cell-cell interactions (15-epi-LX; Fig. 1). Co-activation of neutrophils with either endothelial cells treated with ASA or certain epithelial cells generates a novel class of 15*R*-containing lipoxins (ATL) that in turn downregulate PMN-endothelial cell interactions as well as epithelial function [8,9,16].

In most clinical arenas ASA is held to act strictly as an inhibitor of prostaglandins. However, the ASA-acetylated form of COX-2 *is still active* and converts arachidonate to 15-HETE, which carries its C15 alcohol in the *R* configuration [16]. The COX-2 substrate channel [17] is larger in this isoenzyme (cf. crystal structures for COX-2 [18]) and gives rise to an unusual L-shaped binding of arachidonic acid that gets oxygenated in the 15*R* position. 15-epi-LXA_4 is more potent and longer acting than its 15*S*-containing form because it is not as rapidly inactivated [8]. It appears that ASA triggers formation of endogenous eicosanoids and related substances that could mediate some of the many beneficial actions of ASA by pirating the native pathway of lipoxin production and signaling. It is important to note that the biosynthesis of 15-epi-lipoxins does not arise from a simple pathway shunt, but rather represents the effect of ASA on the oxygenating function of COX-2 at foci of inflammation.

The biological importance of this difference in the enzyme structure (COX-1 versus COX-2) is not clear, but the presence of an additional binding pocket in COX-2 for NSAID was exploited to make COX-2-inhibitors [18]. Acetylation of COX-1 by ASA does not permit substantial amounts of arachidonate conversion to 15*R*-HETE. Once formed, 15*R*-HETE is rapidly esterified in inflammatory cells, altering signal transduction as well as priming the supply of LX precursors [3]. The endothelial cell production of 15-HETE is highly effective *in situ* [19] and *in vivo* at sites of inflammation. Given the vast size of the vasculature and its role in host defense and inflammation, the vascular endothelium is likely to contain focal regions or "hot spots" under stress that express COX-2 and can generate substantial amounts of COX-2-derived products with ASA treatment.

Novel Anti-Inflammatory Signals and Pathways

Over the past 25 years, numerous studies reported that dietary supplementation with omega-3 polyunsaturated fatty acids (ω-3 PUFA) has beneficial effects in disease. Recent reviews discuss potential antithrombotic, immunoregulatory, and anti-inflammatory responses relevant in arteriosclerosis, arthritis, and asthma as well as anti-tumor and anti-metastatic effects [20]. The possible preventative or therapeutic actions of ω-3 PUFA supplementation in infant nutrition, for cardiovascular diseases, and for mental health led an international workshop to call for recommended dietary intakes [20], and data from one large trial (GISSI—Prevenzione, which included over 11,300 subjects) that evaluated the benefits of aspirin with or without ω-3 PUFA supplementation for patients surviving myocardial infarction found a significant decrease in death in the group taking the supplement [21].

Fish oils or n-3 PUFA per se are proposed to act by one or several possible mechanisms [20]. None of the proposed explanations are widely accepted, largely because of the supra-pharmacologic amounts, usually milligram to microgram range of ω-3 PUFA, that are required *in vitro* to achieve the supposed beneficial effects. Because compelling molecular evidence has been lacking and in view of beneficial profiles attributed to dietary ω-3 PUFA and those of aspirin in a variety of diseases, we sought evidence for possible new lipid-derived signals that could explain the epidemiological findings from humans.

A Protective Role for Vascular COX-2 in Micro-inflammation

Inflammatory exudates formed in murine dorsal pouches treated with ω-3 and ASA generate several novel compounds [19], including 18*R*-hydroxy-eicosapentaenoic acid (18*R*-HEPE) and several trihydroxy-containing compounds derived from the ω-3 fish oil eicosapentaenoic acid (EPA) (C20:5) used as an n-3 PUFA prototype. Human cells also generate these new 18*R* and 15*R* series of compounds from EPA, which carry intriguing bioactivities. When human endothelial cells expressing COX-2 are pulsed with EPA and treated with ASA, they generate 18*R*-HEPE or a mixture of 18*R*-HEPE and 15*R*-HEPE. A role for COX-2 in this biosynthetic pathway was confirmed with recombinant human COX-2, in which acetylation by ASA dramatically increased the production of both 18*R*-HEPE and 15*R*-HEPE, findings that could be of clinical significance [19].

When engaged in phagocytosis, activated human polymorphonuclear leukocytes (PMN) process the intermediates derived from acetylated recombinant COX-2 to produce two series of trihydroxy-containing compounds; one series carries an 18*R*-position hydroxyl group, and the other series in the 15*R* position that are related to 15-epi-LX_5. Trout macrophages and human leukocytes can indeed convert endogenous EPA to 15S-containing LX also denoted as 5-series LX_5 [22]. Briefly, we found that human PMN take up and convert 18*R*-HEPE via 5-lipoxygenation to insert molecular oxygen and, in subsequent steps, form 5-hydro(peroxy)-18*R*-DiH(p)EPE and a more labile intermediate 5(6)epoxide that gives rise to 5,12,18*R*-triHEPE. In a similar biosynthetic pathway, 15*R*-HEPE released by endothelial cells is converted by

activated PMN via 5-lipoxygenation to a 5-series LXA_5 analog that also retains their C15 *R* or *epi* configuration, namely 15-epi-LXA_5 [19]. The stereochemistry of compounds in this pathway is different from those of the LO-LO driven pathways that give predominantly C15 S containing LX_5 structures (so-called 5-series of five double bonds) as with endogenous sources of EPA in trout macrophages (cf. [22] and references therein). The chirality of the precursor with ASA-COX-2 (predominantly *R*) is retained when converted by human PMN to give 15-epi-LXA_5 [19].

The new 18*R*-series members might serve as dampers for inflammatory responses, since 18*R*-HEPE gave some inhibitory activity and its product 5,12,18*R*-triHEPE potently inhibits PMN transmigration and infiltration [19]. These results raise the question of whether arachidonate is the sole substrate for COX-2 in physiologic settings in human tissues or whether EPA or other PUFA are important as well [19]. Despite the many reports of possible beneficial impacts of ω-3 PUFAs and EPA in humans [20,21], oxygenation by COX-2 to generate bioactive compounds, as referenced herein, has not been addressed. In fish leukocytes and platelets, EPA (C20:5) and arachidonic acid are both mobilized and converted to both 5-series and 4-series eicosanoids (including PG, LT, and LX) with roughly equal abundance [22]. Given the gram amounts of ω-3 PUFA taken as dietary supplements by humans, as in [20,21], and the large area of the vasculature that can express COX-2 (vascular "hot spots" during local inflammation), the conversion of EPA by vascular endothelial cells and neighboring cells could represent a significant *in vivo* source.

Concluding Remarks

Inappropriate control of inflammation and its resolution is now recognized to contribute to many diseases. Aspirin as well as other NSAIDs that affect these signaling systems (Fig. 1) are in wide use, yet these agents are not without *unwanted* side effects, particularly in kidney and stomach. The discovery of the second isoform of COX (reviewed in [23]) sparked a large-scale search for safer aspirin-like drugs, namely COX-2 inhibitors, that would bypass the unwanted side effects. Results reviewed here indicate that lipoxins, their aspirin-triggered epimers (ATL), and broader arrays of aspirin-triggered lipid mediators derived from omega-3 PUFA reveal previously unappreciated *endogenous* anti-inflammation and pro-resolution signaling mechanisms (Table I) that could offer new treatment approaches. The finding that lipoxin counters inflammatory events led to more general concepts, namely that aspirin-triggered lipid mediators could serve as local mediators of anti-inflammation or endogenous agonists that favor resolution of inflammation. Additional support for this notion that lipoxins are protective and that ATLs share this property [3,19] comes from finding that LXA_4 stimulates macrophages to clear apoptotic PMN [12], and that LXA_4 receptors regulate gene expression, cytokines, and metalloproteases (see [24] and references within). These signaling pathways add a new dimension to the well-established use of low-dose ASA as a specific COX-1 inhibitor in platelets, which also triggers COX-2 generated protective products, thus underscoring the importance of transcellular biosynthetic signaling pathways.

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CHAPTER 170

Cholesterol Signaling

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SREBP, sterol regulatory element binding protein
LXR, liver X-activated receptor
FXR, farnesoid X-activated receptor
PXR, pregnane X receptor
VDR, vitamin D receptor
GR, glucocorticoid receptor
ER, estrogen receptor
MR, mineralocorticoid receptor
PR, progesterone receptor
AR, androgen receptor
FPP, farnesyl diphosphate
GGPP, geranylgeranyl diphosphate
PE, phosphatidylethanolamine

Introduction

Numerous intermediates are formed either during the biosynthesis or catabolism of cholesterol that function as important components in many cell signaling events (Fig. 1). In this review we will briefly discuss some of the recent studies that have revealed the importance of these newly identified signaling molecules.

Cholesterol Precursors

HMG-CoA reductase is the rate-limiting enzyme of cholesterol biosynthesis. The expression level of this membrane-bound enzyme is controlled by many factors that in turn regulate cholesterol synthesis and cellular cholesterol homeostasis (reviewed in [1]). However, the most important mechanisms involve those that control the stability of the protein and transcription of the gene. Studies utilizing both mammalian cells and yeast have shown that increased degradation of HMG-CoA reductase occurs when cellular levels of either the 15-carbon isoprenoid farnesyl diphosphate (FPP), farnesol (dephosphoryled FPP), or an unidentified derivative of FPP are increased [2–4]. The isoprenoid-dependent increase in degradation of both mammalian [2] and yeast [5] HMG-CoA reductase also requires an oxysterol.

Some of the many biologically active oxysterols that are synthesized from cholesterol are illustrated in Fig. 1 (reviewed in [6] and discussed below). However, 24(*S*), 25-epoxycholesterol is synthesized in many tissues from squalene, and not from cholesterol (Fig. 1) [7]. Recent studies have shown that this epoxysterol is one of the most potent activators of the nuclear receptor LXR (discussed below) [8]. Nonetheless, the physiological importance of the endogenous pathway that generates this epoxysterol is currently unknown.

FPP lies at a critical branch point in the cholesterol biosynthetic pathway, since it is a precursor of several important compounds (Fig. 1) (reviewed in [1]). One such compound is the 20-carbon isoprenoid geranylgeranyl diphosphate (GGPP) (Fig. 1). Both FPP and GGPP are important isoprenoid donors that are subsequently covalently linked, via a thioether bond, to a cysteine, located at or near the carboxy terminus of many proteins. This prenylation reaction is necessary for both the intracellular location and function of many proteins, including many members of the ras, rab, or rho family of small G proteins, kinases, and G-protein-coupled receptors [1].

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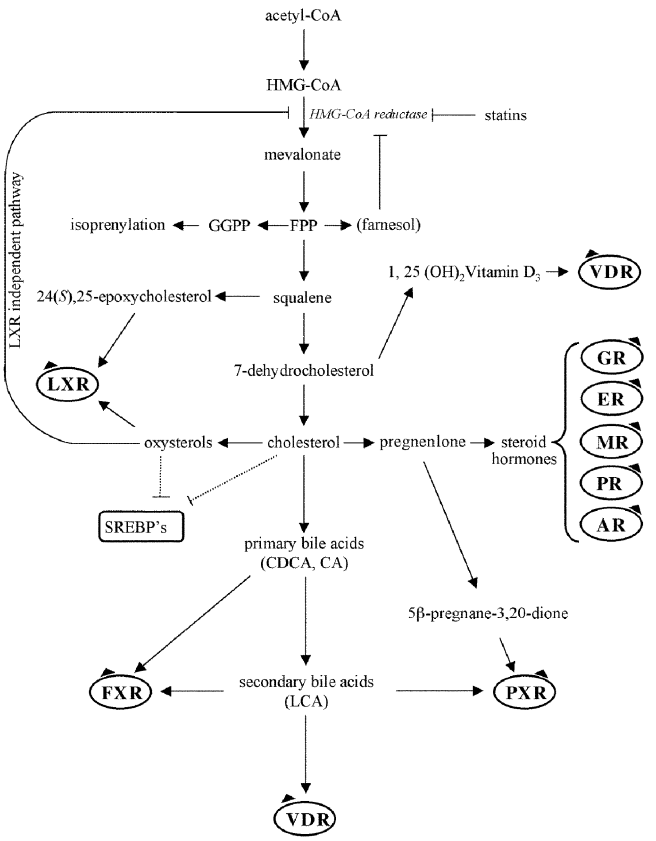


Figure 1 *Signaling molecules generated during the synthesis and catabolism of cholesterol.* Only some of the intermediates in the synthesis and catabolism of cholesterol are shown. The regulatory enzyme HMG-CoA reductase is indicated in italics. The catabolism of cholesterol to primary bile acids, chenodeoxycholic acid (CDCA), and cholic acid (CA) occurs only in the liver. The subsequent synthesis of the secondary bile acid lithocholic acid (LCA) from CDCA occurs as a result of a 7α-dehydroxylation pathway present in certain intestinal bacteria. These bile acids activate FXR, PXR, or VDR, as indicated. The synthesis of steroid hormones that activate the steroid receptors (GR, ER, MR, PR, and AR) primarily occurs in steroidogenic tissues. The conversion of 7-dehydrocholesterol to 1,25 $(OH)_2$ Vitamin D_3, the ligand for the vitamin D receptor (VDR), is discussed in the text. Oxysterols derived from cholesterol or squalene can either activate the nuclear receptor LXR or inhibit the cleavage and maturation of SREBPs by a process that is independent of LXR. Ligands/hormones that function to activate members of the nuclear receptor family are indicated (▲).

7-Dehydrocholesterol, one of the late intermediates in the cholesterol biosynthetic pathway, is found in high concentrations in the skin. Exposure of the skin to UV radiation results in cleavage of the B ring of 7-dehydrocholesterol to produce cholecalciferol. The latter is subsequently converted in the liver and kidney to 1,25(OH)2 vitamin D_3, the biologically active form of vitamin D. Until very recently, 1,25(OH)2 vitamin D_3 was considered to be the major endogenous ligand that activates the vitamin D receptor (VDR). This activated nuclear receptor is essential for the normal absorption of dietary calcium and for the control of calcium homeostasis. However, the secondary bile acid lithocholic acid has recently been shown to function as a potent agonist of VDR [9]. Secondary bile acids are synthesized by bacteria in the intestinal lumen from the primary bile acids that are secreted from the liver. These results imply that secondary bile acids, via activation of VDR in enterocytes, may have a heretofore unrecognized role in maintaining calcium absorption and metabolism.

Cholesterol

In May 1953, Gould *et al.* reported that hepatic cholesterol synthesis decreased when dogs were fed a cholesterol-rich diet [10]. Forty-nine years later we know much about the mechanisms involved in this feedback inhibition. One mechanism involves the accelerated degradation of pre-formed HMG-CoA reductase protein by a process that requires oxysterols and a derivitive of FPP (see preceding section, Cholesterol Precursors).

A second mechanism involves transcriptional repression. Studies initiated in the 1970s demonstrated that the enzymatic activity of many enzymes involved in cholesterol synthesis, including HMG-CoA reductase [11], HMG-CoA synthase, and FPP synthase, was repressed when cells were exposed to oxysterols but not pure cholesterol. It is now clear that cellular accumulation of cholesterol and/or oxysterols results in decreased transcription of these and many other genes that encode enzymes involved in cholesterol biosynthesis. In addition, transcription of the low-density lipoprotein receptor is also repressed by these sterols (reviewed in [12–14]). Goldstein and Brown and colleagues have identified a novel mechanism that controls the transcription of these genes. Transcription is dependent on the nuclear localization of a transcription factor termed sterol regulatory element binding protein (SREBP) (reviewed in [12–15]). In brief, there are two mammalian SREBP genes that, as a result of the use of alternative promoters and splicing, encode three proteins: SREBP1a, SREBP1c, and SREBP2. Each of these proteins is synthesized as a larger precursor that is embedded in the endoplasmic reticulum via a central hairpin loop containing two transmembrane domains [12]. When levels of cellular sterols are reduced, SREBP is escorted from the endoplasmic reticulum to the Golgi by the membrane-bound chaperone SCAP (SREBP-cleavage activating protein) [16–19]. Once in the Golgi, the SREBPs are sequentially cleaved by the site 1 and then the site 2 proteases (S1P and S2P, respectively) to release the mature amino terminal fragment of SREBP [19–21]. This protein fragment translocates to the nucleus, binds to SREBP response elements (SREs) in the promoters of target genes, and activates transcription [12]. These target genes encode enzymes that control the synthesis of cholesterol, fatty acids, triacylglycerides, phospholipids, and NADPH [13,14]. Space limitations prevent further discussion of this area, but the reader is referred to several reviews [1,12,15,32].

The cleavage and maturation of SREBPs is prevented by specific oxysterols but is relatively unaffected by pure cholesterol [22]. However, since exogenously added oxysterols have been shown to enhance the translocation of

cholesterol from the plasma membrane to the endoplasmic reticulum [23], it is still unclear whether cholesterol or oxysterols are the active lipid that interferes with the movement of SREBPs and SCAP out of the endoplasmic reticulum.

A recent publication has shed light onto the possible mechanism by which cholesterol/oxysterols inhibit this translocation in mammalian cells; Dobrosotskaya *et al.* reported that the *Drosophila* SREBP protein undergoes a similar translocation and cleavage prior to the entry of the mature protein into the nucleus [24]. However, in contrast to the sterol-regulated translocation and cleavage of SREBPs in mammalian cells, the translocation of the *Drosophila* SREBP was prevented by phosphatidylethanolamine (PE) [24]. This effect specifically required PE containing the saturated fatty acid palmitate [25]. A surprising finding is that cholesterol had no effect on the processing of the *Drosophila* SREBP [24]. Based on these studies, the authors suggest a mechanism by which cholesterol and PE regulate the translocation of SREBP in mammals and flies, respectively; they propose that excess cellular cholesterol or PE may alter or distort the lipid phase of the endoplasmic reticulum membrane [24]. Such a change in the lipid phase may be sensed by SCAP (possibly via its "sterol sensing domains") and result in altered conformation of the SCAP protein, such that it can no longer bind and chaperone SREBP to the Golgi [24,26]. Since three other membrane-bound proteins (HMG-CoA reductase, Neimann-Pick C and Patched) are also reported to contain homologous sterol sensing domains [27], these data suggest that the function of all four proteins may be dependent upon their ability to "sense" the fluidity of the membranes in which they reside.

In addition to cholesterol/oxysterols, long-chain unsaturated fatty acids also repress the maturation of mammalian SREBPs [28–31]. In contrast, unsaturated fatty acids do not regulate the maturation of the *Drosophila* SREBP [25]. Taken together, these data suggest that a phospholipid containing one or more unsaturated fatty acids may function to regulate the maturation of mammalian SREBP, whereas in flies, this process is regulated by PE containing the saturated fatty acid palmitate.

Cholesterol Derivatives: Ligands for Nuclear Receptors

As shown in Fig. 1, cholesterol can be metabolized to many steroid hormones, including estrogen, testosterone, dihydrotestosterone, progesterone, aldosterone, and glucocorticoids. Each of these steroids activates specific members of the steroid receptor family (Fig. 1) (reviewed in [33,34]). Cholesterol can also be metabolized to a number of other biologically active compounds that function as potent agonists for other members of the nuclear receptor superfamily (Fig. 1). These agonists, which include oxysterols, primary bile acids (chenodeoxycholic acid and cholic acid), secondary bile acids (lithocholic acid), and 5β-pregnane,3,20-dione, activate LXR, FXR, PXR, and/or VDR, as illustrated in Fig. 1 [35,36]. Each of these latter nuclear receptors form functional heterodimers with RXR and bind to specific DNA sequences termed hormone response elements (reviewed in [34,37]). In general, agonists must bind to these DNA-bound nuclear receptor factors in order to activate transcription (reviewed in [38]).

The recent identification of novel nuclear receptors, which include FXR, LXR, CAR, and PXR, their natural ligands (see Fig. 1), their activated target genes [35,39–41], and the generation of nuclear receptor null mice, has led to a wealth of information about the physiological importance of each receptor. The results indicate that these nuclear receptors may represent useful targets for pharmacological intervention. For example, activation of LXR results in decreased cholesterol absorption [42]; activation of FXR results in decreased plasma triglyceride levels [43–45]; and activation of PXR results in the catabolism of a myriad of drugs, xenobiotics, and natural compounds (reviewed in [41]). The recent observations that St. John's Wort (taken as an antidepressant) contains a potent agonist of PXR [46] and that gugulipid (taken as a hypocholesterolemic agent) contains guggulsterone, an antagonist of FXR [47], are particularly intriguing. Based on these reports, it seems likely that many other natural compounds will be discovered that function as agonists or antagonists for these nuclear receptors.

When mevinolin, a natural fungal metabolite, was discovered in 1976 and shown to inhibit HMG-CoA reductase [48] (Fig. 1), few investigators would have guessed that this would lead to a class of drugs called statins that are used extensively worldwide to lower plasma cholesterol levels. Perhaps new agonists-antagonists of the recently discovered nuclear receptors that are activated by derivatives of cholesterol will prove to be equally useful in the clinical arena in the next few years.

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SECTION E

Protein Proximity Interactions

John D. Scott, Editor

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CHAPTER 171

Protein Proximity Interactions

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Introduction

Location, location, and location are the three most important aspects of real estate. The same may be true for intracellular signaling as the subcellular localization of protein kinases and phosphatases are key determinants that control the response time and specificity of many signal transduction pathways. This is equally true for growth factor, phosphotyrosine, and second messenger regulated signaling events where the localization of enzymes in close proximity to substrates ensures an efficient relay of information and directs the signal toward a subset of target proteins. This "protein proximity" section of the handbook highlights a range of cellular mechanisms that contribute to the compartmentalization of signaling enzymes and the assembly of multiprotein networks. Three general areas are covered:

1. Techniques for the analysis of protein-protein interactions,
2. Subcellular structures and multiprotein complexes that contribute to cell signaling and
3. Kinase and phosphatase targeting proteins.

Techniques for the Analysis of Protein-Protein Interactions

The first four chapters describe some of the methods that are used to define protein signaling neworks. Advances in mass spectrometry techniques have revolutionized the analysis of multiprotein complexes. The opening chapter by Shao-En Ong and Mathais Mann (chapter 172) introduces this approach and provides a practical step by step explanation of how signaling complexes are dissected. Paul Graves and Tim Haystead (chapter 173) expand on this theme by discussing some innovative approaches that use affinity chromatography and fluorescent tagging to isolate and characterize native signaling complexes. Peter Verver and Philippe Bastiaens (chapter 174) introduce Fluorescence Resonance Energy Transfer (FRET) and optical techniques that provide sensitive means to quantify and detect protein interactions in living cells. Finally, Gary Bader and colleagues (chapter 175) describe a combined phage display and yeast two-hybrid approach that has been successfully used to map protein-protein interactions within the yeast proteome.

Subcellular Structures and Multiprotein Complexes

The next six chapters define some of the specialized subcellular structures where signaling networks are organized and describe certain cellular events that are controlled by multi protein transduction units. Benjamin Geiger and colleagues (chapter 176) describe the molecular architecture of focal adhesions, where cells attach to the extracellular matrix and transfer signals to the actin cytoskeleton. Karl Saxe (chapter 177) presents evidence for SCAR/Wave and WASP proteins in the coordination of signals from Rho family GTPases to the actin remodeling machinery. Mary Kennedy (chapter 178) defines NMDA receptor signaling complexes at the postsynaptic densities of neurons. Hana Bilak and colleagues (chapter 179) describe the Toll family of receptors, which detect pathogen-associated materials and activate the innate immune response. Andrey Shaw (chapter 180) introduces a protein/membrane compartment called the immune synapse that provides a molecular basis for T cell signaling. Mark Hochstrasser (chapter 181) outlines the ubiquitin-proteosome system, an important group of cellular proteins and enzymes that target degradation, and removal of selected proteins. Finally Guy Salvesen (chapter 182) describes the role of caspase cascades in the interleukin mediated apoptosis.

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Kinase and Phosphatase Targeting Proteins

The final seven chapters of this section introduce some of the anchoring, adapter and scaffolding proteins that organize broad specifity protein kinases and phosphoprotein phosphatases. Elaine Elion (chapter 183) provides a historical perspective by describing the identification and analyses of scaffolding proteins that maintain Mitogen Activated Protein (MAP) kinase cascades in yeast. A complementary chapter by Roger Davis (chapter 184) defines MAP kinase and Jun kinase scaffolds in mammalian cells. Mark Dell'Acqua introduces the A-kinase Anchoring Proteins (AKAPs) that localize the cAMP-dependent protein kinase at specific sites inside cells in chapter 185. This theme is expanded in chapter 186 by Lorene Langeberg and myself as we explore the cellular roles of AKAP signaling complexes containing PKA, PKC and signal termination enzymes such as phosphatases and phosphodiesterases. Peter Parker and colleagues (chapter 187) cover the topic of PKC binding partners and document the various classes of interacting proteins that compartmentalize this enzyme family. Roger Colbran (chapter 188) reviews the extensive literature on protein phosphatase localization with special emphasis of the role of targeting subunits that direct their enzymes to synaptic sites. Finally, in chapter 189, Marc Mumby and colleagues catalog the numerous families of targeting subunits that compartmentalize and modulate the activity of protein phosphatase 2A.

CHAPTER 172

Protein Interaction Mapping by Coprecipitation and Mass Spectrometric Identification

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MS	mass spectrometry
Y2H	yeast-two-hybrid
GST	glutathione S-transferase
TAP	tandem affinity purification
TEV	tobacco etch virus

Introduction

The complex web of signaling pathways and their mechanisms of action rely heavily on protein-protein interactions. These interactions often involve large signaling complexes containing many different protein kinases, protein phosphatases, their substrates, and scaffold proteins. For instance, the scaffold proteins, kinase suppressor of Ras (KSR) and Mek partner 1 (MP-1), bind members of signaling cascades in close proximity and help maintain the stoichometric binding of these molecular scaffolds to kinases, which can determine the amplitude of the transduced signal[1].

The classical approach to the study of protein-protein interactions has been to employ antibodies and glutathione S-transferase (GST) fusions for the coprecipitation of proteins. These studies often make use of Western blotting as readout and as such are limited to use in verification rather than discovery, since antibodies or epitope tags specific to each potential interaction partner are required.

As studies of protein-protein interactions developed, new genetic approaches like the yeast two hybrid (Y2H) method [2] appeared that allowed the screening of large libraries of proteins for interacting partners. This approach is now commonly used to test for protein-protein interactions in functional characterization of proteins and has also been used in several large-scale Y2H studies (see Chapter 53, this volume).

With the advent of sensitive mass spectrometric technologies that allow the rapid identification of proteins in complex mixtures, protein interaction mapping on the scale of whole organisms has now become technologically feasible. In this chapter, we discuss the general principles that govern the study of multiprotein complexes by coprecipitations and subsequent analysis by mass spectrometry (MS), highlighting the role of MS as an especially useful and powerful tool in the field of cellular signaling.

General Considerations of the Coprecipitation Experiment

The coprecipitation experiment consists of three fundamental steps—presentation of the bait, providing the "hook" or affinity tag, and employing a particular mass spectrometric technology for binding partner identification (Table I). The examples described below (and in references cited therein)

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Table I The Three Tiers of the Coprecipitation and Mass Spectrometric Approach to the Study of Protein-Protein Interactions

Introduction of bait		
In vitro	*In vivo* with transient/ inducible overexpression	*In vivo* with endogenous promoter
Separate expression of bait (e.g. with GST vector in bacterial expression, incubation of lysate with GST-protein for pulldown)	Bait is epitope tagged expressed directly in system studied under the control of a inducible promoter (e.g. *GAL1*)	Bait is epitope tagged, homologous recombination of tagged gene to achieve genomic replacement.
Choice of affinity separation/elution		
Single tag	Single tag with enzymatic cleavage	Multiple tags
Many options available: Can be large tags (GST), small moieties (biotin) or specific reactivities (6xHis). Monoclonal antibody specific epitopes (Flag, Myc, Hemagglutinin)	Affinity selection is similar to single tag, release from column is performed with an enzyme specific to the tag to increase specificity. (for example, Protein A or 3xMyc and TEV cleavage [29])	Several variants now exist incorporating different affinity tags and often an internal enzymatic cleavage site. Increased specificity, though more steps perhaps resulting in sample loss.
Mass spectrometric methods		
Gel with MALDI	Gel with tandem MS	Gel free with LC-MS/MS
Matrix assisted desorption ionization time-of-flight MS (MALDI-TOF MS) and peptide mass fingerprinting. Analysis of bands containing only one protein. Less specific than tandem MS.	Prefractionation of sample with SDS-PAGE Nanoelectrospray and tandem MS and MS/MS. Analysis of simple mixtures. Sequence information allows unambiguous protein identifications.	Liquid chromatography and MS/MS allows analysis of complex mixtures. Separation of peptides in time as well as automated data acquisition will make this tool highly suited for large-scale approaches.

Depending on the experimenter's needs and the system studied, some combinations may prove more useful. Refer to text for further discussion.

illustrate the use of coprecipitation and mass spectrometry in a variety of ways and are not intended to be an exhaustive review. The pros and cons of each choice (bait format, type of tag, type of MS used) should be evaluated for each study. Important variables that govern these choices include the amount and type of starting material available (cell lysates versus tissue); the type of complexes to be purified, the strength of their interactions; the inherent tradeoff between specificity of purification and the loss of weaker interacting proteins; and the degree of prefractionation and the scale of the experiment (single bait versus proteome-wide).

GST-Tagged Proteins

Glutathione S-transferase (GST) fusions with bait proteins are well established as a means for coprecipitating interacting proteins. The major advantage of GST-fusions is that large amounts of the bait can often be easily made in bacterial expression systems. These fusion proteins can be used for coprecipitation studies in an *in vitro* format; that is, large volumes of cell lysate are incubated with the GST-fusion and subsequently purified over glutathione-agarose. Fig. 1 shows an example in which GST-fusions with an interaction domain like SH2 yielded promising results in coprecipitation experiments. Other examples of coprecipitation experiments with GST-fusions and mass spectrometry include the identification of novel binding partners for Shc [3] and the Cdk5 activator protein $p35^{nck5a}$ [4].

Although the use of GST fusions is widespread and it is a tremendously useful technique, there are some disadvantages in its application to large-scale proteomics studies. First, the large size of GST (220 aa) may sterically inhibit complex formation. Second, the production of GST fusion proteins in bacterial expression systems may also mean that the protein may not be folded in its native state. Third, bacterially expressed recombinant proteins are sometimes unstable and/or less soluble and do not carry mammalian posttranslational modifications. Fourth, bait presentation is not done within the cell and so the physiological relevance of the protein interactions is less certain than with *in vivo* methods.

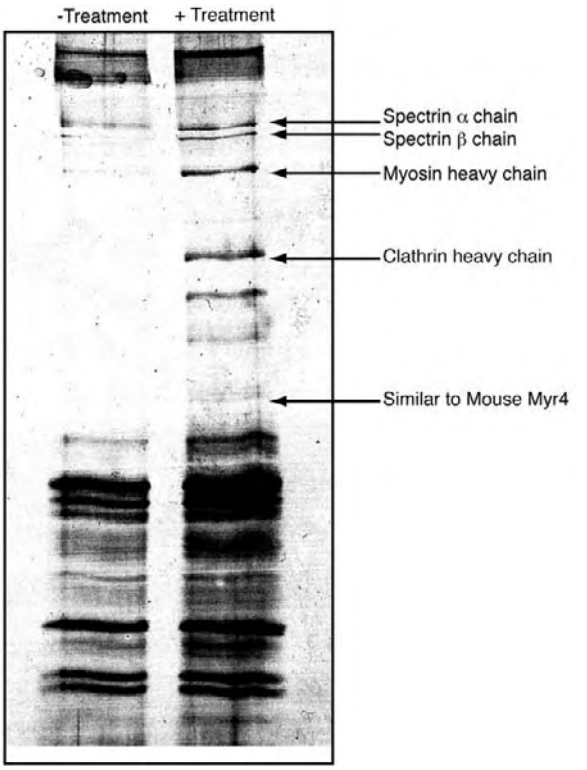


Figure 1 GST-SH2 coprecipitation experiment in mammalian cell lysates. Proteins in the right lane (marked with an arrow) are pulled down by the GST-fusion with SH2 following treatment, suggestive that these bands are specific. Gel bands were excised and then analyzed by Nano ES tandem MS.

For the above reasons, this approach is most useful as a validation method for protein interactions suggested by other evidence. For large-scale fishing strategies where coprecipitation is used as a discovery tool, we suggest considering other tags (refer to Table I for a summary of features in some of the more commonly used tags).

Antibodies

In discovery-oriented approaches—that is, where the interacting partners were not already suspected—the coupling of coimmunoprecipitation and mass spectrometry has proven to be an elegant and effective approach for finding novel substrates and novel proteins in growth factor and cytokine receptor signaling [5]. In addition to directing an antibody against a single "bait" protein, the antibody can also be directed against a common feature of a group of proteins, such as a phospho-residue. Pandey *et al.* were able to identify novel substrates of both the epidermal growth factor receptor (EGFR) and the platelet derived growth factor receptor (PDGFR) signaling pathway with the use of antiphosphotyrosine antibodies to immunoprecipitate phosphoproteins from cell lysates [6–8]. In these pull-down experiments, gel bands resolved by one-dimensional SDS-PAGE from immunoprecipitates of experimental samples treated with cytokine or growth factor were compared with untreated controls. Gel bands specific to treated cells were excised, digested, and analyzed by tandem mass spectrometry. In the example of the analysis of immunoprecipitated proteins from EGFR signaling, nine proteins were identified. Of these two were previously undescribed substrates in EGFR signaling and seven others were previously known.

The use of antibodies against endogenous bait protein is attractive because the bait is in its *in vivo* state, which makes this experiment the closest to the *in vivo* situation. However, it should be noted that it can be quite time consuming to develop antibodies against a large set of proteins and that the subset of antibodies capable of immunoprecipitating proteins is small. The specificity and ability to pull down proteins is also exceedingly variable (for example, the antiphosphoserine and antiphosphothreonine antibodies are not as effective as currently available antiphosphotyrosine antibodies). Large-scale collections of validated antibodies against all human proteins would solve these problems.

Epitope Tags

Epitope tags were historically derived by mapping the interaction site of monoclonal antibody (linear epitope of less than ten amino acids), and then fusing this epitope to bait proteins of interest. A commonly used epitope tag is the FLAG peptide [9,10]. Its salient features are its small size (8 aa), its hydrophilicity, and the availability of good monoclonal antibodies for highly specific purification. The size and hydrophilic nature of the tag were specifically designed to minimize the tag's effect on the formation of native protein complexes. Plasmid vectors for adding N- and C-terminal versions of the FLAG tag to the bait of interest exist, and each have specific conjugated monoclonal antibodies raised against them. Although these antibodies are highly specific, they do not have a large binding capacity. In MS-coupled approaches, this high specificity is a desirable tradeoff versus the lower cost of the GST-based approach, and the high sensitivity of MS may mean that large preparations are unnecessary. As shown in Table II, other epitope tags, such as Myc and HA, have also been used, but they may not be appropriate for use for their own reasons: Myc tagged proteins are difficult to elute from the monoclonal antibody (9E10) and should be used in conjunction with enzymatic cleavage (see below); anti-HA (12CA5) antibodies are not as specific as anti-Myc or anti-Flag antibodies.

In an effort to increase specificity of the affinity purification without the use of monoclonal antibodies, the combination of multiple epitopes in a single tag has recently been popularized (for example, TAP for tandem affinity purification [11]) and has been used successfully in several recent studies [12,13]. In the TAP example, the tag consists of a calmodulin-binding peptide and an IgG-binding region (ProtA) with a tobacco etch virus (TEV) cleavage site separating the two epitopes. Briefly, the tagged protein and its associated proteins are first purified on IgG beads, washed, and then eluted by

Table II A Summary Table of Common Tags Used in coprecipitation Studies

Types of Tags[a]	GST	Biotinylated	6xHis	Epitope	Affinity tag with enzymatic cleavage
Size of tag	Large, 26 kDa protein (220 aa residues)	Biotin plus linker region is small	Small	Less than 10 amino acids; (FLAG, Myc; HA)	Large, bulky (e.g. TAP is 184 aa residues)
Features	Many commercial vectors available in different expression systems	Biotin as a reactive hook for fishing with avidin conjugated beads Requires a chemical modification that attaches biotinylated tag to reactive moieties on peptide chain	Polyanionic	FLAG has an enterokinase cleavage site, hydrophilic tag; Myc binds tightly and should be combined with enzymatic release; HA is less specific	ProtA and TEV (29); TAP tag as a modification-Multiple affinity tags Protein A, Calmodulin binding peptide, Internal cleavage site (11, 30)
Purification	Fairly specific, glutathione conjugated agarose for affinity purification	Avidin binding is extremely strong, fairly specific depending on the type of avidin	Commonly Immobilized Metal Affinity Chromatography (IMAC) like Ni-NTA resin, not very specific.	Monoclonal antibodies-very specific	Enzymatic cleavage is one of the first steps to elute bound material. In TAP tagging, first bound to one affinity matrix (IgG beads), cleave purification tag internally to release, bind to second affinity matrix (calmodulin beads) elute with EGTA
Ease of elution	Competitive elution with glutathione; enzyme cleavage (e.g. thrombin)	Requires harsh elution conditions, typically organic solvent/acid.	Mild elution conditions with 100 mM imidazole or with L-histidine	Gentle–competitive elution with the peptide constituting the epitope	Enzymatic cleavage is generally specific. Dual elution steps– both fairly mild but might be biased against specific classes of proteins (EGTA)
Potential Pitfall	Large size of tag may interfere with native protein or protein complexes	Chemical modification is not specific–requires purification of complex to homogeneity which is generally impractical; harsh treatment of sample.	Affinity matrix is not very specific to His-tagged proteins. Might also pull down other proteins.		More complicated purification procedure; Complex needs to be stable under enzymatic release

[a]See also [31,32], for a good discussion of available tags.

incubating with the TEV protease. The enzyme will cleave the tag, resulting in the elution of the protein complex. The eluate is affinity purified in the next step over calmodulin beads and subsequently eluted with EGTA. The TAP tag and other purification methods based on two orthogonal principles significantly reduce background. Often, a single tag, for example Protein A or n x Myc, achieves similar specificity when combined with enzymatic release of the complex and is simpler to perform. Limitations of the TAP method include the large size of the tag (184 aa residues), the requirement for stability of the complex during enzymatic cleavage, and the fact that some mammalian proteins contain the recognition site for the TEV protease.

Mass Spectrometric Approaches

Since the mid-1990s, mass spectrometry (MS) has been the definitive method used for protein identification. Technological developments have brought significant advances in the sensitivity and throughput of MS approaches [14,15]. Quantitative approaches using stable isotopes together with MS greatly enchance the utility of such experiments [15].

A well-established and still commonly used approach is the identification of proteins following separation by gel electrophoresis. Proteins are visualized by staining with either silver or Coomassie-based stains. Gel bands from one-dimensional SDS-PAGE gels or spots from two-dimensional

IEF-SDS-PAGE gels are excised, reduced, and alkylated, then digested with a proteolytic enzyme (typically trypsin) in order to yield peptides that are more easily characterized by mass spectrometric methods [16].

The MS method used for the identification of proteins may vary depending on the degree of prefractionation of the sample. For instance, it may be acceptable to use matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) instruments and peptide mass fingerprinting (PMF) for protein identification of well-resolved proteins on two-dimensional gels. Analyses of large proteins (and correspondingly large numbers of peptides) or mixtures of proteins may require the use of tandem mass spectrometry in order to yield unambiguous identifications from femtomole (nanogram) amounts of protein.

Two recent studies in yeast involving the use of epitope tags for the study of multiprotein complexes by mass spectrometry have demonstrated the feasibility of large-scale approaches [17–19]. Notably, up to 25% of the yeast genome was identified in immunoprecipitations of tagged proteins in both studies. An important finding was that datasets from both of these interaction mapping studies did not show considerable overlap with known protein interaction data obtained from Y2H experiments [20]. Datasets from Y2H experiments have also shown that these derived interaction maps show variability even when the same approach is used [21,22]. This may be an indication that such studies, though large in scale, still only sample a small area of the interaction space in the overall scope of protein interactions.

It is important to bear in mind the principal differences between the Y2H approach and the pull-down experiment. Y2H experiments are more suited to the discovery of binary or tertiary (with yeast-three hybrid) of moderate to strong binding strengths. The pull-down experiment is more likely to yield protein complexes that interact strongly within a complex but may consist of a large number of interacting proteins that individually might have a much weaker binding affinity for other proteins within the complex. Another attractive feature of the coprecipitation approach is that the complex is pre-assembled *in vivo* and subsequently affinity purified. It is likely that the maintenance of the physiological conditions where these interactions form would have a positive impact on the biological relevance of the interaction data obtained. In contrast, the Y2H experiment is based on the inherent assumption that the cDNAs will express in yeast and that the interactions persist (or form) in possibly nonphysiological contexts.

Outlook

The combination of affinity purification of proteins and subsequent detection by mass spectrometry has become a very powerful tool over the last few years. Although different tags are continually being developed, the goal remains the same: the specific enrichment of certain proteins either in a modification-dependent fashion or as a member of a larger protein complex. The choice of the tag depends on the experimental context.

Recently, the move toward high-throughput analyses of complex protein mixtures has led to the increased use of liquid chromatographic separation as an orthogonal method of separation before mass spectrometric analyses [23,24]. In this approach, complex mixtures of proteins (e.g. a cell lysate) are digested in-solution and loaded onto a reverse phase C_{18} column, desalted, and eluted with a gradient of organic solvent directly into the mass spectrometer for analysis. Present approaches require that one determine the validity of a coprecipitating protein by visualizing the differences with a "control" sample (Fig. 1). It would therefore be necessary to incorporate quantitative information directly in protein complexes studied. This is also necessary because mass spectrometry is becoming ever more sensitive and can detect trace amounts of proteins in a pull-down. Labeling of protein complexes before mass spectrometric analyses with chemical reagents such as ICAT [25] or other derivatizing agents [26,27] may serve this function. In addition, recent methods for the labeling of cells *in vivo* [28,33] may be more suitable as proteins are quantitatively coded at the amino acid level even before any purification steps are performed. By using quantitatively labeled proteins from two different states in coprecipitation experiments, specific protein interactions can be easily distinguished from non-specific 'background' with a high degree of confidence and sensitivity [34,35]. Furthermore, this quantitative data can facilitate the determination of stoichiometries of protein binding within complexes.

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CHAPTER 173

Proteomics, Fluorescence, and Binding Affinity

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Introduction

In this chapter, we discuss how affinity chromatography can be used to purify protein complexes or isolate specific proteomes for further analysis. This approach can facilitate the investigation of protein-protein interactions, enable the identification of novel binding proteins, or allow changes in protein expression or modification to be monitored under different conditions.

Isolation of Specific Proteomes

Proteomics, the study of all proteins expressed in a cell, can provide insight into complex cellular processes. However, studying the entire proteome of a typical eukaryotic cell can be difficult because (1) the number of proteins may exceed the capacity of the systems used to analyze them, and (2) abundantly expressed proteins can dominate the analysis, obscuring less abundant yet potentially interesting proteins. Therefore, we have used affinity chromatography to select for specific types of proteins to simplify and direct the analysis.

Gamma-Phosphate Linked ATP-Sepharose

Several years ago we developed gamma-phosphate linked ATP-sepharose for the affinity purification of protein kinases from complex mixtures [1]. The design of this resin was based upon the orientation of ATP when bound in the active site of a protein kinase. The crystal structure of cyclic-AMP-dependent protein kinase revealed that the adenine portion of ATP is buried within the binding pocket of the enzyme while the gamma-phosphate group of ATP is exposed to the solvent [2]. Therefore, we linked ATP to sepharose via its gamma-phosphate group to enable binding of protein kinases. An important property is that if ATP is linked through adenosine at N6, the resin becomes nonfunctional [P. R. Graves *et al.*, in preparation].

In addition to protein kinases, ATP-sepharose is capable of binding a large number of other purine-utilizing enzymes including the NAD^+/$NADP^+$ utilizing dehydrogenases, DNA ligases, nonprotein kinases, mononucleotide ATPases, and nonconventional purine utilizing enzymes. Indeed, the number of proteins that utilize purines (the purine binding proteome) is quite large and has been estimated to represent about 4% of the human genome [3]. Characterization of proteins that specifically bound ATP-sepharose from a whole mouse lysate revealed that a significant portion of the purine-binding proteome was captured (P. R. Graves *et al.*, in preparation).

The following experiment illustrates how ATP-sepharose (or another type of affinity matrix specific for certain types of proteins) can be used (Fig. 1). Two sets of cells are prepared, one as a control, and one that undergoes some form of treatment (stimulation with growth factors, drug treatments, and so forth). The cells are lysed, and the extracts passed over a column of ATP-sepharose to capture the purine-binding proteome from each sample. After washing to remove nonspecific proteins, proteins are eluted with ATP and resolved by one- or two-dimensional gel electrophoresis. Proteins that are altered in their expression levels or have undergone

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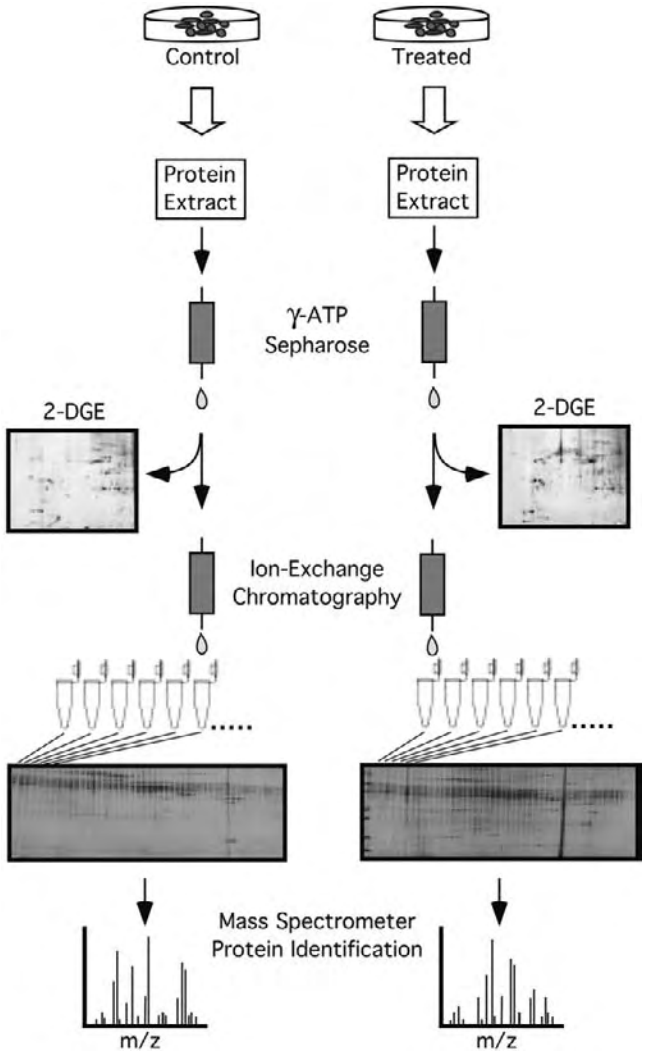


Figure 1

changes in modification (such as phosphorylation) are excised from the gel and identified by mass spectrometry (Fig. 1). Alternatively, proteins isolated by ATP-sepharose can be further purified by more specific techniques before analysis.

Phosphoprotein Enrichment

Protein phosphorylation is a reversible and widespread mechanism for regulating protein function. One goal of proteomics is to allow study of the phosphoproteome, or all the phosphorylated proteins expressed in the cell, under different conditions. However, many phosphorylated proteins are in low abundance and cannot be detected in a whole cell extract. Therefore, a number of techniques have emerged to enrich for phosphorylated proteins. Two methods were recently reported that capture phosphorylated proteins by converting the phosphoamino acids in proteins to groups that allow attachment of affinity ligands for their subsequent purification [4–6]. However, because of the chemistry and the purification strategy involved in these methods, it is still unclear whether low-abundance phosphoproteins can be recovered [4,5]. We developed a method to label phosphoserine in proteins or peptides with a fluorophore [7] based upon the work of Meyer and colleagues [8]. The labeling of phosphorylated amino acids with fluorescent moieties could serve as an alternative to labeling with the radioisotope ^{32}P and be used to study phosphorylation in animals, intact tissues, and humans [7].

Affinity Chromatography for the Isolation of Protein Complexes

Microcystin-Sepharose Chromatography for the Study of Protein-Protein Interactions

Microcystin is a cyclic heptapeptide known to inhibit type 1 and 2 protein phosphatases by binding to their catalytic subunits [9]. Microcystin-sepharose was developed by Sugimura and colleagues [10] and has been used for the purification of PP-1 and PP-2A [11,12]. We have used this resin for the identification of novel protein phosphatase interacting proteins [11,12]. Skeletal muscle lysates were applied to microcystin-sepharose and bound proteins analyzed by one-dimensional gel electrophoresis and protein sequencing. Using this approach, 36 protein phosphatase 1-binding proteins were detected and sequenced [12]. In addition to the recovery of many known protein phosphatase binding proteins, a novel PP-1 regulatory subunit was defined [12].

Gamma-Phosphate Linked ATP-Sepharose

Because ATP-sepharose is capable of binding protein kinases, it can also be used to characterize protein kinase complexes. For example, the subunit structure of AMP-activated protein kinase was established by purifying native AMP-kinase with ATP-sepharose [13]. ATP-sepharose was also used for the purification of Cdc28p kinase complexes from budding yeast [14]. Since ATP-sepharose binds protein kinases in the catalytic domain, it offers another method for the isolation of protein kinase interacting proteins.

Specificity of Protein-Protein or Protein-Ligand Interactions

When starting with a cell extract, the recovery of proteins with an affinity matrix will depend on the solubility of the protein target in the lysis buffer, the binding constant between the protein and the ligand, the abundance of the protein, and the stability of the protein in the cellular extract. Regardless of which affinity approach is used, proteins will be isolated that are not specific for the protein or ligand used because of interaction with the affinity ligand support. Therefore, in each case the appropriate controls need to be performed. If two samples are being compared, ideally, an identical affinity

column should be used for both samples. If a "bait" protein is being used to isolate interacting proteins, then it is a good idea to use a scrambled or irrelevant protein of the same mass as a control. In some cases, specific mutations can be introduced into the bait protein to prevent protein interactions. Double epitope tagging (also known as tandem affinity purification, or TAP) has also been used to increase the stringency of protein complex isolation [15].

One of the most important steps in affinity chromatography is the elution step. Elutions should be performed with an excess of the ligand used for the affinity matrix. In this way, the risk of nonspecific protein elutions is likely to be eliminated. As an example, to increase specificity, we linked microcystin to biotin for the capture of phosphatase interacting proteins [11]. This provides two advantages. First, since mild conditions can be used to elute the bound proteins, holoenzyme complexes remain intact. Second, since the elution is performed with biotin, only proteins that bind to microcystin are eluted, eliminating proteins that bind to the column matrix. Some additional factors to be considered are (1) Can the binding of the recovered proteins to an affinity column be rationalized? For example, if a protein is recovered with ATP-sepharose, is it a purine-utilizing enzyme or does it have a binding site for a molecule resembling ATP? (2) Does pretreatment of the extract with free ligand prevent binding of proteins to the column? (3) Does the protein binding survive stringent washing conditions? (4) Can the result be confirmed by other methods such as far western or co-immunoprecipitation experiments? Because of the complexity of the proteome of a eukaryotic cell and the fact that many proteins of low abundance cannot be detected in a cell lysate, affinity chromatography will continue to be an important method for the isolation of specific types of proteins.

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CHAPTER 174

FRET Analysis of Signaling Events in Cells

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Introduction

Detection of fluorescence resonance energy transfer (FRET) by optical techniques provides a sensitive means of detecting and quantifying molecular interactions and protein modifications in cells. Several strategies are available to develop sensors for use in FRET assays based on fluorescent labeling or green fluorescent protein (GFP) fusions. By using these sensors, techniques such as ratio imaging, sensitized emission measurements, photobleaching methods, or fluorescence lifetime imaging can be employed to spatially and temporally resolve the occurrence of FRET in cells. In this contribution, the strengths and weaknesses of the different sensors and measurement methods are discussed and compared and their use illustrated by reviewing the recent literature. We conclude that the spatially and temporally resolved measurement of FRET in cells has opened new opportunities to image biochemistry in intact cells and expect that these techniques will play an increasingly important role in cell biology.

Optical microscopy provides a sensitive, specific, and noninvasive approach to localize fluorescently labeled macromolecules in cells with high spatial and temporal resolution. Moreover, the spectroscopic properties of fluorescence probes can be used to obtain information on their molecular environment. With the advent of genetically encoded variants of green fluorescent proteins, the observation of biochemistry in cells has become feasible *in vivo* [1–3]. Fluorescence resonance energy transfer is one photophysical phenomenon that has been put to good use to detect and quantify molecular interactions and protein modifications [4]. FRET cannot be measured directly, but the resulting changes in the fluorescence properties of the fluorophores can be detected by several optical techniques with spatial and temporal resolution inside cells. FRET is a photophysical effect whereby energy is transferred from an excited donor fluorophore to an acceptor fluorophore. This does not involve the emission and subsequent absorption of a photon but occurs by a direct electromagnetic interaction. The efficiency of transfer depends on the spectral properties of donor and acceptor, and on their relative orientation and distance. An important factor is that the energy transfer efficiency has an inverse sixth order dependence on the distance between the two fluorophores, and typically FRET only occurs when the distance is less than 10 nm, which is generally only achieved when donor and acceptor are attached to the same macromolecule or to interacting molecules. For this reason FRET can be applied to specifically image such events as molecular interactions or conformational changes. FRET can be observed by its consequences, which are reflected by a change in the fluorescence kinetics of both the donor and the acceptor. Due to the transfer of energy the rate at which the donor returns to its ground state increases, and hence its fluorescence lifetime decreases. As a consequence the quantum yield of the donor, and therefore its steady-state fluorescence intensity, also decreases. The steady-state fluorescence of the acceptor, however, is increased by the sensitized emission that is emitted when the acceptor returns to its ground state.

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Fluorescent Probes for FRET

The design of the fluorescent probes for FRET measurements must match the problem at hand. Single component molecule sensors, consisting of two fluorophores flanking a protein domain or subunit, change FRET efficiency upon a change of conformation or cleavage. These types of sensors are commonly constructed by fusing cyan and yellow variants of GFP to the reporter domains. They can be used to detect physiologically relevant ions [5–7] and small organic compounds [8–10], or report on protein activity or conformational or covalent state [11–14]. They can be targeted to specific cellular compartments by incorporating suitable localization signals. Another class of sensors is based upon the interaction between different compounds tagged with a donor or acceptor fluorophore [15–20]. One application is the detection of covalent modification of a protein, in which case the protein is tagged by a donor, and an acceptor-tagged reagent interacting with the conjugated group is present in large excess. The state of donor-tagged proteins can then be probed by FRET. An example is the use of generic phosphoamino-acid-specific antibodies to probe the phosphorylation state of a protein, whereby the protein is tagged with a donor fluorophore (e.g. by fusing a GFP molecule) and the antibody with the acceptor fluorophore (e.g. Cy3). Another application, not necessarily employing an excess of one species, is using cyan and yellow GFP variants to measure interactions between different proteins, or homo- or hetero-dimerization *in vivo*. The use of GFP variants is attractive for live cell applications, but FRET measurements can also be done via labeled antibodies. This may seem potentially problematic due to the antibody size, but depending on the donor/acceptor configuration and the type of FRET measurement this may in fact be an advantage. Consider an assay to probe the state of a protein based on the observation of the donor fluorophore (e.g. a GFP) only and an antibody labeled with multiple acceptor fluorophores (e.g. Cy3; see [20]). In this case, the increased density of acceptor fluorophores implies a higher probability that upon antibody binding, the donor is in close proximity of an acceptor. However, using an antibody labeled with multiple donor fluorophores would lead to an increased probability of detecting a donor that is too far away from an acceptor for efficient FRET and therefore a lower average signal. In general, the choice of sensor, fluorophore (GFP or fluorescent dye), and where donor and acceptor are located is also determined by the measurement method that is used. The different techniques are described in the next section, where the types of sensors that are appropriate for the respective techniques will also be discussed.

FRET Detection Techniques

Ratio Imaging

Upon the occurrence of FRET the steady-state fluorescence of the donor is quenched, since part of the excited state energy is transferred to the acceptor rather than emitted as photons. Simultaneously, emitting this energy as photons increases the steady-state fluorescence of the acceptor. Therefore, a change in FRET is reflected in an increase in the ratio of sensitized emission over donor emission. This type of measurement is straightforward, as it requires only measurements at two filter settings, and is therefore well suited to live cell imaging.

In an ideal situation, the sensitized emission and donor emission would be directly measured by choosing appropriate combinations of excitation and emission filters, exciting the donor specifically, and detecting the intensities through filters specific for donor and acceptor emissions. Indeed, the donor emission can be imaged specifically; however, in practice the measured acceptor channel contains significant contributions of leak-through of the donor emission and of direct excitation of the acceptor. This implies that the signal in the acceptor channel is strongly dependent on the relative concentrations of donor and acceptor, and that interpretation of the ratio as a diagnostic for FRET is problematic. However, if the relative concentrations of donor and acceptor are fixed, then a change in the donor/acceptor fluorescence ratio can be attributed to a change in FRET. Therefore this approach should mainly be used with sensors that consist of donor and acceptor fluorophores attached to a single molecule. A change in FRET due to a conformational change can then be reliably detected. Another situation is presented by a sensor where donor and acceptor disassociate, for instance by proteolytic activity. In such a case the relative concentrations are known before cleavage and, if no significant differential relocation of the cleaved products is expected, the ratio may still be a good measure of FRET. Also, if one is only interested in the total signal integrated over a cell, the relative total concentrations can be considered to remain the same, although this assumption may be incorrect in a confocal microscope, where molecules may relocate to a position outside the focal plane.

In general, quantification of ratio measurements in terms of the concentrations of the species that exhibit FRET is not straightforward, due to the unknown contributions of leak-through and direct excitation. However, it is possible to externally calibrate the ratio values to physiologically relevant quantities by using reference samples where a known concentration of the species of interest can be related to the measured ratio.

Ratio measurements of FRET have been utilized in a wide spectrum of applications. They have been used as an indicator for adenosine 3′,5′-cyclic monophosphate (cAMP) [8,10], guanosine 3′,5′-cyclic monophosphate (cGMP) [9], and Ca^{2+} [5,6]. More recently sensors for protein kinase [11,12,14], and GTPase [13] activity have been described.

Sensitized Emission Measurements

Whereas ratio measurements are difficult to quantify, it is possible to make use of reference measurements to further quantify results. Generally three measurements are made: (1) using a filter set where the donor is excited ands the donor

emission is measured (donor filter-set); (2) using a filter set where the acceptor is excited and the acceptor emission is measured (acceptor filter-set); (3) using a filter set where the donor is excited and the acceptor emission is measured (FRET filter-set). The images from the donor and acceptor filter sets are multiplied with correction factors and subtracted from the image taken with the FRET filter-set to obtain the sensitized emission, corrected for contributions of donor leak-through and direct excitation. The correction factors are determined via reference samples that contain only donor or only acceptor molecules. The resulting estimation of the intensity of the sensitized emission is then normalized for donor and/or acceptor concentration by using an expression for apparent energy transfer, for which several approaches have been taken [21–23]. Ideally, quantification should provide the relative fraction of molecules that are exhibiting FRET. Up to a scalar factor, it is indeed possible to determine the fraction of acceptor molecules that are exhibiting FRET, since the acceptor concentration can be directly related to the acceptor fluorescence. Determining the fraction of donor molecules that exhibit FRET is much more difficult, since the donor fluorescence is not proportional to the donor concentration, owing to the quenching of the donor fluorescence by FRET. The donor concentration can, however, be measured using acceptor photobleaching, as described below.

In contrast to ratio measurements, this approach is suited to applications where donor and acceptor are not on the same molecule, due to the correction for leak-through and direct excitation. They are also suitable to live cell imaging since only three measurements need to be made. Mostly these approaches are used when donor- and acceptor-tagged molecules have concentrations that are in the same order of magnitude [17]. This FRET method may become less effective in cases where the acceptor is in large excess, since the corrections for direct excitation become large and the result is more susceptible to noise. Similarly, a large excess of donor leads to large corrections for donor leak-through, with associated problems to estimate the sensitized emission. Thus this approach is likely to be less suitable for sensors where a protein state is determined by a large excess of a probe, although sensors to probe the state of a protein have been reported [18]. Even if donor and acceptor concentrations are similar, the corrections can be large, depending on the spectral properties of the donor/acceptor pair that is employed. The different variants of GFP are examples in which the corrections may be substantial, and in such cases this method works best with a high energy transfer efficiency between donor and acceptor, implying a large relative contribution of sensitized emission.[18]. We note that the correction factors are generally determined from averages of images of reference samples, and are therefore scalar factors. It is not known how much these factors change as a function of the environment or whether scalar correction factors are sufficient.

Sensitized emission measurements have been used to study the interactions between different molecules. Examples are the studies by Sorkin and colleagues, who looked at the interaction of the EGF receptor with Grb2 in living cells [17], and by Mahajan *et al.*, who investigated interaction of Bcl-2 with Bax [24]. The sensitized emission method was also used to localize the activity of the GTPase Rac [18].

Methods Based on Photobleaching

Photobleaching of either the donor or acceptor molecules can be utilized to detect the effects of FRET on the kinetics of the fluorescence of either. Photobleaching is the process whereby a fluorophore is converted to a nonfluorescent species, for instance in the presence of oxygen. This essentially happens only when the donor is in the excited state and therefore the rate at which photobleaching occurs is proportional to the average time it spends in the excited state, which in turn is inversely proportional to the rate at which the molecule returns to its ground state. Hence, an increase in the latter due to FRET can be detected by a decrease in the photobleaching rate. Thus, one approach to measure and quantify FRET is to measure the kinetics of photobleaching of the donor [25,26]. Generally, the kinetics are described by a sum of exponentials, and it is possible to quantify the fraction of donor molecules exhibiting FRET. Although this is a potentially precise approach, it is not in much use nowadays for several reasons. First, the requirement to photobleach the donor over extended time periods leads to long acquisition times. Therefore the method is mostly useful on fixed samples, although presumably live cells could be used if one is only interested in the integrated response of a complete cell. Second, the mechanisms of photobleaching are not understood very well, although the assumption of a multi-exponential model is reasonable in first approximation.

Rather than examining the photobleaching kinetics of the donor, one can utilize photobleaching of the acceptor [16,27]. Acceptor molecules can be excited specifically, since their absorption spectrum generally does not overlap with that of the donor. One makes use of the simple property that destroying all acceptor fluorophores abolishes FRET. Thus, after photobleaching the acceptor, the donor intensity should increase, since it is not quenched anymore by FRET. Therefore, comparing the intensity of the donor before and after acceptor photobleaching should indicate whether FRET was occurring. Dividing the difference of the donor intensity before and after photobleaching by the intensity after photobleaching yields an apparent energy transfer measure that is directly proportional to the fraction of donor molecules exhibiting FRET. Obviously this approach is attractive since it is experimentally easy, and also interpretation does not require extensive analysis. Acceptor photobleaching does suffer from the same drawback as donor photobleaching in that it is a time-consuming approach less suitable for imaging of FRET in live cells. A point of possible concern that has not been addressed much so far is that photobleaching of the acceptor may create a different species of molecule that fluoresces in the donor channel, leading to an overestimation of the unquenched donor signal. Alternatively, a dark species could be created that absorbs light and

still acts as an acceptor, leading to an underestimation of the unquenched donor fluorescence.

Acceptor photobleaching has been used as an independent technique to measure FRET. For instance, it was used to study the localization of the A- and B-subunits of cholera toxin [16], and recently to image the three-dimensional distribution of receptor tyrosine kinases interacting with protein tyrosine phosphatase 1B [28]. It is also increasingly being used in combination with other techniques as a standard control for the occurrence of FRET [10,14,29,30].

Fluorescence Lifetime Imaging Microscopy

The kinetics of fluorescence can be measured by fluorescence lifetime imaging microscopy (FLIM). This makes it possible to detect whether the rate at which an excited donor molecule returns to the ground state increases by FRET, since the fluorescence lifetime of a fluorophore is inversely proportional to the sum of rates of all possible pathways by which an excited molecule returns to the ground state [31].

FLIM has been applied in a qualitative fashion where an average lifetime is measured that decreases upon FRET [31]. In such a case, photobleaching the acceptor may serve as an internal control since after destruction of the acceptor fluorophore the lifetime of the donor should attain its normal value [30]. In this case, the acceptor photobleaching can also be applied in live cell imaging by photobleaching after FLIM measurements are made. Since the fluorescence lifetime of the donor is independent of concentration and light path-length, the average donor lifetime can then serve as the control value. FLIM has also been applied quantitatively by resolving the multi-exponential decay kinetics of the donor to determine the fractions of donor molecules exhibiting FRET [20].

So far, FLIM has been applied mostly to donor-only imaging. It is therefore well suited to applications in which the acceptor is present in excess, e.g. to probe the state of a donor-tagged molecule [19,20]. However, it is also suited to cases in which the donor and acceptor are available in comparable concentrations [28]. FLIM has also been applied by imaging both donor and acceptor simultaneously. In this case, the kinetics of the acceptor come into play, and it becomes possible to use fluorophores that are difficult to separate spectrally, such as GFP and YFP [32]. In principle, it is then possible to quantitatively determine both the fractions of donor and acceptor that are participating in FRET, but this has not been demonstrated yet. FLIM requires the acquisition of multiple images but is rapid enough to enable live cell imaging. One drawback of the method in comparison with other FRET methods is that it is more technically involved and equipment cost is higher.

FLIM has been used in a wide variety of applications, among others to probe the phosphorylation state of proteins such as PKCα [19] and the EGF-receptor [20,30] and to study the proteolysis of PCKβ1 [15], the oligomerization of EGF-receptors [33], and the interactions between PKCα and ezrin [29]. In a rather different type of application, Murata *et al*. [34] studied the organization of DNA in cell nuclei by visualizing FRET between the AT-specific donor Hoechst 33258 and the GC-specific acceptor 7-aminoactinomycin D.

Conclusions and Prospects

Exploitation of the physical phenomenon of FRET in biomolecular systems has opened new ways to image biochemistry in live cells. Several optical techniques to measure FRET have been developed in recent years and the number of applications of these techniques to relevant biological systems has been increasing steadily. All of these techniques have their strengths and weaknesses, and it is to be expected that the technological developments will continue for some time. In addition, the development of novel sensors that are based on FRET measurements promises to open more fields of applications, not in the least due to the large variety of GFPs that is becoming available [35,36]. FRET is complementary to biochemical approaches for investigating the complex signaling systems that are encountered at the cellular level in the fundamental biological sciences. We therefore expect that FRET measurements will play an increasingly important role in cell biology in the future.

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CHAPTER 175

Peptide Recognition Module Networks: Combining Phage Display with Two-Hybrid Analysis to Define Protein-Protein Interactions

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Introduction

Many of the protein-protein interactions of macromolecular signaling complexes are mediated by domains that function as recognition modules to bind specific peptide sequences found in their partner proteins [1]. For example, SH3, WW, and EVH1 domains bind to proline-rich peptides [2–4], EH domains bind to peptides containing the NPF motif [5,6], and SH2 and FHA domains bind to peptides phosphorylated on Tyr and Thr, respectively [7,8]. For particular modules within the same family, specificity is determined by critical residues in the binding partner flanking the core peptide motif [9,10]. A major challenge is to construct protein-protein interaction networks in which every module within the predicted proteome of a sequenced organism is linked to its cognate partner.

To address this problem, we developed a four-step strategy for the derivation of protein-protein interaction networks mediated by peptide recognition modules [11–13]. First, the consensus sequences for preferred ligands for each peptide recognition module are defined by isolating 10 to 20 different peptide ligands from screens of phage display libraries. Second, the consensus sequences resulting from the phage display experiments are used to computationally derive a protein-protein interaction network that links each peptide recognition module to proteins containing a preferred peptide ligand. Third, a protein-protein interaction network is experimentally derived via large-scale two-hybrid analysis.

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Fourth, the intersection of the predicted and experimental networks is determined.

As a test of this strategy, we constructed a protein interaction network for the SH3 domains of the yeast *Saccharomyces cerevisiae*. The SH3 domain is one of the more commonly used protein recognition modules. In fact, over 1500 different SH3 domains have been identified in the protein databases of eukaryotic organisms [14]. The yeast proteome contains a total 28 SH3 domains, found in 24 different proteins [15], the majority of which had been implicated in signal transduction (Bem1, Boi1, Boi2, Cdc25, Sdc25, and Sho1) or reorganization of the actin cytoskeleton (Abp1, Bud14, Cyk3, Hof1, Myo3, Myo5, Rvs167, Sla1) [16,17]. A set of eight SH3 proteins remained to be characterized (Bbc1, Bzz1, Nbp2, Yfr024c, Ygr136w, Yhl002w, Ypr154w, and Ysc84).

We were able to express 24 different SH3 domains in a soluble form as glutathione S-transferase (GST)-SH3 fusion proteins in *Escherichia coli*. Because some of the SH3 domains did not select a ligand from the nonapeptide library, we were able to obtain a consensus sequence for only a subset of 20 different SH3 domains. Most SH3 domains bind to a core PxxP ligand motif (P = proline, x = any amino acid), with particular residues that occur on either side of the core determining binding specificity. Two general classes of SH3 ligands have been defined; class I peptides conform to the general consensus RxxPxxP (R = arginine) and class II peptides conform to PxxPxR [2], Most of the yeast SH3 domains selected peptides that aligned to yield a class I or class II consensus ligand, with one to six domain-specific residues constrained outside the PxxP motif (Table I).

The consensus sequences were used to search the yeast proteome for proteins that contained potential SH3 ligands. Because hundreds of the predicted yeast proteins contain an SH3 class I and class II consensus ligand, we used a position specific scoring matrix (PSSM) to rank the peptides present in yeast proteins based upon their similarity to the peptides selected from the phage display libraries. The peptides within the top 20% of the PSSM scores captured most of the literature-validated SH3 domain interactions, and therefore this set was considered as potential ligands. The predicted protein-protein interactions were imported into the Biomolecular Interaction Network Database (BIND) [18] and formatted for visualization in the Pajek package [19], a program originally designed for visualization of social networks. The resulting phage display protein-protein interaction network contained 394 interactions among 206 proteins (Fig. 1A). Proteins are represented as nodes on the graph and the interactions represented as edges connecting the nodes.

Proteins found within highly connected subgraphs can be extracted from more complex networks by using graph

Table I Consensus Sequence of Yeast SH3 Peptide Ligands

	Class I	Class II	Unusual
Bem1-1			P P x V x P Y
Fus1			R x x R st st S l
Abp1		rk x x p x x P x rk P x w #	
Myo3	P x @ p P P x x P		
Myo5	P x @ p P P x x P		
Pex13	R x l P x # P		
Sla1-3	h R x p P x p P		
Sho1	s kr x L P x x P		
Ygr136w	R x rk #@ x l P	P x # P x R p	
Ypr154w	@ kr R P p # x l P	P P # P x R P	
Yhl002w	y R p # P x x P		f R x x x h Y t
Ysc84		P x L P x R	
Yfr024c		P p L P x R P	
Rvs167	R x # P x p P	P P # P P R	
Bzz1-1	K kr x P P p x P		
Bzz1-2	kr kr p P P P p # P		
Bbc1	R kr x P x p P	P kr # P x R P	
Boi1	R x x P x x P	p P R x P r R #	
Boi2		p p R n P x R #	
Nbp2	P x R P a P x x P		

The consensus peptides were derived from an alignment of the selected phage-display peptides (x, any amino acid; lowercase letters, residues conserved in 50 to 80% of the selected peptides; uppercase letters, residues conserved in more than 80% of the selected peptides). Abbreviations for the amino acid residues are as follows: A, Ala; H, His; K, Lys; L, Leu; N, Asn; P, Pro; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; #, hydrophobic residues; @, aromatic residues. The consensus sequences corresponding to Class I peptides, first column, Class II peptides, second column; unaligned, third column.

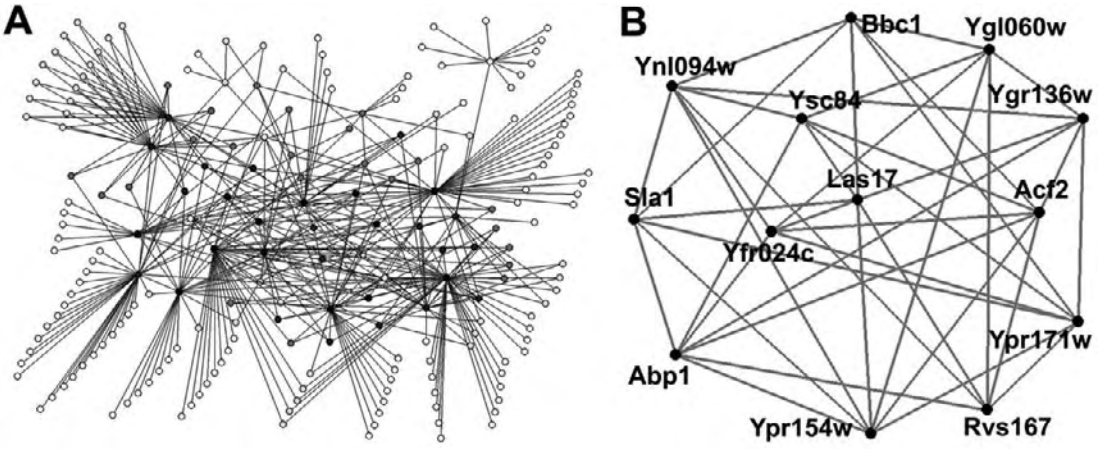


Figure 1 (A) Yeast SH3 domain protein-protein interaction network predicted via phage display selected peptides; 394 interactions and 206 proteins are shown; a network with each gene name labeled is included in the supplementary material [7]. The proteins are colored according to their k-core value (six-core = black, five-core = cyan, four-core = blue, three-core = red, two-core = green, one-core = yellow), identifying subsets of interconnected proteins in which each protein has at least k interactions. By definition, lower core numbers encompass all higher core numbers (e.g. a four-core includes all the nodes in the four-core, five-core, and six-core). The interactions of the six-core subgraph are highlighted in red. (B) The six-core subgraph derived from the phage display protein-protein interaction network, expanded to allow identification of individual proteins. The six-core subset contains eight SH3 domain proteins (Abp1, Bbc1, Rvs167, Sla1, Yfr024c, Ysc84, Ypr154w, and Ygr136w) and five proteins predicted to bind at least six different SH3 domains (Las17, Acf2, Ypr171w, Ygl060w, and Ynl094w).

theoretical algorithms. The phage display network contained a highly connected six-core subgraph, in which each protein has at least six interactions with the other proteins in the subgraph (Fig. 1B). Because the phage display network represents an integration of all potential interactions and does not take into account temporal expression or protein localization information, the six-core is subject to various biological interpretations. It may represent a single complex, provided all the proteins are co-expressed *in vivo* and all of the interactions occur simultaneously; however, it may represent multiple dimers or other oligomers, each of which forms independently under some cellular state. In any case, the presence of a highly connected core suggests a functional association between the interacting proteins. We examined 1,000 random model networks, in which a similar number of random proteins were linked to each SH3 domain. The model networks were not as highly connected as the phage display network and at most contained a four-core subgraph, indicating that the six-core within the phage display network was unlikely to occur by chance. Indeed, the six-core contains a number of functionally related proteins. At the center of the six-core is Las17, the yeast homolog of human Wiscott-Aldrich syndrome protein, which binds and activates the Arp2/3 actin nucleation complex and assembles the filamentous actin of yeast cortical actin patches [20–23]. The six-core also contains Acf2, a protein required for Las17-dependent reconstitution of actin assembly *in vitro* [24] and a set of proteins that were either implicated previously in the endocytotic role of cortical actin patches (Abp1, Sla1, Rvs167) [25–27] or found to localize to cortical patches (Bbc1, Ysc84, Ynl094w, and Ypr171w) [11,28]. Thus, the construction of a protein-protein interaction network from *in vitro* peptide binding information and the graphical analysis of its connectivity revealed known components of the yeast cortical actin patch complex.

To construct a two-hybrid protein-protein interaction network for comparison to the phage display network, we screened 18 SH3 domain baits against conventional two-hybrid libraries and an ordered genome-wide array of yeast Gal4 activation domain–open reading frame fusions [29]. The results from these screens were assembled into a network containing 233 interactions and 145 proteins (Fig. 2A). Only a subset of the interactions within the phage-display network and the two-hybrid network are expected to overlap. In particular, the phage display and two-hybrid methodologies will lead to different sets of false positives, which should exclude them from the overlap network. A total of 59 interactions in the phage display network also occurred in the two-hybrid network (Fig. 2B). All of the overlap interactions are mediated directly by SH3 domains; the precise ligand of the binding partner was predicted by the phage display analysis. Three lines of evidence suggest that the interactions within the overlap network are meaningful. First, the phage display network was highly enriched for overlap interactions when compared to the random model networks, which contained an average of 0.84 overlap interactions (SD = 1.01). Second, the overlap network was enriched for interactions validated previously in the literature, over three-fold compared to the two-hybrid network and over five-fold compared to the phage display network. Third, a focused analysis of the proline-rich peptides within Las17 revealed that the phage display ligand analysis consistently predicted the ligand fragment that showed the strongest binding.

Future experiments of this type may be able to achieve better results by optimizing specific steps. For example, some false positives in the phage display approach undoubtedly arise because the predicted ligand peptide is, in fact, buried in the core of the protein. This aspect of the analysis could be improved by assessing surface accessibility with a program

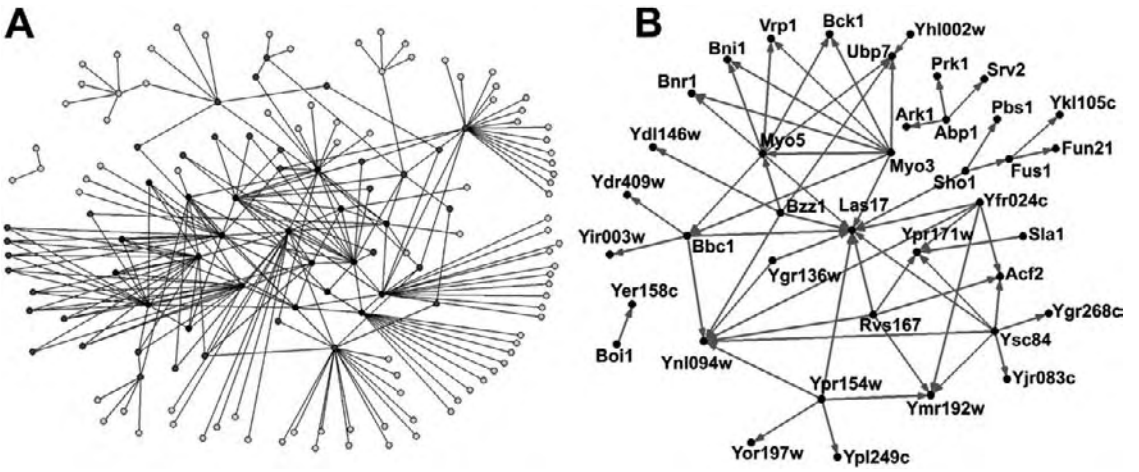


Figure 2 (A) Two-hybrid SH3 domain protein-protein interaction network. Two-hybrid results, based largely on screens with SH3 domains as baits, generated a network containing 233 interactions and 145 proteins. Proteins are colored according to their k-core (see Fig. 1A). The largest core of the two-hybrid network is a single four-core (blue nodes). Interactions common to the phage display network are highlighted in red. (B) Overlap of the protein-protein interaction networks derived from phage display and two-hybrid analysis. Expanded view of the common elements of the phage display and two-hybrid protein-protein interaction networks, 59 interactions, and 39 proteins. All of these interactions are predicted to be mediated directly by SH3 domains. The arrows point from an SH3 domain protein to the target protein. Additional evidence to support the relevance of several of these interactions is provided as supplementary material.

such as PHDacc [30], or homology models [31] of the protein could be scanned. Another means to improve proteome scanning would use a specificity and sensitivity analysis to assess what PSSM score threshold would retain the largest number of physiologically relevant interactions (true positives) and discard as many potential false positive interactions as possible. In this case, false positives can be defined operationally as those not identified within the literature or the yeast two-hybrid network. Thus, the optimization could be based on maximizing overlap with the yeast two-hybrid network or a set of confirmed interactions from a literature-based benchmark.

The overlap step could be improved in a number of ways. While the reasons for the false-positives and false-negatives of yeast two-hybrid screens seem satisfyingly orthogonal to those of the phage display predicted network, other protein interaction experimental methods, such as co-immunoprecipitation coupled with mass spectrometry [32,33], should also be evaluated. The current network representation, with a single node corresponding to a protein and a single edge corresponding to an interaction, could be much improved by making it probabilistic. The attachment of a probability value as a weight on the edges could enter into the overlap calculation to result in a more realistic model. For instance, a weight value on an edge could be high if the interaction has been characterized by several different methods, or found by multiple laboratories. These highly probable edges could be made to appear in the weighted combination of networks; in this fashion, "textbook" interactions would be included even if they were not found by both the phage display and two-hybrid derived networks. A review by Gerstein *et al*. (2002) addresses some of these points in more detail [12]. A better visualization tool that could draw networks with probabilistic information and allow one to examine parameter changes (for example, in the PSSM score threshold) in real-time would complement these method improvements and facilitate evaluation of the results.

Many of these future improvements depend on the availability of a literature-based benchmark, a manually curated collection of high-quality, expert-validated interactions. Sources of more stringently validated interactions are MIPS [17], YPD [34] and PreBIND [33]. Collecting these together in a nonredundant set creates a benchmark of over 3,300 protein-protein interactions for yeast. Because some experimental methods are more likely to yield physiologically relevant information (for example, interactions detected with full length proteins expressed at native levels), the literature benchmark could also include a reliability score for each record.

A set of over 15,000 unique protein interactions collected for yeast from the literature and from all available large-scale studies contained 519 interactions involving 364 proteins in which one interaction partner has an SH3 domain [18]. Because many of these proteins are highly conserved, it will be of interest to determine the extent to which the connectivity of the network is conserved. The prospects for applying this interaction network mapping approach to other organisms are reasonable; for example, *Caenorhabditis elegans* has only 99 SH3 domains in 77 proteins, according to the SMART database, whereas the mouse has on the order of 327 SH3 domains in 172 proteins. A map of peptide-binding module-mediated interaction networks across organisms will provide a powerful dataset to study the specificity of domain-mediated interactions, the evolution of complexity, and the biology that these interactions dictate. Finally, the systematic analysis of binding properties and

protein-protein interaction networks for peptide recognition modules will enable the development of sets of dominant interfering small molecules for systematic functional interrogation of the network [35].

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CHAPTER 176

The Focal Adhesion: A Network of Molecular Interactions

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Introduction

Focal adhesions (FA) and related structures are specialized subcellular sites through which cells in culture or in tissues attach to the extracellular matrix (ECM). The molecular structure and dynamics of these plaque-like adhesion sites [1–4], as well as their roles in adhesion, motility, and signaling, have been established in numerous studies over the past decades [5–8]. Although various types of cell-ECM adhesions are formed, affecting anchorage, motility, or ECM assembly, FA are still the best-studied type of adhesion.

The overall structural organization of FA consists of three major domains: transmembrane receptors, the attached cytoskeleton, and interconnecting plaque molecules. These three domains are structurally and functionally interlinked, and mutations that perturb their interactions disrupt the adhesive process. For example, mutations of integrin receptors that truncate the cytoplasmic domains through which they interact with cytoplasmic partners produce functional inactivation. Similarly, disruption of the actin cytoskeleton by use of drugs that inhibit actin polymerization or block actomyosin contractility leads to the loss of FA.

At the molecular level, both the transmembrane and cytoskeletal domains (but not the plaque domain) are relatively simple, with limited numbers of molecular components. The primary molecular constituents of the membrane domain are members of the integrin receptor family that bind specifically to the ECM molecule to which the cell is adhering. For example, when a fibroblast adheres to fibronectin, clusters of both the fibronectin receptor $\alpha_5\beta_1$ and the multipurpose receptor $\alpha_V\beta_3$ accumulate initially in FA, whereas only $\alpha_V\beta_3$ accumulates on a vitronectin substrate [4,9–11].

The cytoskeletal domain of FA consists mainly of actin filaments, with a few associated proteins, such as α-actinin and filamin. In contrast, the submembrane plaque between integrins and cytoskeleton is considerably more complex, consisting of a large number of linker and signaling molecules (more than 50 reported components). Some major actin-associated proteins are also potent linkers of actin to the membrane at FA. For example, α-actinin and filamin may bind to an integrin and also serve as an actin-integrin linker. Because the molecular components of FA have been extensively described and discussed in a number of recent reviews [8,12], they will not be addressed here in detail. Instead, we describe some key properties of the various constituents of the plaque and their interactions.

FA plaque constituents include both structural molecules, which can physically cross-link different FA molecules to each other and stabilize the adhesion complex, and signaling molecules, such as a variety of kinases, adapter proteins, and other signal transduction proteins. Some FA proteins contain specific motifs involved in protein-protein interactions. The best-characterized involve the SH2 domains, which interact with tyrosine-phosphorylated motifs, and the SH3 domains, which bind proline-rich motifs. Other types of interactions can be mediated by LIM, PDZ, and PH domains. Many of these proteins can bind to more than one partner, and thus they can potentially serve as "nodes" in the formation of multi-molecular adhesion complexes.

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Although the binding affinity between many of these components might be weak, they form aggregates or molecular complexes that cumulatively generate high-avidity interactions. Their low intrinsic affinity may facilitate dynamic rearrangements of molecular complexes, for example during cell migration. Information on the modes of interaction of the different FA molecules with each other *in vivo* is quite limited. Consequently, "wiring diagrams" [12,13] or "network hierarchy schemes" (Fig. 1) can provide a general idea about potential interactions. Yet future research is needed to determine which interactions actually occur *in vivo* and in various physiological states (see below). It is also noteworthy that the actual complexity of FA in specific cell types is not clear, and that it may be less than expected by simply combining all information on the many plaque components in various cell types described in the scientific literature.

Connectivity-Based Ordering of FA Components

Although network schemes currently involve all potential interactions, rather than only interactions that actually occur in adhesion sites, they can shed novel light on molecular connectivity within FA. For example, Fig. 1A shows that actin and integrins can interact with many partners. High connectivity is also seen for several plaque proteins, such as FAK, vinculin, paxillin, α-actinin, and talin, which have at least nine potential interactions each. Less connected are a variety of signaling molecules, which have relatively few links. It is interesting that there are a number of molecules that have only one known link to another FA molecule, suggesting that even though they may be associated with the protein network, their interaction is not as an integral component.

This network type of analysis is presently preliminary, since the published data used for the construction of the network are incomplete and of variable quality. Nevertheless, integration of the currently available data on the players and their interactions into a network presentation allows one to gain some new information. For example, some insight can be gained about the effects of gene knockout (or mRNA inactivation), overexpression, or inhibition of specific certain signaling processes on the network. In Fig. 1B, a vinculin-null version of the network is shown. Such analysis predicts that in vinculin-null cells, vinexin and ponsin will be excluded from FA whereas other vinculin-binding molecules (e.g. talin, α-actinin, and paxillin) may remain via interactions with other components. Similarly, based on a FAK-null network, PLC-γ is predicted to be lost from FA (Fig. 1C). Obviously, such predictions can be challenged experimentally.

In order to illustrate a situation in which one molecular interaction dominates others, we have tested restricting the interactions mediated by the cytoplasmic domain of the integrin to only one molecule: talin (Fig. 1D). As a consequence, some molecules might lose their specific anchorage in FA (DRAL and uPAR) and many others are downshifted in their connectivity. Other scenarios presented here are the changes in molecular connectivity in networks in which phosphotyrosine-SH2-based interactions are excluded, either with or without the presence of FAK (Fig. 1E and 1F, respectively). Again, in these networks some proteins lose their connection to any FA component whereas others are lowered in their connectivity.

Despite its rather speculative and preliminary nature, this approach provides a novel tool for molecular modeling of FA interactions. It makes a number of specific predictions that can be directly tested experimentally. Note that even the deletion of the most highly connected proteins, namely FAK or vinculin, resulted in a surprisingly minor loss of other components (at most two). Furthermore, none of these network modifications caused a split into two unconnected sub-networks. These findings emphasize the robust nature of the connections between most components of FA.

Molecular Switches in FA

An obvious limitation of a network scheme, such as that shown in Fig. 1, is that all of the intermolecular links are presented as though they can all take place simultaneously. In fact, the structure and function of FA, and hence the interactions between its components, need to be regulated physiologically in many processes, including cell spreading, migration, and response to different growth factors and cytokines. By using different modes of regulation (defined here as "switches"), cells can employ a number of potentially important regulatory mechanisms, some of which are illustrated in Fig. 2.

Transcriptional Switches The level of a specific component can be regulated at the level of gene transcription, which in turn affects FA composition. For example, mRNA levels of vinculin can be modulated by matrix adhesiveness.

Protein Stability Switches The dynamic turnover of FA can be initiated by proteolytic cleavage of specific plaque components such as talin and FAK by proteases like calpain.

Tyrosine Phosphorylation Switches Dynamic changes in phosphorylation of plaque components such as FAK are thought to be involved in FA assembly and turnover.

Other Posttranslational Switches Changes in serine/threonine phosphorylation can also regulate integrin and plaque protein (e.g. paxillin) functions [14,15].

Conformational Switches The molecular conformation of proteins or their clustering can be regulated by phosphorylation or binding of other molecules. For example, vinculin can be transformed from a folded state to an open state with exposed intermolecular interaction sites after binding the signaling lipid PIP_2. Tensin conformation may also be regulated by tyrosine phosphorylation, and integrin clustering might trigger its binding to cytoplasmic partners and activate FAK autophosphorylation.

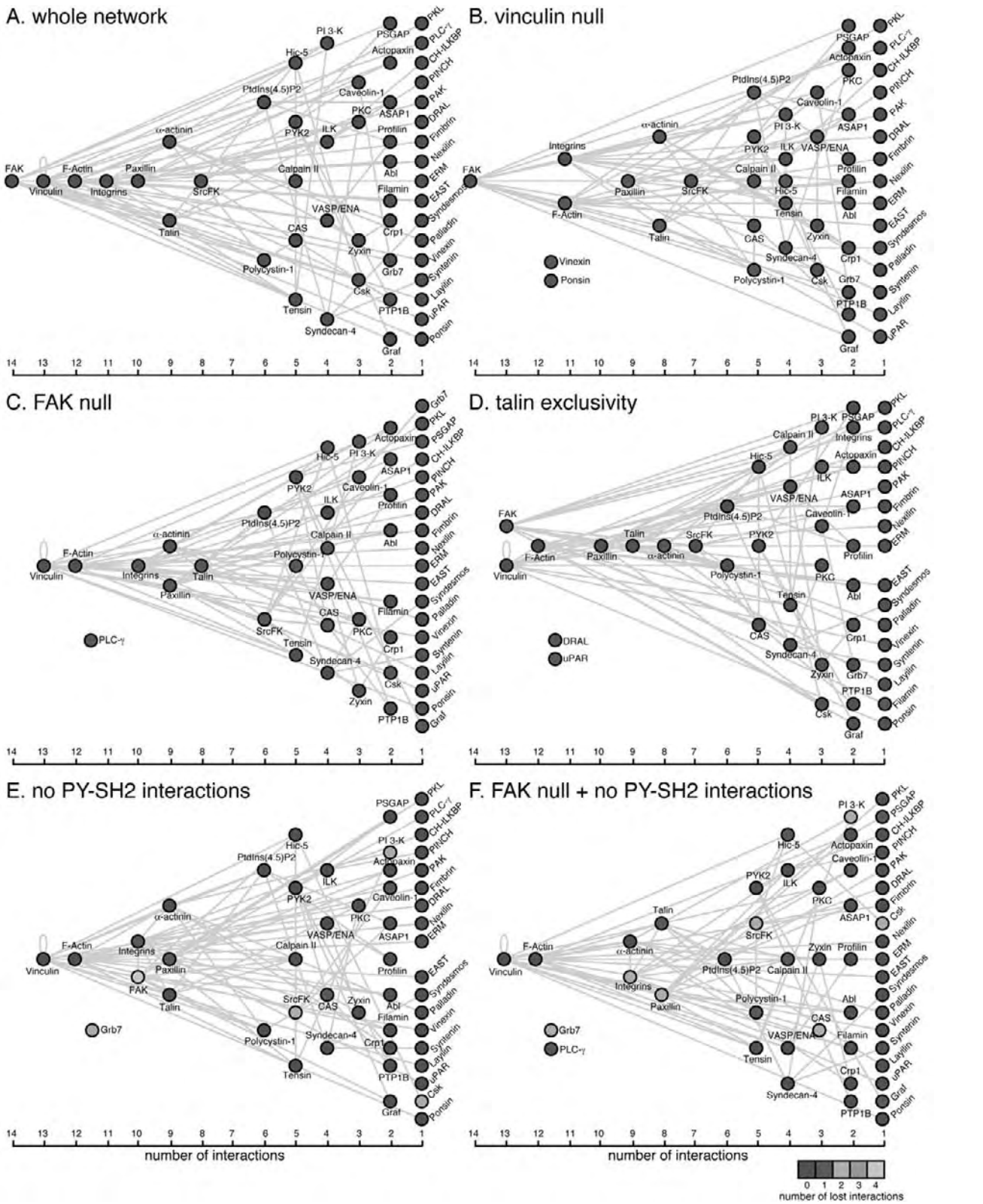


Figure 1 Connectivity-based ordering of FA components. In each of the network diagrams, the proteins were ordered from the most connected to the least connected, as indicated by the scale on the horizontal axis. Proteins listed in the lower left corner of each diagram have no known connections with the network. The connectivity data were taken from previous reviews [12,13] and analyzed via the Pajek program (http://vlado.fmf.uni-lj.si/pub/networks/pajek). (A) The whole network. (B) The network from which vinculin was removed. (C) The network from which FAK was removed. (D) The network in which talin dominates over all of the other cytoplasmic interactions with integrin. Thus, integrin is connected exclusively to talin and to membrane caveolin. (E) The whole network after removing all connections that are mediated by phosphotyrosine-SH2 motifs. (F) The same as (E) with the exclusion of FAK. The color of each vertex (circle) indicates the number of connections lost in comparison to the whole network; For each component, the number of lost connections can be realized by comparison with the whole network, or by the color of its node (a color representation of this figure is available on the CD-ROM version of *Handbook of Cell Signaling*).

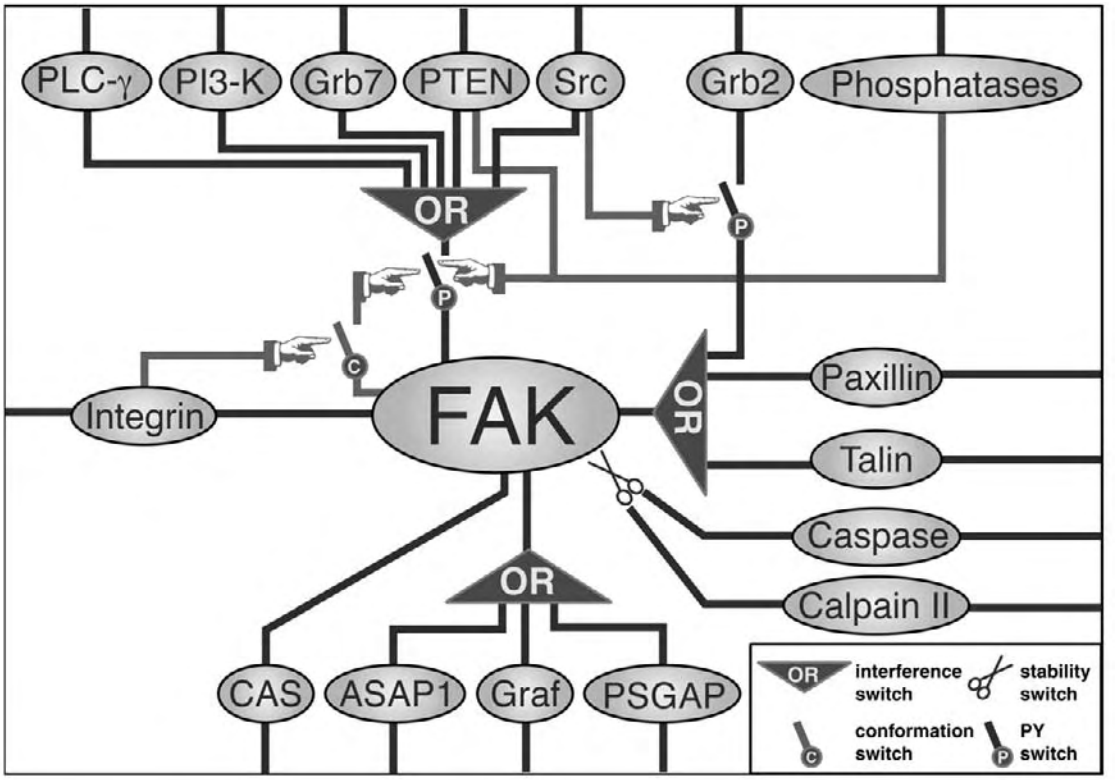


Figure 2 Molecular switches that might regulate protein-protein interactions in FA. The example shown here involves FAK (for general reviews about FAK see [20,21]), which can interact with multiple FA molecules (Fig. 1A). It is proposed that several types of "switches" can significantly constrain the possible combination of interactions with individual FAK. Molecular interference prevents more than one protein from interacting simultaneously with the same site or a nearby site. Thus, an exclusive "OR gate" can restrict the interactions of FAK with Src, Grb7, PTEN, PLC-γ, and PI3-K (all bind FAK Tyr397). Similarly, ASAP1, Graf, and PSGAP interact via their SH3 domain with the proline-rich motif located between amino acids 778–881 of FAK, and thus may interfere with each other. However, CAS interacts with a distinct proline-rich motif, located between amino acids 715–718 of FAK, and thus may be unaffected by other FAK interactions. An additional interference is predicted among paxillin, talin (both interact with the FAT domain of FAK), and Grb2 (which interact with Tyr925 at the FAT domain of FAK). Tyrosine phosphorylation of FAK at residues Tyr397 and Tyr925 is an additional "switch" activating binding sites for several SH2-containing proteins such as Src or Grb7 (Tyr397) and Grb2 (Tyr925) ("P-switch"). The phosphorylation at Tyr397 is performed by FAK itself (autophosphorylation) and can be induced by the clustering of integrin receptors, which aggregates FAK molecules together ("conformational (C) switch"). Phosphatases such as PTEN and Shp2 have been reported to dephosphorylate FAK, presumably targeting Tyr397, which could turn off all FAK interactions mediated by that phosphorylated residue. The phosphorylation of Tyr925 is mediated by Src and may play a role in the recruitment of Grb2, which, in turn, can interfere with FA localization. Finally, caspases and calpain II, in response to various signals, can mediate the proteolytic cleavage of FAK ("stability switch").

Tension Switches Although the mechanisms are still poorly understood at the molecular level, external tension on cells and intracellular actomyosin contractility can dramatically regulate the initiation and sizes of FA [16–18]. Shear stress can also trigger FAK tyrosine phosphorylation [19].

Molecular-interference Switches The binding of one component to a site may block the binding of other molecules to the same or neighboring sites in a dominant-negative fashion. A related switch involves the dominant-negative inhibition of FAK binding of its target molecules by a truncated form of the molecule, termed FRNK, the levels of which are regulated by alternative splicing.

In conclusion, it appears that in constructing meaningful interaction networks, such as those shown in Fig. 1, the various switches described above should be taken into account. We provide here one example how a set of switches can affect the molecular interactions of one of the busiest components of FA, namely FAK (Fig. 2). We show, for example, that many of FAK's partners compete with each other for binding to the same or adjacent sites, and that conformational, phosphorylation, and stability switches can all affect the actual connectivity of this molecule and its signaling activity. Obviously, it would be highly desirable to incorporate such switches into molecular interaction maps.

Future Challenges

Focal adhesions have been studied intensively for the past decade, and there is now a large body of literature on their composition and functions. Even so, our current understanding of the mechanisms involved in FA organization and function is still highly preliminary and speculative. Moreover, even

though FA are thought to serve as signaling centers initiating signaling cascades (ranging from activation of MAP kinases to phosphoinositol lipid signaling), the evidence that signaling comes directly from interacting components located within FA is still minimal. Rigorous studies will be needed to establish which integrin-triggered signals come directly from FA or other cell-matrix adhesions.

A further challenge comes from the fact that many FA components and adapters have multiple potential binding sites. Therefore, it seems likely that FA contain complex mixtures of interacting components with variable binding to different components. That is, a given protein may be bound to multiple combinations of five to ten different proteins, and each of these complexes may behave differently. In addition, local heterogeneity of protein complexes within FA will also need to be evaluated. The approaches for probing these different sources of complexity will have to include experimental molecular perturbation of each component alone and then in various combinations. Obvious methods will include regulated overexpression, anti-sense and RNAi methods, enzymatic activity modulation, and dominant-negative inhibition approaches. However, because of the complexity of FA, computer-based network analysis seems essential. A combination of "wet" biochemical and molecular biology approaches with computerized analyses and generation of specific predictions for experimental testing should provide a powerful approach to understanding the role of these adhesive structures in regulating cell signaling and function.

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CHAPTER 177

WASp/Scar/WAVE

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Introduction

The regulation of actin dynamics is central to a variety of biological events, including cell motility, chemotaxis, nerve growth cone extension, and establishment of cell polarity. A number of signaling mechanisms control these events and play a role in determining the position and extent of new actin assembly. Our understanding of the details is still incomplete, but many of these signals involve receptor stimulation and activation of members of the rho family of small GTPases [1]. One mechanism by which signaling pathways regulate actin polymerization is by controlling the formation of new actin filaments. Activation of the Arp2/3 complex appears to be critical in the nucleation step of this process. In turn, activation of members of the Wiskott-Aldrich protein (WASp) family leads to activation of the Arp2/3 complex (reviewed in [2]). WASp family members appear to play the role of signal integrator, responding to inputs from a variety of extracellular and intracellular signals and converting those signals into effects on actin dynamics.

The WASp protein family has two major branches, WASp and Scar/WAVE. WASp and Scar/WAVE proteins share a distinct modular architecture (Fig. 1). Conserved among all members of the family is a central proline-rich region, followed by a monomeric actin-binding domain (alternatively referred to as a Wiskott-Aldrich homology 2 domain, WH2, or verprolin homology domain, V; Fig. 1). Also common to all members of the family is a C-terminal acidic region shown to be essential for binding the Arp2/3 complex. The precise number and position of the acidic amino acids at the C-terminus are believed to influence the activity of the protein [3]. Between the WH2 and Acidic domains is a region that connects the two (C) that is important for WASp family proteins to activate Arp2/3. What distinguishes WASp (and the closely related N-WASp) from the Scar/WAVE subfamily is the N-terminal region. WASp and N-WASp have, at their most N-terminus, a region termed a WH1 domain, with structural similarities to the EVH1 domain of Ena/VASP proteins [4]. WASp and N-WASp also have in their N-terminus a basic region followed immediately by a small GTPase-binding domain (GBD or CRIB motif). The former, basic, region interacts with acidic phospholipids, particularly phosphatidylinositol 4,5-bisphosphate (PIP_2). The latter CRIB domain binds Cdc42 (and weakly rac1), and both regions are thought to be important in regulating the activation of WASp (see below). Scar proteins have neither a WH1 domain nor a conserved GBD, and though a distinct basic region is present, the role of PIP_2 in Scar function remains unclear. The difference in N-terminal domain structure has led to speculation that WASp and Scar proteins are regulated by different mechanisms. Recent evidence supports this idea (see below). For a more complete discussion of the role(s) and regulation of WASp/Scar/WAVE proteins see the extensive reviews of Higgs and Pollard [2] and Takenawa and Miki [5].

WASp

WASp was originally described as the protein defective in patients with Wiskott-Aldrich syndrome (WAS), an X-linked hematopoietic disease characterized by abnormalities in platelets and lymphocytes [6]. Hematopoietic cells from WAS patients have defects in actin-associated structures such as podosomes and microvilli and motility defects in cells such as macrophages. Independently, a related, more ubiquitiously expressed protein, N-WASp, was identified as a binding partner for Ash/Grb2 [7]. It was recognized that both proteins contained a Cdc42-binding site, suggesting that they might provide a connection between the rho family of small GTPases and actin polymerization. More distantly related proteins, Las17p/bee1p and Wsp1p, were subsequently identified in *Saccharomyces* and *Schizosaccharomyces*, respectively,

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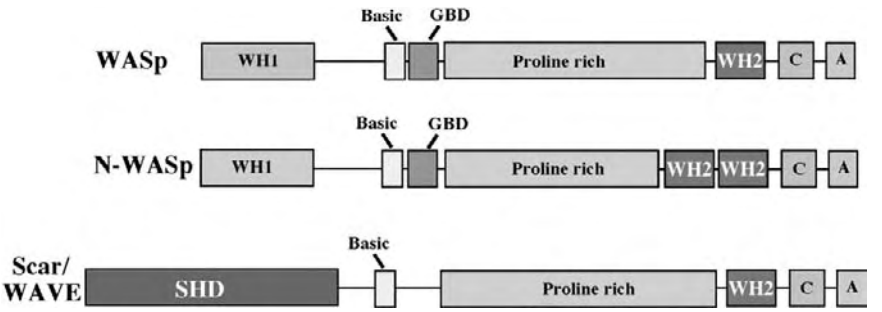


Figure 1 Diagram of domain structure of WASp family proteins. Abbreviations: WH1, Wiskott-Aldrich homology region 1; GBD, GTPase binding domain (also called CRIB motif); WH2, Wiskott-Aldrich homology region 2 (also called verprolin (V) domain); C, connecting domain (also called cofilin-like domain); A, acidic region.

and shown to be essential for actin patch formation and endocytosis [8,9].

The connection between WASp and the Arp2/3 complex came when it was determined that the C-terminal acidic domain of WASp (and hScar1) bound, in a yeast two hybrid screen, to the p21-Arc subunit [10]. Subsequently it was shown that C-terminal fragments of WASp, N-WASp, and Scar could all facilitate the ability of the Arp2/3 complex to nucleate new actin filament formation *in vitro* (reviewed in [2]). Further studies showed that full length WASp (and N-WASp) were significantly less effective at activating Arp2/3 than a C-terminal fragment. Addition of activated Cdc42 enhanced the ability of full-length N-WASp to activate Arp2/3. These data and other studies in cell culture led to a basic model whereby WASp/N-WASp is normally autoinhibited in the cell due to interaction of the C region of the protein with the GBD domain. Activation of WASp/N-WASp involves binding of Cdc42 and/or PIP_2 and unmasking of the carboxyl-terminal region, allowing it to bind actin and Arp2/3 (with an ancillary role for profilin binding to the proline rich region). There are subtle, but important, differences in the way WASp and N-WASp react in the presence of GTP-Cdc42 and PIP_2 suggesting that the model is incomplete. This notion is discussed in more detail in Higgs and Pollard [2]. In addition to Cdc42, a number of other proteins bind to various regions of WASp and/or N-WASp and are believed to be critical in regulating WASp/N-WASp function (Table I). The WH1 region provides a binding site for WASp-interacting protein (WIP) and two related proteins, CR16 and WICH (also called WIRE) [11–14]. These proteins bind not only WASp and N-WASp but also actin and isoforms of the adaptor Nck. The cellular role of these proteins is still unclear, but ectopic expression studies suggest they may couple WASp and N-WASp to SH3-containing proteins such as Nck and regulate subcellular localization, as well as affecting actin polymerization. The protein WISH (WASp-interacting SH3 protein) also binds to the proline-rich region of N-WASp and stimulates actin polymerization in an N-WASp, Arp2/3-dependent, but Cdc42 independent manner [15]. Cdc42 and PIP_2-independent signaling also has been reported in *Drosophila* where rescue of a WASp null mutation can be accomplished by expressing a form of WASp missing both the PIP_2- and Cdc42-binding domains [16]. SH3-containing tyrosine kinases also bind WASp and/or N-WASp (Table I). The importance of these interactions is incompletely understood. It appears, however, that there are many layers to WASp/N-WASp regulation. How this relates to specific function(s) is still unknown.

Some aspects of WASp function are relatively clear. WASp and N-WASp affect Arp2/3 activation and thus actin polymerization, and they can be found localized to filopodia, consistent with their being regulated by Cdc42. In humans the lack of WASp results in defects in filamentous actin formation that leads, among other things, to abnormal T-cell activation, abnormal formation of microvilli and podosomes, and cell motility defects in macrophage. Not so clear is why constitutively activating WASp should result in a severe neutropenia and monocytopenia [17]. Also, GTP-Cdc42 is still able to induce filopodia in fibroblasts derived from N-WASp null mice [18]. Likewise, the loss of WASp in *Drosophila* results in errors in cell fate determination, largely as a result of defects in asymmetric cell division. Defects in other fundamental aspects of actin function are not disturbed [19]. Clearly there is much more about WASP/N-WASp function and regulation that needs to be understood.

Scar/WAVE

Scar family proteins were originally identified in a genetic suppressor screen of a G-protein-coupled receptor mutation in *Dictyostelium* (*S*uppressor of *cA*MP *r*eceptor defect; [20]). Though the original report described the existence of Scar-like proteins in a variety of other organisms, the first study of a vertebrate ortholog was by Machesky and Insall [10] followed shortly by Miki *et al*. [21]. The latter group identified the protein, based on a database search, as WAVE (*WA*Sp family, *Ve*rprolin homology protein). All three early reports provided evidence that, like WASp, Scar proteins affect actin polymerization. In *Dictyostelium, C. elegan*, and *Drosophila* single isoforms of Scar exist. In the vertebrates reported to date there are (at least) three isoforms, Scar1, Scar2, and Scar3 (WAVE1, WAVE2, and WAVE3, respectively). The distribution

Table I Know Binding Interactions of WASp, N-WASp, and Scar/WAVE Proteins

WASp/Scar member	Binding partner	Binding region	Effect on WASp/Scar	References
WASp	Cdc42	GBD domain	"Activate"	2, 5
	PIP_2	Basic domain	"Activate"	2, 5
	WIP	WH1 domain	"Retards"	11
	WIRE(WICH)	WH1	Localization	14
	Ash/Grb2	Proline-rich	?	5
	Src family kinases	Proline-rich	?	2
	Btk family kinases	Proline-rich	?	2
	Profilin	Proline-rich	"Accelerates"	2, 5
	p21-Arc	Acidic domain	"Facilitates"	10
N-WASp	Cdc42	GBD domain	"Activate"	2, 5
	PIP_2	Basic domain	"Activate"	2, 5
	Calmodulin	IQ domain	?	5
	Ash/Grb2	Proline-rich	?	5
	WISH	Proline-rich	Activate	15
	Nck	Proline-rich	Stimulates	2, 5
	Profilin	Proline-rich	"Accelerates"	2, 5
	PSTPIP	Proline-rich	?	2
	Syndapin	Proline-rich	?	2
	p21-Arc	Acidic domain	"Facilitates"	10
	WIP/CR16/WICH	WH1	"Retards/?"	11–14
Scar/WAVE	PKA	Partial WH2	?	26
(interactions may not	Abl kinase	Proline-rich	?	26
occur with all isoforms)	NCKAP1	?	"Inhibits"	24
	PIR121	?	"Inhibits"	24
	HSPC300	?	?	24
	IRSp53	Proline-rich	"Activates"	23
	WRP	Proline-rich	"Terminates"?	25
	Profilin	Proline-rich	"Accelerates"	21
	p21-Arc	Acidic domain	"Facilitates"	10

Binding protein and domain on WASp family member are indicated. Domains are as shown in Fig. 1. Effects are meant as a broad description of the consequence based on either *in vitro* or *in vivo* interaction.

of each is somewhat different, though none seems as restricted as WASp. Although all three isoforms share the same basic domain structure there is evidence that their cellular roles and regulation may be somewhat different (see below). Mutations in Scar genes in *Dictyostelium* and *Drosophila* have established the importance of Scar in regulating actin assembly. In *Dictyostelium*, basic cell motility is abnormal in Scar mutants, and actin assembly in response to chemoattractant stimulation is severely impaired (Steiner *et al.* submitted). In *Drosophila*, Scar mutants are impaired in several aspects of development and oogenesis owing to defects in filamentous actin assembly. Scar, rather than WASp, appears to be the mediator of most Arp2/3-dependent events in this organism [22].

The presence of Scar proteins at the leading edge of cells suggests that they might be regulated by rac-like small GTPases, and Miki *et al.* showed that transvection of a nonfunctional Scar into cells blocked the activity of an activated rac1 but not an activated Cdc42 [21]. There is no obvious rac-binding region in Scar, and there is no evidence that Scar proteins directly bind rac proteins. Evidence does exist for at least two indirect mechanisms by which rac proteins regulate Scar activity. Using a yeast two-hybrid screen Miki *et al.* found that the rac-binding protein IRSp53 binds directly to the proline-rich region of hScar/WAVE proteins [23]. They further found that the interaction of IRSp53 and Scar2 was necessary for the formation of membrane ruffles induced by ectopic expression of activated rac1 in COS7 cells. It is interesting that interaction with IRSp53 seems to be largely (though perhaps not exclusively) restricted to the Scar2/WAVE2 isoform, and IRSp53 can associate with Scar2 in the absence of rac. In another recent study, Eden *et al.* reported the existence of a Scar1-containing complex that includes the rac1-binding proteins NCKAP1 and PIR121, as well as

Scar and the 8 KDa protein HSPC300 [24]. When a purified form of this complex is added to Arp2/3 complex and actin monomers *in vitro*, little actin polymerization occurs unless GTPγS-rac1 is added to the reaction. In the same assay purified Scar alone activates Arp2/3 complex to the maximal extent. The data suggest that in the cell Scar1 is retained in an inactive complex until GTP-bound rac1 binds either, or both, NCKAP1 and PIR121. The complex then separates into two components, one containing NCKAP1 and PIR121 and the other containing Scar and HSPC300. In this form Scar is able to activate Arp2/3 complex and facilitate formation of new actin polymers [24]. It should be noted that Nck can substitute for rac1 in this experiment. Whether this same inhibitory mechanism is common to all the isoforms of Scar is unknown, but it appears to be a possibility [24]. The relationship between the complexes containing IRSp53 and the one containing NCKAP1/PIR121/HSPC300 is not yet clear. It may be that different isoforms of Scar are retained in different complexes or that Scar proteins can each be sequestered in multiple ways. Further work is necessary to clarify this issue. What is true is that activation of WASp/N-WASp and Scar by small GTPases is performed by different mechanisms. Scar proteins are indirectly regulated by rac-like GTPases and can involve disassembly of a multiprotein complex that inhibits Scar function. WASp is autoinhibited and it is the direct binding of Cdc42 (and or other proteins) that can relieve the inhibition. The difference in regulation may provide a basis for the difference in localization seen for Scar and WASp proteins.

Another mechanism by which Scar function may be regulated involves a novel rac-GAP, WRP [25]. This GTPase-activating protein was identified as a binding partner of Scar through immunoprecipitation of rat brain extracts. The interaction is direct and involves the SH3 domain of WRP and the proline-rich region of WAVE-1. The direct interaction between a racGAP and Scar/WAVE proteins may provide at least part of the mechanism for terminating Scar-mediated signaling. It is interesting that in the same report, a number of other signaling/cytoskeleton proteins were reported to immunoprecipitate with WAVE-1. These included the Abl kinase interacting proteins, abi-1 and abi-2, α-tubulin, and SNAP-25 interacting protein. The *in vivo* significance of these interactions awaits further study.

Another level at which Scar/WAVE proteins may be regulated involves cAMP-dependent protein kinase (PKA). Scar1 binds directly to the regulatory subunit (RII) of PKA, and the binding site overlaps the region of monomeric actin binding [26] (Table I). Actin and PKA compete for this binding site, and the interaction is unique to Scar1 and does not occur with Scar2 or Scar3. Scar1 may, therefore, act as an A-kinase anchoring protein (AKAP) and be involved in regulating PKA activity at the sites of new actin assembly. The same study found that the tyrosine kinase abl also bound Scar. To date, there is no report of tyrosine phosphorylation of Scar (though phosphorylation downstream of MEK has been found; [27]), so the significance of abl binding remains to be determined.

Whereas WASp and N-WASp are enriched throughout extending lamellipods, in the general cell periphery and in the tips of filopodia, Scar proteins are localized in a very narrow region at the edge of a cell and at the tips of pseudopodia and lamellipodia ([28,29], Steiner *et al.*, submitted). The use of Scar-GFP in *Dictyostelium* has shown that Scar does not localize to the rim of the whole pseudopod but only to subregions that presumably reflect points of active actin filament assembly. The localization is thus transient and dynamic. Much has been learned about WASp and Scar/WAVE proteins during the last several years, but much remains to be determined. Even though they share a basic structure at the C-terminal end, how similar is the mechanism of activation of Arp2/3 complex? How do the mechanisms affect the branched structure of actin filaments associated with Arp2/3 activation? Are there proteins besides PKA that can compete for binding by the core components to that region of these molecules? What is the mechanistic significance of being able to bind multiple SH3-containing proteins? Does binding of each have a subtly different effect on WASp/Scar activation? Is binding of individual partners competitive or is it used to integrate responses via WASp/Scar proteins? How does all of this relate to the different localizations of Scar/WASp proteins and their potentially different roles in the formation of lamellipodia and filopodia. Although the field has progressed very rapidly, there is still a long way to go.

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CHAPTER 178

Synaptic NMDA-Receptor Signaling Complex

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Introduction

Glutamate is the neurotransmitter released from presynaptic terminals at most excitatory synapses in the central nervous system. It binds to two major classes of postsynaptic ligand-gated ion channels called AMPA (α-amino-3-hydroxy-5-methylisoxazole 4-propionic acid)-type glutamate receptors and NMDA (*N*-methyl-D-aspartate)-type glutamate receptors (further abbreviated NMDA receptor). Each of these receptors can trigger influx of ions across the membrane to produce an excitatory postsynaptic potential, but the NMDA receptor is also linked to a complex signaling pathway that produces biochemical changes in the postsynaptic neuron. The linkage is both physical [1], through direct interactions with cytosolic signaling molecules and scaffolds, and metabolic, through influx of Ca^{2+} ion through its ion channel [2,3].

The NMDA receptor is specialized to initiate and control changes in synaptic strength, based on the firing patterns of the synapse. When its channel opens, a large portion of the current across the membrane is carried by influx of Ca^{2+} ions [4,5]. The Ca^{2+} interacts with a variety of signaling molecules present just below the postsynaptic membrane. Opening of its channel is delicately controlled by the combination of glutamate-binding and concurrent depolarization of the postsynaptic membrane. At resting membrane potentials, the pore of the channel is blocked by Mg^{2+} ions. The block is relieved in a graded way by depolarization of the membrane. Thus, the NMDA receptor can "titrate" the amount of Ca^{2+} influx through its channel depending on how much the membrane is depolarized when glutamate is bound to it [6–8]. The required membrane depolarization can come from "back-propagating" action potentials that spread into the dendrites when the neuron fires an action potential, or from activation of several nearby synapses at the same time [9]. Ca^{2+} influx is highest when the synapse is activated a few milliseconds after the neuron has already fired an action potential that is spreading back into the dendrites [10]. Because of this "coincidence detection" property [11], the NMDA receptor can trigger synaptic changes when the presynaptic neuron and postsynaptic neuron are activated concurrently, fulfilling the prediction of Donald Hebb for a synaptic learning mechanism [12].

Here, I will discuss the structure and function of the signaling complex assembled in the cytosol around the NMDA receptor.

Structure of the NMDA Receptor Signaling Complex

Like other ligand-gated channels, the NMDA receptor is assembled from four (or five) distinct subunits that fall into structural and functional classes. However, it differs significantly from other ligand-gated channels because one major class of subunits, the NR2A-D subunits, have long (~300 residues) carboxyl terminal tails that extend into the cytosol and associate with a variety of proteins. The signaling complex that assembles around this tail does not appear to be a rigid structure with a fixed stoichiometry. Instead, it is believed to be assembled stochastically by associations with a set of scaffold proteins and, in some instances, by direct association of the tails of the NMDA receptor with signaling molecules. The meshwork of proteins that results is often referred to as the postsynaptic density, a structure that can be observed at synapses in the electron microscope [13].

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Scaffold Proteins

At least three classes of scaffold molecules participate in organizing the interactions of signaling molecules associated with NMDA receptors in the postsynaptic density. A fifth class, the GRIPS/ABPs, associate primarily with AMPA receptors.

PSD-95

PSD-95 is the only known scaffold protein that associates directly with the NMDA receptor in the postsynaptic density. It was discovered as a prominent component of the "postsynaptic density fraction" recovered after detergent extraction of synaptosomes [14]. PSD-95 comprises three amino terminal "PDZ-domains" named after three proteins in which they were first recognized (PSD-95, Discs-large, and ZO-1), an SH3 domain, and a carboxyl terminal guanylate kinase domain. The first two PDZ domains interact directly with a terminal T/SXV motif at the carboxyl terminus of the NR2 class of NMDA receptor subunits [15,16]. The SH3 domain and guanylate kinase domains are fused into a structure that also appears to mediate a variety of protein interactions [17,18].

Immunocytochemical studies show that PSD-95 is highly concentrated in postsynaptic densities at glutamatergic synapses where it strongly co-localizes with the NMDA receptor [16,19]. Nearly all synapses that contain NMDA receptors appear to also contain PSD-95, indicating that it is an ubiquitous scaffold and associates with a variety of NMDA-receptor complexes. Several cytosolic signaling molecules have been shown to bind to PSD-95, including neuronal nitric oxide (NO) synthase; the protein kinase scaffold AKAP (next section); synGAP, a synaptic Ras GTPase activating protein; and GKAP, a protein that appears to link PSD-95 to an additional scaffold protein, shank (see below). At least one family of transmembrane proteins, the neuroligins, also binds to PSD-95.

Nitric Oxide Synthase. The neuronal form of NO synthase (nNOS) contains a PDZ domain near its amino terminus. Bredt and co-workers have shown that this PDZ domain associates directly with the second PDZ domain of PSD-95 by an atypical PDZ/PDZ interaction [20,21]. Thus, PSD-95 can link nNOS to the NMDA receptor. Neuronal NOS is activated by Ca^{2+}/calmodulin, and in cerebellar synapses nNOS is preferentially activated by Ca^{2+} flowing through NMDA receptors [22]. It seems likely that association of nNOS with PSD-95 plays an important role in controlling the specificity of its activation in cerebellar and other synapses.

SynGAP. SynGAP was discovered both as a prominent component of the PSD fraction [23] and in a two-hybrid screen for proteins that interact with SAP-102, a homologue of PSD-95 [24]. Its GTPase-activating (GAP) domain is highly homologous to that of the canonical p120 RasGAP, and the protein has RasGAP activity. Thus, it is assumed that its principal function is to inactivate Ras that has been activated by GTP exchange factors (GEFs) or by the action of protein tyrosine kinases such as Trks or Ephrin receptors. Although it is not a transmembrane protein, SynGAP nevertheless interacts strongly with the membrane via a combined PH/C2 domain near its amino terminus. It interacts with PSD-95 through a T/SXV motif at its carboxyl terminus. Immunocytochemical studies have shown that SynGAP is almost as highly concentrated at synaptic sites as PSD-95 itself [23,25], but it is also located on small vesicles throughout the cytosol and dendrite. Its precise functions are still mysterious. However, there are some clues. SynGAP is a prominent target for phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in the postsynaptic density (see below) [23]. Furthermore, deletion of synGAP is lethal to mice a few days after birth, producing abnormalities in development of certain brain areas and altering the number and size of synaptic sites in cultured hippocampal neurons (H.-J. Chen, L. Vazquez, Knuesel, and M. B. Kennedy, unpublished).

C. Neuroligins. Neuroligins are a large family of alternatively spliced transmembrane molecules that are located primarily in postsynaptic membranes, where they are believed to interact with the presynaptic neurexin proteins to mediate heterophilic adhesion [26–28]. Neuroligins were found to bind in a two-hybrid screen to PSD-95 through a terminal T/SXV motif [29]. Recent evidence suggests an important role for neuroligins in synaptic differentiation [30,31]. However, it is not yet known how their interaction with PSD-95 contributes to their function.

GKAP. GKAP (Guanylate kinase associated protein), a 70 kD protein with no identified functional domains, was isolated from a two-hybrid screen for proteins that bind to PSD-95 [32]. GKAP interacts specifically with the guanylate kinase homology domain of the PSD-95 family of proteins. GKAP was soon found to interact directly with an additional scaffold molecule termed Shank (or ProSAP; see below) [33,34]. Thus, one function of GKAP appears to be the formation of a link between the two scaffold proteins PSD-95 (and its family members) and Shank.

AKAPs (A-Kinase Interacting Proteins)

The AKAPs are a family of scaffold proteins that bind to the regulatory subunit of the cAMP-dependent protein kinase (PKA) and direct the kinase holoenzyme to particular subcellular compartments [35]. Many of the AKAPs also bind other enzymes in the cAMP pathway, forming discretely localized signaling complexes. AKAP79/150 provides a scaffold for PKA, protein kinase C, and calcineurin, a calcium-dependent protein phosphatase, positioning them adjacent to one another. AKAP79/150 binds to the SH3 and GK domains of both PSD-95 and SAP-97 (a relative of PSD-95 that associates specifically with the AMPA-receptor rather than with the NMDA receptor) [36]. It is localized together with glutamate receptors in most, but not all, excitatory synapses in hippocampal neurons in culture, and evidence suggests that it helps orchestrate phosphorylation of the AMPA receptor

by PKA [36]. Calcineurin, a Ca^{2+}-dependent phosphatase that is localized to the PSD by its association with AKAP79/150, is a likely target of Ca^{2+} flowing through the NMDA receptor and may play a crucial role in control of LTP and LTD (see below).

SHANKS

The shank family was discovered in a two-hybrid screen for proteins that interact with GKAP (mentioned above) [33,34,37]. They are a particularly interesting family because they interact with other scaffold proteins and appear to be "scaffolds of scaffolds" linking the NMDA receptor complex, metabotropic glutamate receptor complexes [38], and perhaps also the AMPA receptor complex [37]. The shank proteins vary in size from ~240 kD to ~120 kD. The largest shank contains multiple ankyrin repeats, an SH3 domain, a PDZ domain, a long proline-rich region that occupies more than half the protein, and a sterile alpha motif (SAM) domain. Many of these domains are known to bind to specific proteins; the SH3 domain binds GRIP (AMPA receptor scaffold), the PDZ domain binds GKAP (linking to PSD-95 scaffold), and the proline rich domain binds homer (metabotropic glutamate receptor scaffold). A different region of the proline rich domain binds cortactin, an actin-associated protein. Thus, shank may provide a link between the postsynaptic density complexes and the actin cytoskeleton of the spine [33]. Overexpression of shank in hippocampal neurons in culture results in early formation of enlarged spines and enhanced recruitment of homer, the IP3 receptor, PSD-95, GKAP, and the NMDA receptor into these spines [39]. Thus, shank plays an important role in shaping the size and structure of spines.

Ca^{2+}/Calmodulin-Dependent Protein Kinase II (CaMKII) Binds Directly to the NMDA Receptor

CaMKII is highly enriched in the PSD [40,41] and is an important target for Ca^{2+} flowing through activated NMDA receptors [42–44]. Mice with a deletion mutation in the α-subunit of CaMKII are epileptic, exhibit deranged long-term potentiation at their hippocampal synapses, and perform poorly in learning tests [45,46].

CaMKII is anchored in the PSD through direct binding to at least two PSD components, the NMDA receptor itself and a transmembrane PSD protein termed densin. Binding of the CaMKII holoenzyme to the tail of the NR2A or NR2B subunits is strengthened by autophosphorylation of CaMKII that occurs upon activation of the kinase [47–49]. Binding to the tail of NR2B *in vitro* stabilizes the kinase in its activated state, suggesting that it may also do so *in vivo* [50].

Association of CaMKII with the PSD is highly dynamic in hippocampal neurons. Application of glutamate to neuronal cultures causes massive movement of CaMKII holoenzymes from dendritic shafts into the PSD over a period of a few minutes [51]. The process can be reversed by removal of Ca^{2+} from the medium. Thus, both activation of CaMKII and its localization at the postsynaptic site are delicately regulated by synaptic activity. Activation of CaMKII in the PSD can lead to phosphorylation and upregulation of AMPA-type glutamate receptors [43], or addition of new AMPA receptors to the synapse through a second process that does not require direct phosphorylation of the AMPA receptor [52].

Orchestration of Responses to Ca^{2+} Entering Through the NMDA Receptor

Two features of activity-dependent plasticity at excitatory synapses in the central nervous system are central to learning mechanisms in the brain and have been the focus of many recent studies. One is the tight dependence of synaptic plasticity on "spike-timing" of pre- and postsynaptic neurons [53]. The second is "metaplasticity" or the adjustment of the sensitivity and sign (LTP or LTD) of synaptic plasticity controlled by patterns of prior activity [54]. These mechanisms depend on the amplitude, time course, and location of origin of Ca^{2+} influx. Thus, the spatial arrangement of calcium-dependent signaling enzymes such as CaMKII, calcineurin, and nitric oxide synthase are likely to be critical determinants of both. Our understanding of the arrangement of signaling molecules at the postsynaptic site will set the stage for a precise quantitative understanding of these mechanisms of learning.

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CHAPTER 179

Toll Family Receptors

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Introduction

In recent years, Toll receptors have emerged as an important family of molecules activating the innate immune response. Mammalian Toll receptors detect pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) from Gram-negative bacteria or peptidoglycan (PGN) from Gram-positive bacteria, and activate signaling cascades that induce the transcription factors NF-κB and AP1.

Structure Function of Toll Receptors

Discovery of Toll and Toll-like Receptors

The *Toll* gene was first identified in the early 1980s by Nusslein-Volhard and Anderson during a mutagenesis screen to identify the genes controlling the establishment of the dorso-ventral (DV) axis of the *Drosophila* embryo, and was found to encode a new kind of type I transmembrane receptor [1]. The eleven remaining genes identified in this screen encode factors acting upstream and downstream of Toll in the signaling pathway. Activation of Toll on the ventral side of the embryo results in a ventral to dorsal gradient of nuclear translocation of the transcription factor Dorsal, thus establishing embryonic polarity. It was also through studies in *Drosophila* that Toll was assigned an immune function in the control of the inducible expression of antimicrobial peptides. The transcriptional activation of the genes encoding these peptides requires transcriptional activators of the Rel family, to which NF-κB belongs. At the time, Dorsal was the only identified member of this family in *Drosophila*. This prompted analysis of the known mutant strains of the Toll pathway for immunodeficiency phenotypes, which led to the demonstration by Hoffmann and collaborators that the Toll pathway controls the response to fungal and Gram-positive bacterial infections [2,3]. These results were rapidly followed by the first description by Medzhitov and coworkers of a mammalian Toll homologue (now known as TLR4) capable of activating NF-κB and the synthesis of cytokines and co-stimulatory molecules [4]. Shortly after, Beutler and colleagues showed that mice from the LPS-hyporesponsive strains C57BL/10ScCr and C3H/HeJ carry mutations in their *tlr4* gene, indicating that Toll-like receptors (TLRs) play an important role in the control of infection in mammals [5]. Since these initial discoveries, a family of 10 TLRs has been described in mammals, the properties of which are described below.

Structure of Toll Family Receptors

The cytoplasmic domain of Toll bears striking similarities to that of the Interleukin-1 type I receptor (IL-1R), and is referred to as the TIR (Toll/IL-1R) homology domain. TIR domains are also present in intracellular signaling molecules such as MyD88 or TIRAP/MAL (see below). Plants also express TIR domain–containing factors [6]. The structures of the TIR domains of human TLR1 and TLR2 have been solved and shown to be composed of a central five-stranded parallel β-sheet surrounded by five α-helices [7], (Fig. 1).

The extracellular domain of Toll family receptors does not contain Ig domains, like the IL-1R, but comprises several leucine-rich repeats flanked by characteristic cysteine-rich motifs. This feature is shared by a number of membrane receptors such as the pattern recognition receptor CD14, the adhesion molecule GpIbα, or the members of the Trk family of neurotrophins receptors. Leucine-rich repeats are generally recognized as a protein-protein interaction domain [8].

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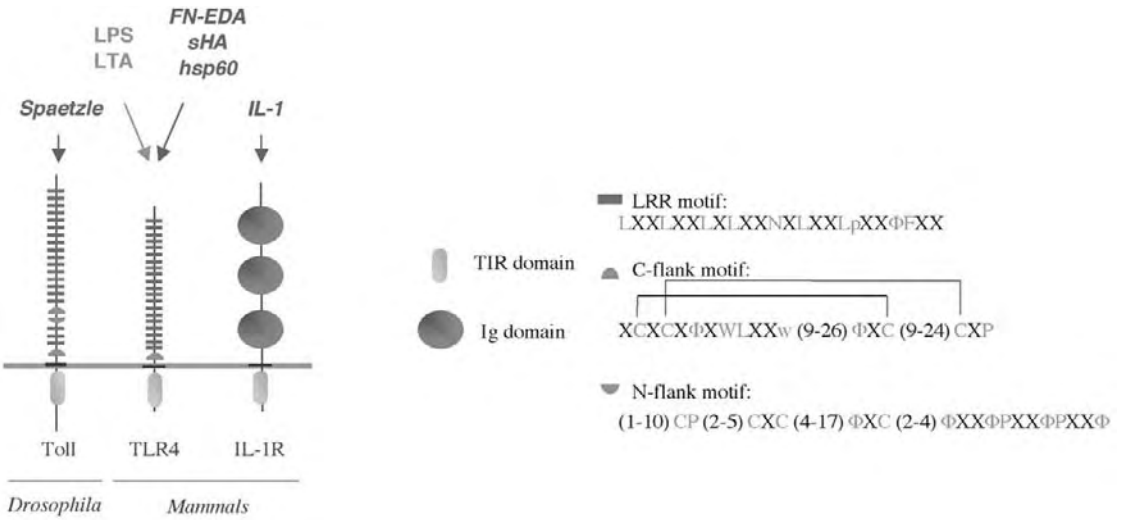


Figure 1 TIR domain receptors in *Drosophila* and mammals. (A) Domain architecture of members of the three groups of TIR domain membrane receptors. TIR domains are represented in yellow, LRR motifs as green rectangles, and flanking cystein-rich regions as orange half-circles. Microbe-derived ligands are in blue, and endogenous ligands in mauve. FN-EDA, fibronectine extra domain A; sHA, soluble Hyaluronic Acid. (B) Amino-acid sequence of the motifs found in the ectodomains of Toll family receptors. Φ, hydrophobic residue; X, any amino-acid [8].

Despite their similarity of structure and function, members of the family of Toll receptors can be subdivided in two different classes, based on phylogenetic analysis of their TIR domain [9]. All mammalian TLRs cluster to one of these subfamilies, together with a single *Drosophila* receptor, Toll-9. The eight other *Drosophila* Toll family members cluster to the second subfamily, together with the *Caenorhabditis elegans Tol-1* gene product. Close examination of the ectodomain of these molecules also reveals differences, the most evident being the presence of a single C-flank cysteine rich motif in TLRs and *Drosophila* Toll-9, as opposed to other Tolls, which most often contain additional C-flank motifs and always share at least one N-flank cysteine rich motif. Thus it appears that most *Drosophila* Tolls have evolved independently from mammalian TLRs, possibly to fulfill different functions. In agreement with this hypothesis, TLRs do not appear to be required for murine development, in contrast to Toll in *Drosophila* [10].

Activation of Toll and Toll-like Receptors

In *Drosophila* embryos, a cascade of proteases is activated on the ventral side of the embryo and promotes proteolytic maturation of the cysteine-knot growth factor Spaetzle, which is structurally related to neurotrophins. The cleaved Spaetzle is thought to bind to the Toll receptor, prompting its dimerization and activation [1]. In adult flies, activation of Toll in response to infections also requires a processed form of Spaetzle, although genetic experiments indicate that the proteases of the embryonic cascade upstream of Spaetzle are largely dispensable for the immune response [2]. Recognition of Gram-positive bacteria is mediated by a member of the PGN recognition protein (PGRP) family, PGRP-SA, which is thought to activate a protease(s) leading to Spaetzle activation [11].

No Spaetzle homologues have been described so far in mammals. Rather, TLRs appear to be directly activated by PAMPs. Analysis of macrophages derived from TLR knockout (KO) mice has revealed that (1) TLR4 is required for the response to LPS and lipoteichoic acid (LTA) derived from Gram-positive bacteria; (2) TLR2 is necessary for activation by several ligands, including PGN, bacterial lipopeptide (BLP), and the mycobacterial lipopeptide MALP2; (3) TLR3-deficient cells do not respond to dsRNA from viruses; (4) TLR5 recognizes flagellin, the principal constituent of bacterial flagella; (5) TLR6 deficient cells do not respond to MALP2; (6) TLR9 is required for cell activation by bacterial DNA containing unmethylated CpG motifs (reviewed in [10,12–14]).

Despite the genetic evidence, biochemical data for a direct interaction between most TLRs and their ligands are still lacking. There is good pharmacological evidence for a direct interaction between LPS and TLR4, or CpG and TLR9. In addition, biochemical experiments using radiolabeled LPS demonstrated that it can be specifically cross-linked to TLR4, thereby indicating that the two molecules directly interact [15].

Some TLRs associate with coreceptors to interact with PAMPs. For example, in the case of LPS, CD14 plays an essential role in the receptor complex. This protein is also required for maximal cell activation by PGN, a TLR2 agonist. In addition, plasma membrane expression of TLR4 requires the coexpression of MD2, a secreted accessory molecule that also seems to interact with LPS (Fig. 2a). In some cases, recognition of PAMPs involves heterodimerization of TLRs. For instance, activation of cells by MALP2 requires both TLR2 and TLR6. TLR6 coimmunoprecipitates with TLR2 in cell lines, and heterodimerization of the TIR domains of TLR2 and TLR6 elicits a cellular response. TLR2 is believed

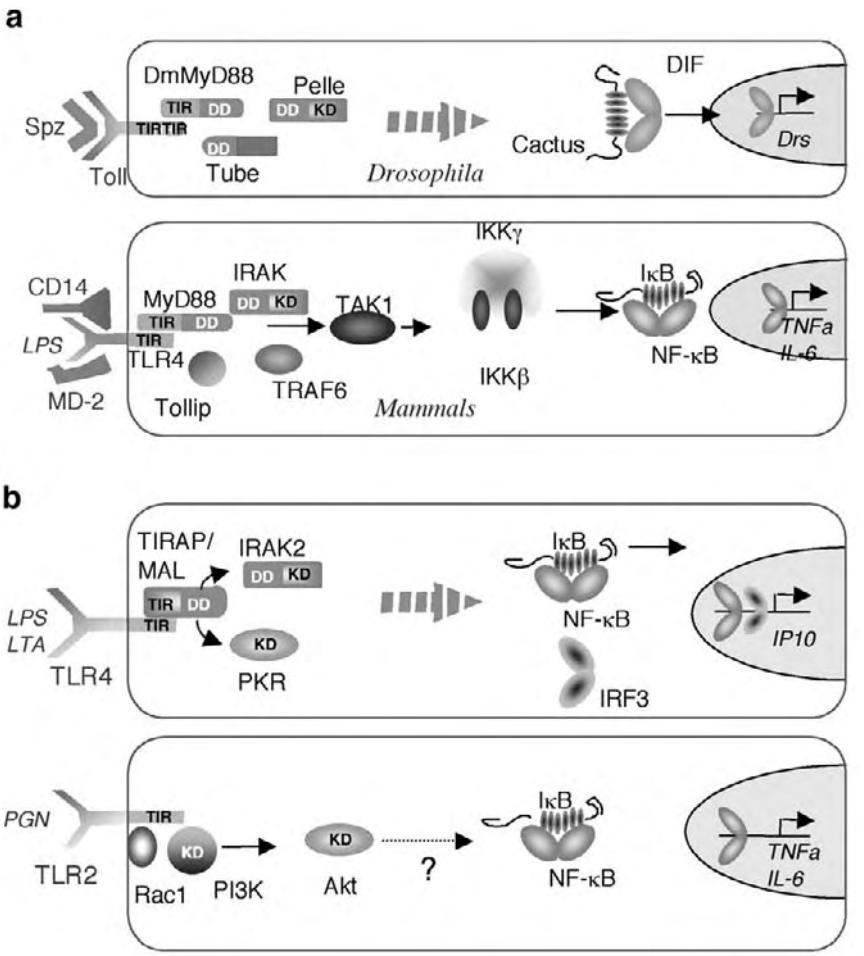


Figure 2 Signaling pathways activated by Toll family receptors. (a) Classical pathway activated by Toll family receptors in *Drosophila* and mammals. IkB and its homologue Cactus are inhibitory factors that retain the Rel proteins DIF and NF-kB in the cytoplasm. Drs, Drosomycin. (b) Examples of subunit-specific alternative signaling pathways downstream of TLR4 and TLR2. IRF3, interferon response factor 3; IP10, chemokine gene induced by TLR4 in a MyD88-independant fashion; DD, death-domain; KD, kinase domain.

to associate with another TLR to recognize BLP. It so appears that cooperation between TLRs is a means of broadening the recognition repertoire of these receptors [10,12,13].

One critical question about regulation of the innate immune response is the distinction by immune cells between harmless commensal microorganisms and infectious microbes, which often share common PAMPs. Cellular distribution of TLRs may contribute to this discrimination. For example, TLR5 is asymetrically distributed in gut epithelial cells: it is absent from the apical side of the cells, which are exposed to the commensal bacterial flora of the gut lumen, and present on the basolateral side of the cells, to which noninvading bacteria do not have access [16]. In addition, it is becoming clear that some TLRs can be activated by host-derived molecules. These include hsp60, oligosaccharides from hyaluronic acid, and a fibronectin fragment [10,12,13]. A common feature of these molecules is that they are produced during stress responses in general and inflammation in particular. One intriguing possibility is that these endogenous products generated in response to infection synergize with PAMPs to activate TLRs and stimulate an efficient innate immune response.

Signaling by Toll Family Receptors

Activation of Toll family receptors results in the transcriptional induction of a number of genes involved in host defense such as antimicrobial molecules or cytokines. A common feature of these genes is the presence of binding sites for the transcription factor NF-κB and AP-1 in their promoters. As described below, a pathway leading from TIR domain receptors to the nucleus has been deciphered. The various ligands that activate TLRs induce overlapping but distinct sets of genes during infection, thus revealing the existence of receptor-specific alternative signaling pathways (Fig. 2b).

The Classical Toll/IL-1R Signaling Pathway

TIR domain receptors are associated with an intracytoplasmic plurimolecular platform in which the MyD88 factor

plays a central role. MyD88 is composed of an amino-terminal death domain (DD) and a carboxy-terminal TIR domain. It interacts in a ligand-dependent manner with the receptors through homophilic TIR-TIR domain interactions. The DD of MyD88 interacts with the DD of the serine-threonine kinase IRAK (IL-1R associated kinase) [10,12,13]. Another adaptor protein, Tollip, is also recruited transiently to the receptor complex upon activation [17]. Tollip does not contain a TIR domain, and its function may be to bring IRAK to the receptor complex. Interaction of IRAK with MyD88 triggers autophosphorylation of the kinase. This progressive phosphorylation affects interaction with the receptor complex and allows IRAK to interact with TRAF6.

TRAF6 belongs to a family of signal tranducers originally identified for their role downstream of receptors of the TNFα family. TRAF6 associates with a dimeric ubiquitin-conjugating enzyme complex composed of Ubc13 and Uev1A. This complex mediates activation of the TAK-1 kinase through a nonclassical ubiquitination mechanism, which does not involve the proteasome. TAK1 then phosphorylates the IκB kinase (IKK) and MKK6 kinases, which in turn activate NF-κB and AP-1 [18].

Significant differences exist in the Toll pathway in flies. The receptor platform contains an additional DD component named Tube, which interacts with the IRAK homologue Pelle. Three TRAF factors are encoded by the *Drosophila* genome, but their role in the Toll pathway is not clear. Curiously, the *Drosophila* homologues of TAK1, Uev1a, Ubc13, IKKβ and γ are not required downstream of Toll during the antifungal response in adults or during embryonic development [19].

Subunit-Specific Alternative Pathways

Analysis of MyD88 KO mice confirmed the importance of this central factor in the signaling pathway downstream of the cytokines IL-1 and IL-18 and several PAMPs including LPS. However, the response to LPS differs between TLR4 and MyD88-deficient cells. Notably, activation of NF-κB and AP1 is only delayed in MyD88$^{-/-}$ cells whereas it is abolished in TLR4$^{-/-}$ cells. Furthermore, maturation of dendritic cells (DC) in response to LPS stimulation, though abolished in TLR4$^{-/-}$ cells, is still functional in MyD88$^{-/-}$ cells [20]. These data clearly point to the existence of a MyD88-independent pathway of cell activation, which also seems to operate downstream of TLR3. Nevertheless, this alternative signaling pathway is not activated by all TLRs. For example, in response to the TLR9 and TLR2 agonists CpG and MALP2, respectively, induction of NF-κB and DC maturation is completely abolished in both TLR(9 or 2) and MyD88 KO cells [10,12].

A new cytosolic factor has recently been identified and is likely to play a role in the MyD88-independent pathway of LPS signal transduction. This factor (MAL for MyD88 adaptor like protein, or TIRAP for TIR associated protein) contains a TIR domain but no DD [21,22]. TIRAP/MAL associates with TLR4 through its own TIR domain but not with TLR9. In addition, a dominant-negative version of the molecule has been shown to block activation of NF-κB by TLR4 but not by TLR9, IL-1R, or IL-18R. Treatment of DCs with a synthetic TIRAP-blocking peptide inhibits their LPS-induced maturation in both wild-type and MyD88$^{-/-}$ backgrounds [22]. These data strongly suggest that TIRAP/MAL controls the MyD88-independent pathway activated by TLR4. By contrast to MyD88, this factor does not interact with IRAK but with the related kinase IRAK2 through which it activates TRAF6. TIRAP/MAL can also associate with the dsRNA binding kinase PKR.

Another subunit-specific pathway seems to operate downstream of TLR2. This pathway is initiated by a ligand-dependent tyrosine phosphorylation of TLR2, which leads to recruitment of the p85 regulatory subunit of phosphatidylinositol 3 kinase (PI3K) and the small GTPase Rac-1, and to activation of the protein kinase Akt (protein kinase B) [23]. Although the effectors downstream of Akt remain to be identified, it is clear that this pathway regulates phosphorylation of NF-κB, rather than targeting its inhibitor IκB. Because this phosphorylation is required for the transactivation properties of NF-κB, it is possible that other TLRs will activate this pathway. However, the PI3-kinase binding motif, which contains the phosphorylated tyrosine residue is only conserved in TLR1, 2, and 6, thus suggesting specificity of this Rac-1 dependent pathway.

The RIP2 serine-threonine kinase was also recently shown to function downstream of some TLRs. Induction of cytokines in RIP2$^{-/-}$ macrophages is reduced upon stimulation with LPS, LTA, PGN, and dsRNA but not CpG. This result indicates that RIP2 is involved in TLR2, TLR3, TLR4 but not TLR9 signaling [24,25].

It is therefore becoming obvious that different combinations of transducing factors can dock to the receptor platform, depending on its molecular composition. This finding probably explains why some TLRs, such as TLR4, TLR3, and TLR9, can signal as homodimers, whereas others, such as TLR2 or TLR6, cannot. These distinct transduction factors initiate discrete signaling events, the combination of which may explain the panel of different responses elicited by the various TLR agonists. The understanding of these regulatory events holds promise for improved vaccination strategies and better control of infectious diseases.

Note Added in Proof

Recent work, including characterization of TIRAP/MAL deficient mice indicate that the MyD88-independent pathway does not involve this molecule, but may rely on a third TIR domain adaptor, TRIF/TICAM-1 (reviewed in Imler and Hoffmann (2003). *Nature Immunol.* **4**,105–106).

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CHAPTER 180

Signaling and the Immunological Synapse

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Introduction

Although considerable progress has been achieved over the last decade in the field of T-cell activation, many interesting questions remain. We now understand the basic biochemical pathways that underlie the basis of T-cell receptor (TCR) signaling, but we don't know how these signals are used to regulate T-cell function. For example, how does the T cell recognize antigens with such high sensitivity and specificity? How are these signals modulated to mediate such diverse processes as T-cell development, T-cell survival, and T-cell effector functions? Understanding these processes will require new approaches that incorporate principles of biochemistry, cell biology, immunology, and systems biology. Recent work using state of the art methods of digital imaging have revealed a process of membrane protein rearrangement known as immunological synapse formation. Immunological synapse formation is intimately associated with the T-cell activation process. Here, I review recent progress in this field (also recently reviewed in [1–4]). Because the study of immunological synapse addresses issues pertaining mainly to the field of cellular immunology, and to put a discussion of immune synapses into a broader context, a brief summary of T cell biology is given first.

Brief Introduction to T Cell Biology

One of the central challenges today is to understand how the TCR can specifically and sensitively sense foreign antigens. The TCR binds to short peptide fragments that are themselves bound to membrane proteins known as major histocompatibility antigens (MHC). These short peptide fragments are generated by antigen-presenting cells via the proteolytic degradation of all proteins (intracellular and extracellular) in the cellular milieu. Thus, at any given time, a large variety of peptides compete for binding to a limited number of MHC molecules expressed on the surface of cells. The ability of the T cell to discern a rare antigenic peptide from an ocean of other, nonrelevant peptides attests to the incredible accuracy of TCR sensitivity. Recent studies verify this sensitivity by demonstrating that for CD8+ T cells, even a single peptide-MHC molecule is sufficient to activate a T cell [5]. The specificity of TCR recognition is also impressive. Conservative changes in the peptide sequence can change a peptide from being a strong agonist to one that is ignored by the T cell [6].

The trafficking of naïve T cells and professional antigen-presenting cells (APC) is designed so that both cells can interact with each other in secondary lymphoid organs like the lymph node or spleen. Although professional APCs include macrophages and B cells, the most important professional APC is the dendritic cell. Professional APCs function to not only carry foreign proteins from the periphery to the secondary lymphoid organs, but each also have specialized roles in the activation of T cells. The expression of membrane proteins like B7 on professional APCs functions to control the activation of naïve T cells via interaction with co-stimulator molecules such as CD28 expressed on the surface of naïve T cells.

Activated naïve T cells proliferate and differentiate into effector T cells. In the CD4+ T cell lineage, the most important effector cells are known as Th1 and Th2 cells and can be

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distinguished by the cytokines that they secrete. Th1 cells secrete IL-2 and γ-interferon and mediate delayed-type hypersensitivity (DTH) responses by activating macrophages. Th2 cells secrete IL-4 and function to stimulate humoral immune responses by controlling B-cell activation and immunoglobulin switching. Similar pathways operate in the differentiation of cells in the CD8 lineage with the development of Tc1 and Tc2 cells. The primary function of CD8 cells is to kill infected target cells. Finally, T cells differentiate into memory T cells, long-lived cells that enhance secondary responses to pathogens.

Initiation of TCR Signaling

How T-cell receptors are initiated by binding to antigenic ligands is still a controversial area [7]. Although receptor clustering is the trigger that initiates signaling in most signaling systems, the small numbers of antigenic ligands present on the surface of the APC *in vivo* and the ability of very small numbers of peptide/MHC molecules to activate a T cell makes receptor clustering an unlikely mechanism. A recent study suggests that ligand binding results in a conformational change that promotes the assembly of signaling molecules with the TCR [8], but such changes have not been seen by structural studies [9]. Another model proposes that the association of accessory molecules with the TCR may be the trigger for T cell activation [10]. Higher-affinity binding would be discerned from low-affinity binding because longer-lived complexes would allow accessory molecules to be recruited to the TCR. The ability of T cells, however, to become activated in the absence of accessory and co-stimulatory molecules suggests that this mechanism is not required for signal initiation [11]. Finally, it has been proposed that T-cell receptors may form microclusters [12,13]. This is supported by structural studies demonstrating that MHC molecules, as well as accessory molecules such as CD4 and CD28, can form dimers [14], but these studies are controversial [15]. One problem with this model is that given the low abundance of antigenic peptides on the surface of the APC, the chances that both halves of an MHC dimer would contain the same peptide are extremely unlikely. But it has also been shown that non-agonist peptides may also play a role in triggering T-cell activation [16]. Thus, it is possible that TCR dimerization could be achieved by combinations of antigenic and non-antigenic ligands.

Definition of the Immunological Synapse

Kupfer and coworkers were the first to demonstrate a specific reorganization of proteins in the contact area between the T cell and the APC [17]. Using digital reconstruction of confocal microscopic images, they noted that T-cell membrane proteins segregated into a bull's-eye pattern during T-cell activation. The center of the bull's-eye, dubbed the C-SMAC, is notable for the clustering of T-cell receptor, PKC-theta, and CD28. The outer ring consists of a zone containing the adhesion molecule, lymphocyte function-associated antigen (LFA-1). In a third zone, proteins excluded from the C-SMAC and P-SMAC, such as CD43 and CD45, are found. Recent work suggests that at least for CD43, there is an active process that targets it outside of the immunological synapse [18–20]. This pattern of a peripheral ring of LFA-1 surrounding a central zone of T-cell receptors is referred to as a classical or mature synapse.

Excitement about this concept and its rapid adoption by those studying T-cell activation is based on the possibility that the morphology of synapses may give important clues about how interactions with APCs lead to distinct T-cell responses (Fig. 1). Since interactions with other cells are fundamental to the T-cell activation process, one possibility is that different synapse morphologies may lead to different outcomes. Thus, recent publications have analyzed synapses during T-cell development [21,22], the activation of Th1 or Th2 cells [23], the synapses of CD8 [24,25] and NK cells [26–30], and those formed using different types of APCs [12,31].

Initial experiments were performed using CD4+ T cell clones and B-cell tumor lines as APCs [17]. These experiments showed that formation of a classical synapse occurs quickly, usually within minutes. Many other groups obtained similar results with T-cell hybridomas and T-cell tumor lines. Using fluorochrome-labeled MHC and adhesion molecules embedded in a planar lipid bilayer, Grakoui *et al*. were able to study the kinetics of synapse formation. Their studies suggested a distinct pattern that preceded mature synapse formation [32]. At early time points, TCRs were first recruited to the periphery of the contact, surrounding a central zone of LFA-1/ICAM complexes. This pattern, the opposite of that seen in the classical synapse has been referred to as an immature synapse. Over the next twenty minutes, this pattern inverts with movement of TCRs into the center of the synapse now surrounded by LFA-1 at the periphery of the contact.

Because mature synapses form very rapidly using T cell clones and cell lines, and the immature pattern is almost never seen, there was some question about the validity of the immature synapse pattern. However, the two-step formation of the immunological synapse was recently confirmed using naïve T cell/APC conjugates [31]. In this system, naïve T cells were incubated with primary APCs (T and B cell depleted splenocytes). Synapses formed very slowly, with the immature pattern achieved between 5–15 minutes after cell contact followed by the pattern of the mature synapse between 15–30 minutes. Because naïve T cells form synapses on lipid bilayers with kinetics similar to T cell blasts [33], the APC is likely to control the rate and pattern of synapse formation. In fact Richie *et al.* showed this directly when they tested the morphologies of synapses formed using blasted T cells or thymocytes with a variety of different APCs [21]. This suggests that synapse formation is strongly influenced by the APC.

Live-cell imaging studies further suggest that interactions with dendritic cells may be distinct from the interactions with other antigen presenting cells [34]. When T cells were

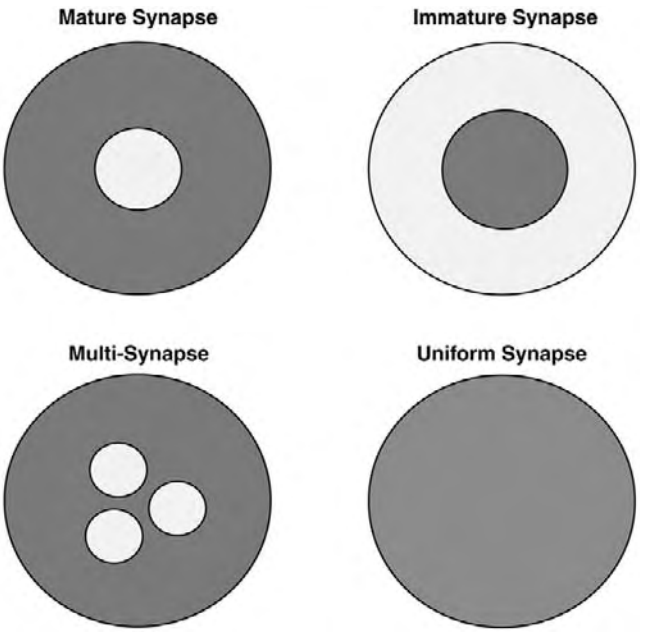


Figure 1 Basic synapse morphologies. Depicted are four basic synapse morphologies. The classical synapse has a central zone known as a C-SMAC surrounded by a peripheral ring of LFA-1 called a P-SMAC. The immature synapse is seen in some systems before the classical synapse. It is also seen in thymocytes undergoing negative selection. The multisynapse consists of multiple clusters of TCRs that are dynamic and stable for long periods. The uniform synapse represents contact areas where there is no visible segregation of membrane protein components. The significance of such synapses is not clear.

incubated with dendritic cells in a collagen matrix, T cell interactions with dendritic cells were transient (minutes) suggesting that a sustained interaction with an antigen-presenting cell might not be required for an activation response. T cells can also from synapses with dendritic cells in the absence of antigenic peptide [35]. But two recent studies imaging T cell/dendritic cell interactions in lymph nodes gave conflicting results. Stoll *et al.* injected fluorescently labeled dendritic cells and T cells into a mouse and imaged movement of T cells in a lymph node using standard one-photon confocal microscopy [36]. They found that T cells formed long-lived stable contacts for about 20 hours after which the T cells become highly motile and started to proliferate. In contrast, Miller *et al.* using two-photon fluorescent microscopy, found instead that the vast majority of antigen-specific T cells were highly motile in the lymph node [37]. T cells were described to be "swarming". Whether differences in the technique of microscopy or immunization procedure account for this difference is unclear. Clearly, this is an area that will require further investigation.

Another active area of interest has been the morphology of synapses formed with APCs displaying altered peptide ligands (APLs). APLs are modified antigenic peptides, in which single amino acids have been changed. These changes can result in a variety of different effects ranging from changing the peptide to a null, or completely non-antigenic peptide, to converting the peptide into an antagonist to having no effect at all. Early interest in the synapse focused on the morphology of synapses formed with APLs. Grakoui *et al.* found that weak agonist peptides still formed mature immunological synapses but accumulated lower levels of MHC-peptide complexes [32]. This suggests that MHC-peptide accumulation is related to the strength of the antigen. Antagonist peptides were able to accumulate MHC-peptide complexes in the junction between the T cell and the APC, however, a C-SMAC did not form and the T cells were unable to stop moving. Consequently, synapses were short lived. In a more recent study, Zal *et al*. used energy transfer techniques to analyze the interaction of the co-receptor, CD4, with the TCR after stimulation with agonist or antagonist peptides [38]. They found that CD4 and the TCR interact in the immunological synapse when stimulated with agonist. Antagonist peptides, interestingly, still recruited CD4 and TCR to the complex, but an interaction between the two never occurred. This is likely related to the shorter half-lives of antagonist peptide/MHC complexes with the TCR preventing association of CD4 with the TCR.

Immunological Synapses and T-Cell Development

During development, T cells must be "educated" to distinguish between self versus foreign antigens. It is thought that T cells learn first to recognize self-MHC in a process known as positive selection. Only T cells that are able to bind to self-MHC molecules are allowed to survive as well as expand. To rid this immature T-cell population of potentially self-reactive, autoimmune cells, cells that react strongly to self-MHC are deleted in a process known as negative selection. This results, at the end of development, in T cells that are weakly reactive against self and that are strongly reactive against foreign antigens. This property is important, for the low self-reactivity is required for the long-term survival of the T-cell population. The foreign reactivity results in the activation, proliferation, and differentiation of T cells into effector T cells. Are the processes of positive and negative selection distinct at the level of the immunological synapse?

Davis and coworkers developed an *in vitro* system to study thymocyte-positive and negative selection and were able to image contacts between thymocytes and thymic epithelial cells during negative selection [21]. They found that thymocytes undergoing negative selection form a variety of synaptic patterns that is most notable by the absence of a C-SMAC. For the most part, the synapses of negative selection are long-lived and are reminiscent of the immature synapse with peripheral localization of the TCR. This pattern was unique to synapses with thymic epithelial cell, as synapses using thymocytes with other types of antigen presenting cells had different morphologies. This suggests that the antigen-presenting cell plays a key role in synapse formation. Hailman *et al*., using a planar bilayer system, also noted a distinct synapse pattern for negative selection [39]. They found that the synapses of thymocytes undergoing negative selection were dynamic, long-lived, and notable for sustained TCR signaling.

The morphology was different from the Richie *et al*. study [21] with synapses showing multiple clusters of TCRs. But common to both studies is the lack of a single, central C-SMAC and the long-lived nature of the synapses. Neither study was able to study positive selection because of inefficient conjugate formation. However, Buosso *et al*. recently imaged cells undergoing positive selection by using two-photon microscopy and a three dimensional organ culture system [22]. They found cell-cell interactions were both stable and highly dynamic. Clearly much more work is necessary to understand whether there is a relationship among cell contacts, synapse morphology and T-cell development.

Synapses and Different Kinds of T Cells

Understanding T cell activation is further complicated by the fact that different types of T cells have different activation requirements. Naïve T cells have relatively high thresholds for T-cell activation, require secondary signals mediated by receptors classified as co-stimulators, and can only be activated by professional antigen-presenting cells in secondary lymphoid organs such as lymph node and spleen. Memory T cells, in contrast, have much lower thresholds for T-cell activation, do not require co-stimulation, and can be activated outside of secondary lymphoid organs. The activation requirements for CD4 T cells versus CD8 T cells also appear to be distinct. Although CD4 T cells appear to require sustained periods of TCR engagement, CD8 T cells can apparently make the decision to fire or not within minutes. Whereas CD4 activation requires 100–1000 peptide-MHC complexes [40], CD8 activation requires only a single peptide-MHC complex [5,41]. Important subgroups of CD4 T cells, the so-called TH1 and TH2 T cells, also appear to have distinct signaling requirements.

Although several groups have imaged synapses of both naïve and previously activated T-cell blasts, there is relatively little analysis about any possible differences. This issue is complicated by the fact that a careful analysis will also require use of a variety of types of antigen-presenting cells. This requirement is critical because *in vivo*, different types of T cells are stimulated by different types of APCs. Both naïve and blasted T cells can form immature and classical synapses, but the kinetics may be slower in naïve T cells [31]. The specific differences between the two will need to be examined closely in the future.

One area that has seen progress in recent years is the nature of CD8+ T-cell synapses. This is an interesting topic because CD8 T-cell activation occurs very quickly and can be stimulated by a single peptide-MHC complex. In addition, multiple targets can be killed within a 10 to 20 minute period. Kupfer and coworkers demonstrated that naïve CD8 T cells form classical synapses even at extremely low levels of peptide-MHC complex [24]. These synapses have a normal P-SMAC but surprisingly have no detectable enrichment of TCRs in the C-SMAC at very low levels of antigen. The C-SMAC, however, can be identified by the recruitment and concentration of PKC-theta in a zone that is central to the P-SMAC. At higher concentrations of antigen, however, visible recruitment of the TCR is seen. This suggests that recruitment and enrichment of the TCR in the C-SMAC is not required for T-cell activation, at least in CD8 cells. A beautiful high-resolution study by Griffiths and coworkers confirmed these findings and also demonstrated that the contact contains a small cleft into which secretory granules empty their contents [25]. This suggests that one important function of the synapse is to help form a target zone for the secretion of cytolytic granules. Electron microscopic studies demonstrated that there is fusion between the plasma membranes of the T cell and the target cell in the synapse. Combined with the tight adhesion of the P-SMAC, the synapse might be important in preventing the spillage of cytolytic proteins into the extracellular space.

Natural Killer Cell Synapses

Three groups have also imaged synapses formed by natural killer (NK) cells and their targets [26,27,39,30,42]. This is an interesting area because NK cells like CD8+ cells must quickly make a decision to kill or not kill a target cell. Unlike CD8+ cells, this decision is not solely dependent on antigen but is negatively regulated by recognition of normal membrane proteins. Thus, the NK cells must integrate information from both stimulatory and inhibitory receptors before they can make a decision to fire or not. First, Leibson and coworkers imaged the recruitment of lipid rafts to synapses in the presence of absence of inhibitory signaling [42]. They found that rafts were recruited when a target cell was killed but that inhibitory signaling blocked raft recruitment. The Strominger and Dupont groups have analyzed these cytolytic and noncytolytic synapses in greater detail. They found that in cytolytic synapses, NK cells formed a P-SMAC as defined as an outer ring of LFA-1 and talin and a C-SMAC as defined by the central accumulation of PKC-theta and other signaling molecules [26,27]. In noncytolytic synapses, while a contact surface is generated, no clearly defined P-SMAC and C-SMAC can be seen, nor is there recruitment of lipid rafts [29]. This is an active process because addition of an inhibitory ligand to the system results in the loss of receptor segregation in the contact area as well as lipid raft recruitment. Thus, in this system, classical synapse formation appears to be linked to not only to antigen recognition but also to the absence of inhibitory receptor signaling.

The Function of the Immunological Synapse

Early studies proposed that the synapse functioned to initiate T-cell receptor signal transduction [32]. This was based on the fact that T-cell receptors were clustered and concentrated in the C-SMAC. In addition, it was noted that lipid rafts, a rich source of signaling molecules, also clustered in the synapse [43]. Thus it seemed logical that the C-SMAC

might function as a way to crosslink and aggregate T-cell receptors. Although early studies suggested that calcium signaling might precede formation of immunological synapse, in many systems, especially those using T-cell clones and T-cell hybridomas, the formation of the immunological synapse occurred quickly, within minutes of contact formation. Thus it was difficult to say definitively whether synapse formation preceded or followed the initiation of signal transduction.

Support for the idea that the C-SMAC serves is an active area of signal transduction was the finding that lipid rafts are enriched in immunological synapses [43]. Since lipid rafts are a rich source of signaling components [44], it was logical to assume that formation of the C-SMAC accompanied by lipid raft recruitment would form a strong trigger and an amplifier of TCR signal transduction. Burack *et al.* were the first to show that lipid rafts are enriched in the C-SMAC and depleted in the P-SMAC [45]. However, the level of raft enrichment is low, about two- to three-fold over basal levels and likely to be due to displacement from the P-SMAC rather than active recruitment from other areas of the plasma membrane.

As discussed above, Lee *et al.*, using naïve T cells and primary antigen-presenting cells, were able to demonstrate that synapse formation occurs in two distinct phases [31]. Up to 15 minutes after conjugate formation, the synapses have an immature morphology; TCRs are found mainly at the periphery of the contact, surrounding a central zone of LFA-1/ICAM-1. Between 15 and 30 minutes, the zones invert, with T-cell receptors moving to the center of the contact and LFA-1/ICAM-1 complexes moving to the periphery. The slow development of the mature synapse in this system allowed a careful analysis of the kinetics of TCR signal transduction via phospho-specific antibodies. The activation of LCK and ZAP-70 occurred in the immature synapse and had largely abated by the time the mature synapse had formed. Not only does this suggest that immunological synapse formation is not involved in initiating T-cell receptor signaling, it also suggests that large-scale aggregation of T-cell receptors is not required to initiate T cell receptor signaling. This in turn suggests that TCR signaling is initiated before mature synapse formation.

Work from Krummel *et al*. examining the recruitment of Lck and CD4 to the synapse is consistent with these findings [46]. They found that CD4 and CD3 zeta, components of the TCR, were both recruited rapidly to the contact area. Their recruitment was coincident with the initiation of calcium signaling. Although CD3 zeta was recruited to the C-SMAC, CD4 surprisingly moved out of the contact area. This result is consistent with the idea that the initiation of TCR signaling does not require the C-SMAC, nor does a significant portion of signaling occur there, and suggests that the C-SMAC is involved in some other biological activity occurring between T cell/ APC conjugates.

Perhaps the immunological synapse functions to sustain T-cell receptor signaling. Several groups have now showed that sustained contact for over two hours is required before a naïve T cell is committed to proliferate [3,47]. The synapse might function, therefore, to help sustain signaling. What signals are required for two hours remains unclear, however. Studies using phospho-specific antibodies suggest that TCR signaling has largely abated by 30 minutes after contact formation [31]. This scenario does not rule out that low-level TCR signaling continues at levels below the level of detection. It is also possible that the immunological synapse may function to facilitate engagement of receptors in addition to the TCR. Important candidates include co-stimulatory molecules as well as cytokine receptors [48].

The engagement of co-stimulatory molecules such as CD28 is required for the activation of naïve T cells, whose sustained signaling may be required in the synapse. The ligand for CD28, B7, is expressed on professional antigen-presenting cells, and CD28/B7 complexes are recruited to the C-SMAC [33]. Although the exact signals transduced by CD28 are still controversial, activation almost certainly will involve more than one signaling pathway. CD28 functions by directly potentiating TCR signaling, enhancing cell survival, and increasing the metabolism of the T cells. One important signaling pathway mediated by CD28 is the PI-3 kinase pathway, and prolonged signaling by the PI-3 kinase/AKT pathways may be required for T-cell commitment [49]. It is also interesting to note that other co-stimulatory molecules, such as CTLA-4 and ICOS, are upregulated hours after the initiation of TCR signaling [50,51]. This finding emphasizes the important role for prolonged co-stimulatory signaling after initiation of TCR signaling. In addition, long-term stability of synapses may play roles in regulating the differentiation of naïve T cells into effector T cells. Cytokine released by the APCs may be more effective if the cells are attached to each other [48]. Thus, it seems logical to consider that the persistence of the immunological synapse would enhance engagement and signal transduction by these accessory molecules.

Immunological Synapses and TCR Downregulation

Because a few peptide MHC molecules are able to downregulate thousands of T-cell receptors, it has been proposed that T-cell activation occurs via the serial triggering of T-cell receptor molecules [52,53]. The immunological synapse might therefore serve to allow multiple receptors to be engaged by the same few MHC peptide molecules clustered into the synapse. Although a provocative model, the serial triggering model is still controversial. First, it has been shown that T-cell receptors can internalize their MHC peptide ligands in the APC plasma membrane [54]. If this occurs *in vivo*, it is difficult to explain how antigen can be maintained in the immunological synapse for many hours. Long-term stability of the peptide-MHC complex in the synapse is also complicated by the half-life of peptide/MHC complexes; sustained signaling could not be maintained for 20 hours if the stability of the peptide-MHC complex is much shorter than this. Finally, it has been shown that

T cells expressing engineered receptors that have very high affinities and that should therefore limit serial engagement are still able to efficiently activate T cells [55]. Thus, whether serial engagement plays an important role in T-cell activation is not currently known.

Still, the formation of a classical immunological synapse might function to direct the internalization and degradation of engaged TCR complexes. In resting T cells, the TCR is recycled continuously from the surface to an early endosomal compartment and back [56]. During activation, TCRs are internalized and instead of being recycled to the plasma membrane are directed for degradation in lysosomes [57,58]. Since the C-SMAC has been shown to be the spot where secretory granules are inserted into the membrane [25], it is likely that it is also the spot where membranes are retrieved. To maintain membrane homeostasis, much of the inserted membrane will need to be retrieved. Thus, it seems possible that the C-SMAC denotes an area of the T-cell plasma membrane where membrane dynamics are particularly active.

Conclusion

More than a contact surface, the immunological synapse is a specific arrangement of proteins in the contact area. Many different patterns of the immunological synapse have been discovered, and it is suggested that these patterns are related to the biological outcome of T-cell activation. Although exactly what determines the morphology of the immunological synapse remains unknown, it almost certainly is due to differences in the antigen-presenting cell as well as differences in the particular T cell studied. It will be important as this field grows to analyze this phenomenon in greater detail. Although the immunological synapse was first proposed to function to initiate T-cell signaling, it is clear now that TCR signaling can occur in the absence of organized synapse formation. Rather, the formation of the immunological synapse appears to be a consequence of activation rather than a requirement for activation. The jury is still out on whether the synapse functions to sustain TCR signaling, but it seems very likely that the synapse is required for several hours to commit the naïve T cell to enter cell cycle. Although much remains to be learned, all can agree that the immunological synapse does represent the polarization of the T cell, a process that is required to allow T-cell effector functions to be accomplished—be that cytolysis or cytokine release. It seems likely, given the rapid progress in this field, that much more will be known about immunological synapses in the very near future.

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CHAPTER 181

The Ubiquitin-Proteasome System

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Introduction

The ubiquitin-proteasome system provides the major route of degradation for most short-lived intracellular proteins in eukaryotes. Ubiquitin is a extraordinarily well-conserved 76-residue polypeptide that is found either free or covalently joined through its C-terminus to a variety of cytoplasmic, nuclear, and membrane proteins. Although the best defined function of ubiquitin is to direct substrate proteins to their destruction by the 26S proteasome, this is not its only role. In recent years, protein ubiquitination has also been shown to regulate endocytosis and intracellular trafficking of membrane proteins, modify proteins in signal transduction pathways, and alter activity of the ribosome. Moreover, we have become aware of a broader set of structurally related proteins—the *ub*iquitin-*l*ike proteins or Ubls—that are also covalently ligated to and removed from other macromolecules by specific enzymatic pathways. These enzymes are generally similar in both mechanism and sequence to those that act on ubiquitin. The purpose of this short review is to give a general description of the ubiquitin-proteasome system, while highlighting some of the key regulatory pathways that depend on it.

Overview of the Ubiquitin-Proteasome System

Ubiquitin is joined reversibly to proteins by an amide (isopeptide) linkage between the C-terminus of ubiquitin and lysine ε-amino groups of the acceptor proteins. A simplified view of the ubiquitin pathway, which is highly conserved among diverse eukaryotes, is depicted in Fig. 1 [1,2]. The C-terminus of ubiquitin must be activated before it can form isopeptide bonds with other proteins. Initially, ubiquitin is adenylated by the ubiquitin activating enzyme, E1. The activated carbonyl in the ubiquitin~AMP intermediate is then attacked by a sulfhydryl group of the E1 enzyme, yielding an E1-ubiquitin thioester. Ubiquitin is subsequently passed to one of a large number of distinct ubiquitin-conjugating enzymes or E2s to form an E2-ubiquitin thioester. The E2s almost always catalyze substrate ubiquitination in conjunction with a specificity factor known as ubiquitin-protein ligase or E3. For proteolytic substrates, assembly of a multiubiquitin chain(s) on the protein is generally necessary for degradation. Ubiquitinated proteins are in a dynamic state, subject to either further rounds of ubiquitin addition, ubiquitin removal by deubiquitinating enzymes (DUBs), or degradation by the 26S proteasome (Fig. 1). The proteasome specifically recognizes multiubiquitin-protein conjugates; it then unfolds the substrate moiety and degrades it into small peptides, while releasing intact ubiquitin for further rounds of protein tagging.

A series of ubiquitin-related proteins (Ubls) that can be ligated to target molecules has also been uncovered, primarily as a result of genomic-scale DNA sequencing efforts. At present, over a dozen proven or putative Ubls are known [3,4]. Some, such as Rub1/NEDD8 and Smt3/SUMO, are known to make important contributions to cell signaling.

Components of the Ubiquitin Ligation and Deubiquitination Pathways

Ubiquitin-Activating Enzyme (E1) The E1 proteins are well-conserved proteins that are about 100 kD in size and

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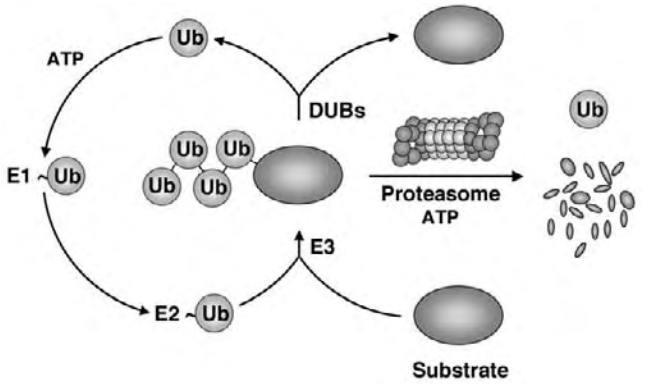


Figure 1 Outline of the ubiquitin-proteasome system. See text for details.

have a recognizable nucleotide-binding motif for ATP binding and a conserved cysteine residue that serves as the site of ubiquitin thioester formation. Most organisms have a single E1-encoding gene, which is essential for survival. No three-dimensional structure for a ubiquitin-activating enzyme has been solved, but a set of structures for an evolutionarily related bacterial protein required for molybdenum cofactor synthesis has been reported recently [5]; the structures suggest a mechanism for ubiquitin adenylation as well.

Ubiquitin-Conjugating Enzyme (E2) There is considerable diversity among the E2 isozymes but they share a core domain of ~120–150 residues with roughly 35 percent sequence identity. A cysteine in this domain accepts ubiquitin from the E1-ubiquitin thioester in a transthiolation reaction. Multiple E2 crystal structures have been reported. Different E2s are specifically required with the ubiquitination of particular substrates. This requirement appears to reflect both specific E2 association with a subset of E3 ligases and direct E2 contacts with the substrate [1].

Ubiquitin-Protein Ligase (E3) The E3s generally make the greatest contribution to substrate recognition and are usually essential for efficient ubiquitin transfer. They can be either single polypeptides or multisubunit complexes. Two mechanistically and structurally distinct classes of E3s have been reported to date. One class appears to function primarily as an adaptor between E2 and substrate, and the most recent structural data are consistent with this view [6]. All E3s of this mechanistic class share a common structural feature, namely, a RING domain. The RING motif utilizes a characteristic arrangement of Cys and His residues to coordinate two zinc ions, and this structural domain can bind directly to E2s. (In a structurally related motif, called the U-box, the same RING-like fold is predicted, but without the coordination of zinc ions; several E3s with U-boxes have been reported [7].) A distinct domain or subunit of the E3 associates with substrate, and the E2-E3-substrate ternary complex is thought to optimize the position or orientation of the E2-linked ubiquitin for attack by a substrate lysine.

In the second class of E3s, an additional ubiquitin transthiolation occurs, in this case between the E2 and E3, and the ubiquitin is then finally transferred from the E3 thiol to the substrate lysine. All E3s of this type bear a conserved ~350-residue stretch called the HECT domain, the key feature of which is the conserved cysteine that forms the thioester with ubiquitin. A crystal structure of a HECT E3 ligase-E2 complex is available, but the relevant cysteines in the E2 and E3 are over 40 Å apart, so very little can be inferred about how ubiquitin is transferred between the two thiols [8]. Although there are many fewer HECT E3s than RING E3s, it was a mutation in a HECT E3, the E6-AP gene mutated in Angelman's Syndrome, that provided the first direct link between the ubiquitin system and a heritable human disease [9].

Deubiquitinating Enzymes (DUBs) In terms of individual physiological functions, most DUBs remain enigmatic. Nevertheless, both the broad requirements for such enzymes and some of their basic molecular features are fairly well understood [10,11]. DUBs are required first of all to process the precursor forms of ubiquitin insofar as all ubiquitin genes encode either head-to-tail fusions of multiple ubiquitin moieties or ubiquitin sequences fused to ribosomal peptides. Because ubiquitin ligation involves intermediates of ubiquitin susceptible to nucleophilic attack, adventitious conjugation to abundant intracellular nucleophiles such as lysine or glutathione is believed to be common, and this must be reversed by DUBs to maintain adequate free ubiquitin pools. DUBs also can negatively regulate ubiquitin-dependent processes by removing the protein modification; for example, they can prevent or limit ubiquitin-dependent proteolysis by the proteasome. Finally, an important positive regulatory function of DUBs is the recycling of ubiquitin from proteins following their commitment to proteolysis by either the proteasome or lysosome/vacuole [10,11].

There are two known classes of DUBs: the ubiquitin C-terminal hydrolases (UCHs) and the ubiquitin-specific processing proteases (UBPs). Both are specialized cysteine proteases. The UCHs, which are usually <40 kD in size, are the less common class, and generally can only cleave ubiquitin from small adducts or disordered protein segments. This substrate constraint reflects the presence of a loop in the UCH enzymes that lies over the active site; segments of the ubiquitin-substrate conjugate may need to be threaded through this loop for efficient cleavage [12]. Less is known about the structure and enzymatic mechanism of the UBPs, which are generally larger than the UCHs. The UBPs are extremely heterogeneous in sequence and are primarily classified together by virtue of two short conserved sequence elements, the Cys box and the His box, which appear to constitute part of the active site of these enzymes. *Saccharomyces cerevisiae*, for example, has 16 such enzymes, and the only regions that can be unequivocally aligned in all of them are the Cys and His boxes. Surprisingly, none of the individual yeast enzymes is required for viability [13].

The 20S and 26S Proteasomes

The 26S proteasome is both the largest and most complex of ubiquitin system components (reviewed in [14]). The most rigorous measurements put its mass at ~2.5 MDa, which is on the same order as ribosome subunits. The complex comprises some 32–35 distinct polypeptides, with additional proteins that either interact with only a subset of proteasomes or associate only transiently with proteasomes. In the absence of ATP, the 26S proteasome readily dissociates into a 19S regulatory particle and a 20S core particle. The 20S proteasome core is a cylindrical structure made of a set of 28 subunits arranged in four coaxially stacked rings. A protected interior chamber houses the protease active sites; access to this hydrolytic chamber is restricted by a set of narrow channels that extend from the two ends of the particle, which at their narrowest are ~13 Å across. Therefore, for a protein to be translocated into the catalytic core, it must be completely unfolded or nearly so. Proteolysis proceeds processively until short peptides are generated and released from the inner chambers.

Unfolding and translocation into the 20S core are controlled by the 19S regulatory particles that are positioned over each axial pore. The 19S complex includes six distinct AAA-type ATPase subunits; these are believed to form a ring that interacts directly with the outer heptameric ring of the 20S proteasome. Functions of the 19S complex include recognition of polyubiquitinated proteins (and less frequently, of nonubiquitinated substrates), protein unfolding, translocation of protein into the 20S proteasome, and recycling of ubiquitin from protein substrates bound to the proteasome. Under some conditions, the 19S regulatory complex can be further dissociated into lid and base subcomplexes. The base, which interacts directly with the 20S proteasome, includes all the ATPase subunits and the two largest non-ATPase subunits.

Degradation Signals or Degrons

A fundamental question about protein degradation is exactly what structural features render a particular protein into a target for ubiquitin-dependent proteolysis [15]. Despite its importance, this remains one of the least well understood aspects of ubiquitin-dependent degradation. Short peptide stretches can sometimes target a protein to an E2/E3 ubiquitin ligase complex, and in some cases their ability to function as targeting signals or degrons is controlled by their phosphorylation. In other examples, it is clear that a higher order structure, which may be made up of discontinuous sequence elements, is required for E2/E3 recognition [15,16]. Virtually any protein that is misfolded or misassembled can become a substrate for the ubiquitin-proteasome system. This implies that there are common features that help a cell distinguish a correctly folded protein from one that is not. The most straightforward idea, for which there is experimental evidence, is that surface-exposed hydrophobic structures, which are normally buried in subunit interfaces or in the hydrophobic core of a protein, can function as folding-dependent degradation signals.

Examples of Regulation by Protein Ubiquitination

Protein ubiquitination is now recognized to be almost as pervasive as protein phosphorylation, and it would be impossible to enumerate here all the known instances in which ubiquitination is an important component of a cellular regulatory mechanism. Instead, a few illustrative examples will be briefly described. Perhaps the most widely appreciated function of ubiquitin-dependent proteolysis is in cell cycle control. Multiple, irreversible transitions in the cell cycle, including G1-to-S, metaphase-to-anaphase, and mitotic exit, require the timed degradation of specific positive or negative cell-cycle regulators. For instance, separation of sister chromatids to initiate anaphase depends on the ubiquitin-dependent destruction of an inhibitor called securin [17]. Securin binds to and keeps inactive a caspase-related protease, called separase or separin. Degradation of securin frees separase to cleave a subunit of the cohesin complexes, which prior to subunit cleavage maintain connections between sister chromatids even while they are under tension from the mitotic spindle.

Another well-studied example of ubiquitin-dependent regulation is in the NFκB signaling pathway. The NFκB transcription factor initiates transcription of multiple genes in response to an array of different environmental signals [18]. Most of these signals work by inactivating an inhibitor of NFκB called IκB, which sequesters NFκB in the cytoplasm by enhancing its export from the nucleus. Signaling leads to the site-specific phosphorylation of IκB, which triggers its multiubiquitination by an ubiquitin-protein ligase and rapid degradation by the proteasome. An interesting finding is that protein multiubiquitination appears to serve an additional, nonproteolytic signaling function upstream of the kinase that phosphorylates IκB, but the mechanism of this regulation remains to be fully elaborated [18].

A final example of how ubiquitin is deployed as a regulator of signaling is the remarkable finding that the ubiquitination of certain transcription factors is both a requirement for their activator function and a prelude to their silencing by proteasomal degradation [19]. A single ubiquitin moiety is sufficient for activation, but a multiubiquitin chain is necessary for subsequent degradation. This latter finding is consistent with the fact that proteasomes only bind efficiently to ubiquitin chains with at least four ubiquitin moieties [20]. Monoubiquitination, however, is also a known signal in other processes, particularly in the endocytosis and trafficking of cell surface receptors [21].

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CHAPTER 182

Caspases: Cell Signaling by Proteolysis

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Protease Signaling

Proteolytic enzymes, including the cell's degrading machine the proteasome, calpains, and integral membrane proteases such as γ-secretase and rhomboid, participate in several intracellular signaling processes (Table I). But given that the human genome contains in excess of 500 genes that encode proteases, it seems odd that only the caspases constitute a formal multistep pathway able to transmit intracellular signals by proteolysis. In contrast, extracellular multistep signaling pathways frequently use the principle of proteolysis extensively for coagulation, fibrinolysis, complement activation in mammals, and gastrulation in flies. What is it about the caspases that makes them so suitable to transmit intracellular signals?

The consensus view of caspases places them in two main camps. First are the cytokine activators related to caspase 1, probably including mouse caspase 11 and its close homologs caspases 4 and 5 in humans. Their main role is to respond to bacterial infection by rapidly converting active cytokines (IL-1β, IL-18) from intracellular stores. Confirmation of the important roles of the caspases in the inflammatory cytokine response comes from gene ablation experiments in mice. Animals ablated in caspase 1 or 11 are deficient in cytokine processing [1,2] but without any overt apoptotic phenotype. The second camp constitutes the apoptotic caspases that transduce and execute death signals. The phenotypes of these knockouts are very gross, evidently anti-apoptotic, and vary from early embryonic lethality (caspase 8) to perinatal lethality (caspases 3 and 9) [3–5] to relatively mild with defects in the process of normal oocyte ablation [6]. Techniques in biochemistry and cell biology have allowed us to place the apoptotic caspases in two converging pathways, such that some are activated by others (Fig. 1). This core pathway probably represents a minimal apoptotic program, and certainly their simplicity is complicated by cell-specific additions that help fine-tune individual cell fates. Nevertheless, the basic order and at least some of the essential functions and, especially important, endogenous regulators of the caspases are now known.

Apoptosis and Limited Proteolysis

Apoptosis is a mechanism to regulate cell number and is vital throughout the life of all metazoan animals. Though several different types of biochemical events have been recognized as important in apoptosis, perhaps the most fundamental is the participation of the caspases [7–9].

The name caspase is a contraction of *c*ysteine-dependent *asp*artate specific prote*ase* [10], thus their enzymatic properties are governed by a dominant specificity for protein substrates containing Asp, and by the use of a Cys side chain for catalyzing peptide bond cleavage. The use of a Cys side chain as a nucleophile during peptide bond hydrolysis is common to several protease families. However, the primary specificity for Asp turns out to be very rare among proteases throughout biotic kingdoms. Of all known mammalian proteases only the caspase activator granzyme B, a serine protease, has the same primary specificity [11,12]. Caspases cleave a number of

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cellular proteins [13], and the process is one of limited proteolysis whereby a small number of cuts, usually only one, are made. Sometimes cleavage results in activation of the protein, sometimes in inactivation [14], but never in degradation, since their substrate specificity distinguishes the caspases as among the most restricted of endopeptidases.

Table I Proteases Involved in Intracellular Signaling

Protease	Signaling function
Caspases	Apoptosis Pro-inflammatory cytokine activation
Proteasome	Cell cycle progression NFκB activation
Rhomboid	EGF signaling
γ-Secretase	Toll receptor signaling
SREB Site2 Protease	Upregulation of sterol synthesis genes
Separase	Anaphase
Calpains	Various signaling events

While not an exhaustive survey, the table highlights the principle of proteolysis as a mechanism of signal transmission.

This is an important distinction from the proteasome, which permits signaling by wholesale destruction of regulatory proteins such as IκB in NFκB signaling and PDS1 in anaphase promotion [see Chapter 291 in Volume 3].

The most primitive organism with a bona fide caspase appears to be *Caenhorabditis elegans*. Indeed, the first apoptotic caspase, Ced3, was identified in this organism, a finding that galvanized the apoptotic research field [15]. As the complexity of primitive cell death pathways developed, so apparently did the number of caspases. *Drosophila* have at least seven caspases [16] and humans have at least 11. Mapping the inherent substrate specificity of caspases has allowed some broad consensuses to be recognized [17]. These consensuses also allow apoptotic caspases to be distinguished from pro-inflammatory caspases, since the latter have a rather distinct specificity that presumably allows them to carry out their job without threatening cell viability. What is interesting, there seems to have been a parallel evolution of apoptotic caspases along with their substrates. Consensus caspase targets in humans such as nuclear lamins and poly (ADP)ribose polymerase have easily recognizable caspase cleavage sites in *Drosophila* but apparently not in organisms such as yeast and plants, which lack an apoptotic pathway.

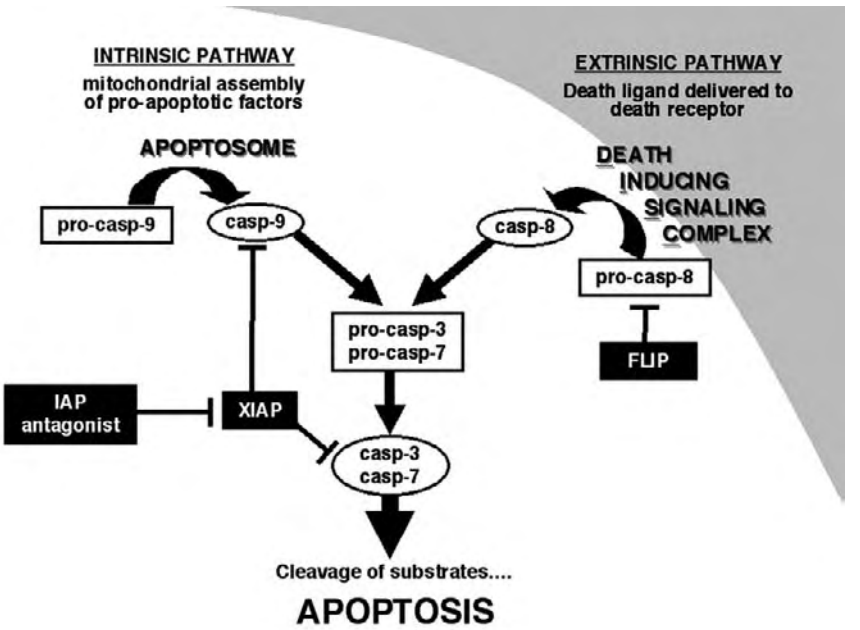


Figure 1 The framework of apoptosis. Death may be signaled by direct ligand-enforced clustering of receptors at the cell surface, which leads to the activation of initiator caspases 8 [46]. This caspase then directly activates the executioner caspases 3 and 7 (and possibly 6), which are predominantly responsible for the limited proteolysis that characterizes apoptotic dismantling of the cell. Alternatively, irreparable damage to the genome caused by mutagens, pharmaceuticals that inhibit DNA repair, or ionizing radiation—transmitted by a mechanism thought to involve the release of cytochrome c from mitochondria—engages the same executioner caspases [47]. The latter events progress through the initiator caspase 9 and its cofactor Apaf-1 [21]. Activation of the extrinsic pathway is regulated by FLIP, which serves to modulate the recruitment of caspase 8 to its adapters [48]. The common execution phase is regulated through direct caspase inhibition by IAPs, some of which can also regulate the active form of caspase 9. In turn, the IAPs are under the influence of antagonist proteins that compete with caspases for IAPs [38]. Though other modulators may regulate the apoptotic pathway in a cell-specific manner, this framework is considered common to most mammalian cells.

Caspase Activation

Induced Proximity

The seminal discovery that death receptor signaling requires, in its most basic form, simply a transmembrane receptor, an adapter molecule, and a caspase [18,19] revealed a solution to the perplexing problem of how the first proteolytic signal was generated during apoptosis, since it implicated a caspase directly in the triggering event. Prior to this work receptors were thought to signal by either altering the phosphorylation status of key signaling molecules or by functioning as ion channels. Death receptors such as Fas signal by direct recruitment and activation of a protease (caspase 8). Concomitantly with this work, groundbreaking studies showed that the intrinsic pathway was activated by a cofactor known as apoptosis protease activating factor (Apaf-1) [20]. Subsequently, Apaf-1 was found to recruit and activate caspase 9, forming the "apoptosome" [21].

In common with most proteolytic enzymes, caspases reside as latent forms that are usually activated by limited proteolysis. It is relatively easy to imagine that the caspases operating at the bottom of the pathway are activated by ones above. Until recently the question of how the first caspase in a pathway became activated, how the first death signal was generated, was a perplexing issue. Now several groups have focused on this issue and a consensus has been arrived at to describe the intriguing operation of the initiation of the proteolytic pathways that execute apoptosis (reviewed in [22,23]). This mechanism is known as the "induced proximity hypothesis" and although it originally referred to death induced by caspase 8 (the extrinsic pathway), it has now been extended to death induced by caspase 9 (the intrinsic pathway); see Fig. 1. Notably, this mechanism does not apply to the executioner caspases.

How exactly does a recruited zymogen become active? To understand this, as a basis for formulating an adequate hypothesis, one must understand the unusual properties of caspase zymogens that set them apart from most other proteases. Unlike most other proteases, simple expression of caspase zymogens in *E. coli* usually results in their activation by limited proteolysis within a "linker segment" that separates the large (~20 kD) and small (~10 kD) subunits of the catalytic domain [24,25]. This activation results from processing that is a consequence of intrinsic proteolytic activity residing in the caspase zymogens. It is not due to *E. coli* proteases, since catalytically disabled C285A (caspase 1 numbering convention) mutants fail to undergo processing.

Initiator Caspases

At the cytosolic concentration in human cells (<50 nM), pro-caspase 9 is a monomer [26] and requires oligomerization within the apoptosome to become active [27,28]. Significantly, unlike the executioner caspases 3 and 7, procaspase 9 does not need to be cleaved in the linker region to become active [29,30]. Not only is cleavage unnecessary, but also it is insufficient to produce an active enzyme. Instead, caspase 9 is activated by small-scale rearrangements of surface loops that define the substrate cleft and catalytic residues [26]. In the simplest model, this is achieved by dimerization of caspase 9 monomers within the apoptosome [31], with the dimer interface providing surfaces compatible with catalytic organization of the active site. More recently, it has been demonstrated that a similar dimerization mechanism activates the caspose 8 Zymogen to trigger the extrinsic pathway [51,52].

Executioner Caspases

Once an initiator caspase has become active, the ensuing activation of the executioners is more straightforwardly explained. At cytosolic concentration in human cells, the cxaspase-3 and 7 zymogens are already dimers, but cleavage within their respective linker segments is required for activation [32,33]. The same re-ordering of catalytic and substrate binding residues occurs in caspase 7 as seen in caspase 9, so the fundamental mechanism of zymogen activation is equivalent. Only the driving forces are distinct, since the linker segment of pro-caspase 7 blocks ordering of the active site, and upon cleavage the new N- and C-terminal sequences so generated aid in active site stabilization. The property that allows the distinct driving forces to converge on the same activation mechanism seems to be the unusual plasticity of the residues constituting the caspase active site, which, rather unusually for proteases, are predominantly placed on flexible loops and not on an ordered secondary structure.

Regulation by Inhibitors

The first level of regulating proteolytic pathways is by zymogen activation, but an equally important level is achieved by the use of specific inhibitors that can govern the activity of the active components. The endogenous inhibitors of caspases, those present in mammalian cells, are members of the inhibitor of apoptosis (IAP) family. In addition to these endogenous regulators are the virally encoded cowpox virus CrmA and baculovirus p35 proteins that are produced early in infection to suppress caspase-mediated host responses. Each of the inhibitors has a characteristic specificity profile against human caspases, as determined *in vitro*, and these profiles, with few caveats [34], agree with the biologic function of the inhibitors (reviewed in [35]). Though IAPs and CrmA would be expected to regulate mammalian caspases *in vivo*, p35 would never be present normally in mammals because it is expressed naturally by baculoviruses.

The best-characterized endogenous caspase inhibitor is the X-linked IAP (XIAP), a member of the IAP family. The IAPs are broadly distributed and, as their name indicates, the founding members are capable of selectively blocking apoptosis, having initially been identified in baculoviruses (reviewed in [36]). Eight distinct IAPs have been identified

in humans. XIAP (which is the human family paradigm) has been found by multiple research groups to be a potent but restricted inhibitor targeting caspases-3, 7, and 9 (reviewed in [37]). Similarly, evidence implicates human cIAPs 1 and 2, ML-IAP, *Drosophila* DIAP-1, as well as ILP-2, as caspase inhibitors (reviewed in [38]). IAPs certainly have functions in addition to caspase inhibition because they have been found in organisms such as yeast, which neither contain caspases nor undergo apoptosis [39].

IAPs contain one, two, or three baculovirus IAP repeat (BIR) domains, which represent the defining characteristic of the family. Currently there is no known function for BIR1; however, domains closely related to the second BIR domain (BIR2) of XIAP specifically target caspases 3 and 7 ($K_i \approx 0.1$–1 nM), and regions closely related to the third BIR domain (BIR3) specifically target caspase 9 ($K_i \approx 10$ nM). This led to the general assumption that the BIR domain itself was important for caspase inhibition. The recent structures of BIR2 in complex with caspases 3 and 7 have surprisingly revealed the BIR domain to have almost no direct role in the inhibitory mechanism. All the important inhibitory contacts are made by the flexible region preceding the BIR domain [40–42]. It is interesting that the mechanism of inhibition of caspase 9 by the BIR3 domain requires cleavage in the inter-subunit linker to generate the new sequence NH_2-ATPF [30]. In part this explains the cleavage of caspase 9 during apoptosis, which as described above is not required for its activation. Paradoxically, it seems required for its inactivation by XIAP.

Neither CrmA-like nor p35-like inhibitors, which operate by mechanism-based inactivation [35], have been chosen for endogenous caspase regulation; rather, IAPs have been adapted to regulate the executioner caspases. Although the reason for this is not certain, it seems likely that the IAP solution provides a degree of specificity that mechanism-based inhibitors cannot achieve. Thus, XIAP inhibition of caspases 3 and 7 requires a nonstandard interaction with the extended 381 loop that is specific to these two caspases (reviewed in [35]). Possibly the 381 loop has evolved to achieve substrate specificity in the executioner caspases [43]. But an equally likely possibility is that the 381 loop has been generated to enable the IAP scaffold to provide a unique control level over the execution phase of apoptosis. Adding to this level of sophistication, IAPs, but not CrmA or p35-like proteins, are subject to negative regulation by IAP antagonists that go by the names of Hid, Grim, Reaper, and Sickle in *Drosophila* and Smac/Diablo, and HtrA2/Omi in mammals (reviewed in [38]).

IAP Antagonists

Biochemical studies led to the identification of a potentially important IAP-interacting protein known as second mitochondrial activator of caspases (Smac) in humans [44] and Diablo in mice [45]. Smac is a nuclear-encoded mitochondrial protein whose 55 amino terminal residues are removed by a proteolytic process during translocation.

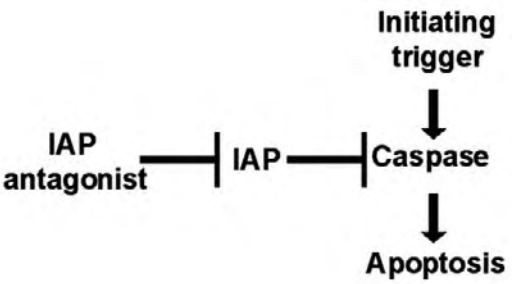


Figure 2 The basis of IAP antagonists as pro-apoptotic proteins. Genetic screens in *Drosophila* initially revealed a genomic region encoding three proteins—head involution defective (Hid), Grim, and Reaper—that together seemed responsible for almost all developmental apoptosis in the fly [49]. These proteins, along with the more recently discovered Sickle, constitute the currently known IAP antagonists in flies. In flies there is evidence for a continuous low-level production of caspases, which are neutralized by *Drosophila* IAP-1 [50]. In this scenario simple upregulation of one or more fly IAP antagonists could send the system into apoptosis, and to this extent the system is transcriptionally regulated. Input from the left of the diagram may be the most important event. In contrast to flies, the currently known mammalian IAP antagonists are mitochondrial proteins and require translocation before they can influence the inhibitory activity of IAPs. This implies a more complex role for IAP antagonists in mammals, since both positive initiator caspase signaling and mitochondrial fluxes would presumably be required, and the system could only be transcriptionally regulated in an indirect manner. In mammals, input from the top of the diagram could be more important.

Smac can be released, apparently along with cytochrome c, in response to apoptotic stimuli. Upon its release from mitochondria, mature Smac binds XIAP and probably several other IAPs, in a manner that displaces caspases from XIAP. Thus, Smac is a negative regulator of IAPs and therefore an apoptosis-enhancing molecule. Residues 56–59 of Smac and DIABLO are homologous to the exposed amino terminal motif utilized by caspase 9 to bind BIR3 of XIAP (see preceding section), and Smac has been found to bind to the same pocket in XIAP, thereby attenuating the affinity and displacing caspase 9 from the complex. The remarkable similarity in binding mode of caspase 9 and Smac to XIAP is not limited to these two proteins. In *Drosophila* the death-inducing proteins Hid, Grim, Reaper, and Sickle all contain homologous IAP binding motifs at their N-terminus (sometimes called the RHG or IBM motif). In distinction to the known mammalian IAP antagonists, the *Drosophila* ones do not have transit peptides and are therefore presumably fully functional following synthesis and removal of their initiator methionine. The mitochondrial versus nonmitochondrial disposition of IAP antagonists between mammals and flies suggests a rather different IAP regulation process (Fig. 2), which may have profound consequences for the interpretation of IAP function.

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CHAPTER 183

MAP Kinase in Yeast

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Introduction

Yeast cells use multiple mitogen-activated protein (MAP) kinases to respond to a wide variety of external stimuli that regulate proliferation, differentiation, survival, and response to stress. As in mammalian cells, yeast MAPKs are activated within MAPK cascades that form the cores of larger signal transduction cascades. Activation of MAPKs leads to the phosphorylation of a variety of effector proteins, including many nuclear transcription factors. This chapter presents a brief overview of the different MAPK kinases in yeast, discusses how physical interactions with regulatory proteins such as scaffolds, activating kinases, and substrates regulate pathway specificity, and elucidates how their function is dynamically controlled at the level of localization and the strength and duration of activation.

Yeast Cells Use Multiple MAPKs to Respond to a Wide Variety of Stimuli

All eukaryotic cells have multiple MAPK cascades that are activated by one or more environmental stimulus and that mediate a variety of cellular responses [1,2]. Much of our knowledge about how MAPK cascades function has come from analysis in yeast. The proliferation, differentiation, morphogenesis, and response to stress of budding yeast *S. cerevisiae* is governed by five different MAPKs (Fig. 1), which function in six MAPK cascades that respond to different nutritional and hormonal inputs (Fig. 2). Three of the MAPKs are expressed in dividing haploid and diploid cells (Kss1, Mpk1/Slt2, Hog1), the fourth MAPK (Fus3) is expressed in dividing haploid cells (Fus3), and the fifth MAPK (Smk1) is expressed in cells undergoing meiosis and sporulation. MAPKs Fus3 and Kss1 function in the mating pathway, which is activated by peptide pheromones and causes cell cycle arrest in G1 phase along with polarized growth and additional differentiative events that prepare cells for cell attachment and fusion (reviewed in [3]). The Kss1 MAPK also functions in the filamentous growth pathways that respond to altered nutritional conditions and induce pseudohyphal diploid cells with higher surface-to-volume ratio and altered budding pattern and invasive haploid cells that can digest the substratum (reviewed in [4]). The Mpk1 MAPK functions in the PKC-regulated MAPK cascade, which is essential for mitotic growth, particularly the G1 to S phase transition, and plays a key role in maintaining cell integrity through cell wall synthesis and the actin cytoskeleton. The PKC-regulated pathway is activated under conditions that perturb the cell wall or plasma membrane (e.g. heat shock) (reviewed in [5,6]). The Kss1 MAPK cascade plays a lesser role in regulating cell integrity in parallel with the PKC-regulated MAPK cascade and is also thought to be activated under conditions of cell wall stress [7]. The Hog1 MAPK functions in the high osmolarity glycerol (HOG) pathway, which responds to hypertonic stress by increasing intracellular pools of glycerol (reviewed in [8]). This pathway is the closest functional equivalent of mammalian p38/JNK pathways. Finally, the Smk1 MAPK functions in a less-defined MAPK cascade that regulates sporulation and meiosis [9]. As in other eukaryotes, the yeast MAPKs phosphorylate many different proteins, including transcription factors in the nucleus (Fig. 2).

The yeast MAPKs are activated by a wide variety of means, including (1) a serpentine receptor and heterotrimeric G protein in the mating pathway, (2) a Ras-linked mechanism for the

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filamentous growth pathways, (3) a plasma membrane sensor and two component system in the HOG pathway, and (4) integrin-like sensors that physically link to Rho guanine exchange factors in the PKC-regulated pathway (Fig. 2). Additional signal transduction enzymes that regulate pathway activity include G-protein regulators, adapter proteins, and phosphatases. G-protein regulators include the RGS protein Sst2 that positively regulates Gpa1, the Gα subunit of the mating pathway G protein [10], Cdc25 (GEF) and Ira1, Ira2 (GAPs) that regulate Ras2 [4], and Rom1, 2 (GEFs) that regulate Rho1 [6]. Adapter proteins include the Ste5 scaffold in the mating pathway [11], Pbs2 MAPKK in the HOG pathway (reviewed in [12]), and the Ste11 MAPKKK regulator Ste50 [3] (Fig. 3).

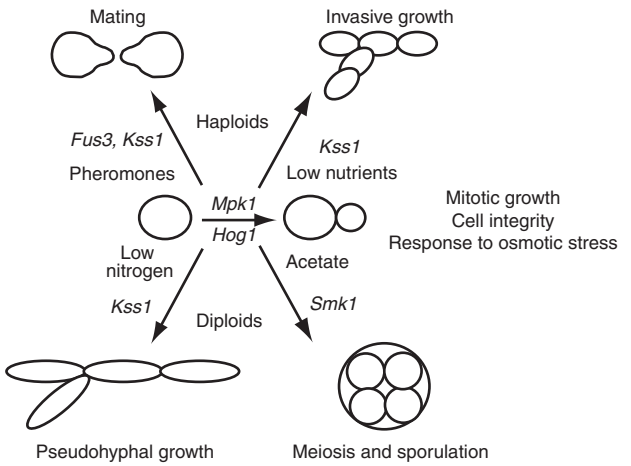


Figure 1 *S. cerevisiae* life cycle is regulated by 5 MAPKs.

Functionally Defining *S. cerevisiae* MAPK Cascades

The *S. cerevisiae* MAPKs and their activating kinases were identified through genetic screens for mutations or high copy plasmids that affect growth and differentiation. The mating MAPK cascade was the first to be identified and is the best characterized. Full assessment of MAPK function should involve analysis of null alleles as well as catalytically inactive mutants. Useful mutations include a lysine→arginine change in the binding site for ATP, which does not block phosphorylation by MKK or protein substrate binding, and threonine→alanine or tyrosine→phenylalanine changes in the T-X-Y sequence, which block phosphorylation by MKK and can affect protein substrate binding. A combined use of these different types of alleles has shown, for example, that certain MAPKs are functionally redundant (e.g. Fus3 and Kss1 for mating, [3]; Fig. 2) and that inactive kinases serve regulatory functions (e.g. Kss1, [13], Fig. 3B).

The core elements of a *S. cerevisiae* MAPK cascade module are three sequentially activated protein kinases [i.e. MAPKK kinase (MKKK) →MAPK kinase (MKK) →MAPK, Fig. 2]. *S. cerevisiae* MAPK modules show remarkably high conservation with MAPK modules in humans [14]. Fus3 and

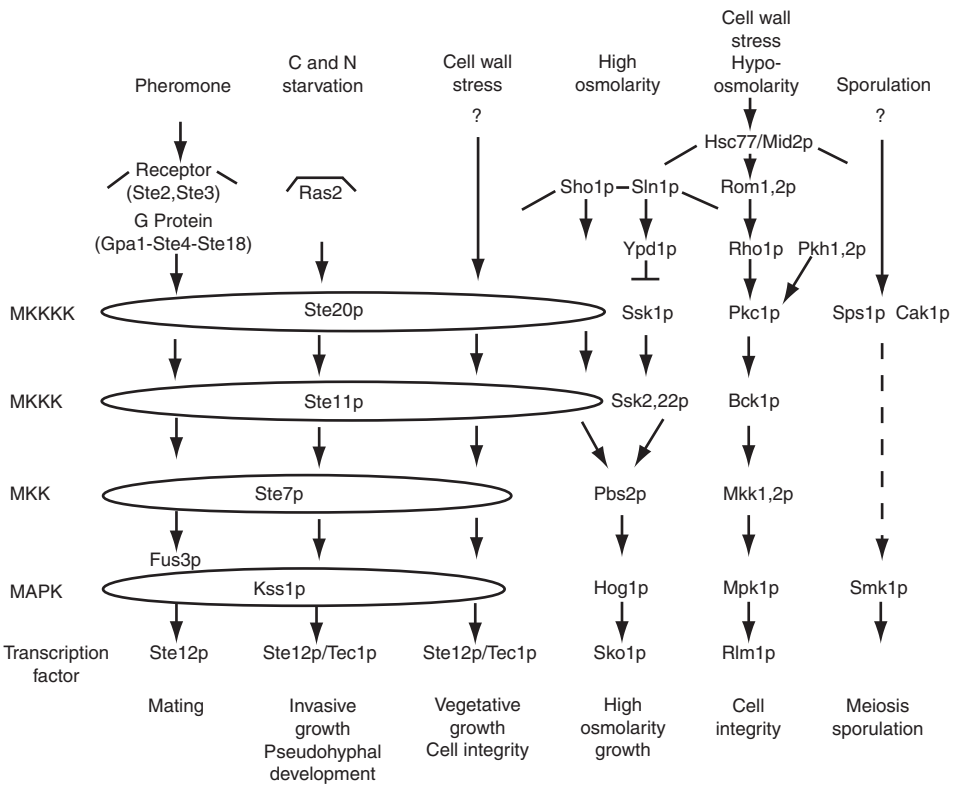


Figure 2 *S. cerevisiae* MAPK cascades.

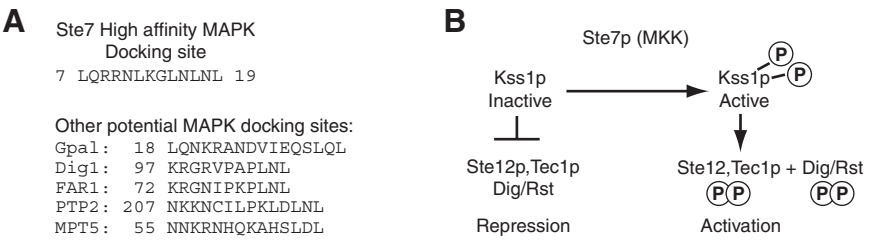


Figure 3 MAPK interactions with activating kinases and targets.

Kss1 are most similar to ERK1, 2, and Hog1 is most similar to p38 MAPK, whereas Mpk1 and Smk1 are distinct [14]. The activation mechanism for the *S. cerevisiae* MAPKs is highly conserved and involves phosphorylation of conserved threonine and tyrosine residues in the signature T-X-Y sequence within the activation (or T) loop at the catalytic cleft by the appropriate dual specificity MKK. Similar to ERK1, Fus3 autophosphorylates on tyrosine 182; however, dual phosphorylation is required for full activation. The yeast MAPKs are tightly regulated and are highly active only in the presence of a stimulus. However, the basal activities of Mpk1, Kss1, and Fus3 perform specific functions in the absence of stimulus [7–8,13]. Biochemical and genetic data support a series of direct phosphorylation events within each MAPK module, leading to serial activation of the MKK and MAPK tiers. A third upstream activating kinase (a putative MKKKK) is thought to activate the uppermost kinase (the MKKK) in a number of MAPK cascades. This third upstream activating kinase includes two subtypes, Ste20 p21-activated kinases (PAK) and protein kinase C (Fig. 2). Ste20 has been shown to directly phosphorylate MKKKK Ste11; its activation involves derepression of a negative regulatory domain and association with a conserved protein called Ste50 through mutual SAM domains [3]. MKK Ste7 may be activated by dual phosphorylation of serine-threonine residues within a conserved domain near the activation loop [15].

Major Regulatory Mechanisms that Control Specificity in *S. cerevisae* MAPK Cascades

A major biological question is how specificity is achieved to assure that the appropriate response takes place for a given stimulus. The individual *S. cerevisiae* MAPK cascades exhibit exquisite specificity based on the phenotypes of cells with mutations in the individual MAPKs. This specificity is remarkable because four of the pathways use subsets of the same kinases (Fig. 2). Pathway specificity occurs through multiple layers of control, which include preferred kinase-kinase and kinase-substrate interactions, the use of scaffold or adapter-like proteins to route signals through specific MAPK modules, cross-regulation between MAPK modules, controlled localization of MAPKs, and control of the strength of the activation. Common themes of signal transduction through yeast MAPK cascades are that they form molecular assemblies within cells and spatial organization at specific locations plays a critical role in mediating efficient activation and specificity [16,17]. The pathways are also highly dynamic at the level of movement of signal transduction proteins [18–20].

Kinase Interactions within a MAPK Cascade

MAPKs prefer to bind to specific activating kinases within a module, providing one level of pathway specificity. For example, the yeast MKK Ste7 preferentially binds to and activates MAPKs Fus3 and Kss1 of the mating and invasive growth and pseudohyphal development pathways, but does not bind to or activate MAPKs Mpk1 or Hog1. Consistent with this, *ste7*Δ null mutants only have defects in mating and invasive growth and pseudohyphal development. A docking site of charged residues has been defined for the interaction of Ste7 with Fus3 and Kss1 [21]; this docking site defines a consensus sequence that is generally applicable for other MAPK binding partners (Fig. 3A; [22]).

Functional analysis in yeast demonstrates that MAPK cascade specificity is enhanced through the use of different MKKs and MAPKs that recognize specific substrates. The mating, invasive growth and pseudohyphal development, and high osmolarity growth pathways use some of the same MAPK module components (i.e. MKKKK Ste20, MKKK Ste11, MKK Ste7, MAPK Kss1), yet elicit very different responses by using a specific MKK (Pbs2) and MAPK (Hog1) for high osmolarity growth and a specific MAPK (Fus3) for mating (Fig. 2). For example, during mating, MAPK Fus3 specifically regulates G1 arrest and mating through phosphorylation of the Far1 protein [23], which is a poor substrate for MAPK Kss1 [24].

Yeast MAPKs form stable complexes with their targets, either as active or inactive kinases, suggesting that such complexes may play a regulatory role [23,25]. The functional importance of catalytically inactive MAPK/substrate complexes was first demonstrated for MAPK Kss1, which exerts both negative and positive control over pseudohyphal development and invasive growth (Fig. 3B; [26,27]). Kss1 forms a stable complex with the Ste12 transcription factor in its unphosphorylated inactive form and acts as a transcriptional repressor. When phosphorylated, Kss1 becomes a positive regulator, presumably through dissociation from Ste12 together with phosphorylation of Ste12, Tec1, and the

Dig1,2 repressors (Fig. 3B). The fact that the same MAPK provides both negative and positive functions for signaling means that a *kss1* null mutation does not block signaling whereas catalytically inactive Kss1 derivatives do. These findings argue that it is important to analyze catalytically inactive kinase mutations in addition to null mutations in assigning function.

Scaffold Proteins

A variety of biochemical and genetic evidence suggests that simple activation of the kinases within a MAPK module is not sufficient for function *in vivo.* Scaffold proteins have been found to play key roles in maintaining MAPK pathway function and fidelity by linking individual components to each other, to upstream activators, downstream targets, and specific cellular locales [16,17]. Many of our ideas about scaffolds are based upon work on the prototype MAPK scaffold Ste5 of the mating pathway (Fig. 4, reviewed in [11]). The criteria for assigning a scaffold function to Ste5 included (1) definition of separable binding sites for each of the three tiers of protein kinases (i.e. Ste11, Ste7, and Fus3/Kss1) and (2) biochemical evidence for a Ste5-multikinase complex that enhances Ste11/Ste7 interactions and increases the specific activity of MAPK Fus3. In addition to tethering kinases, Ste5 plays a direct role in the activation of MKKKK Ste11 by MKKKK Ste20, which is normally associated with the Rho-type G protein Cdc42 at the cell cortex (Fig. 4). Ste11 must be properly targeted to Ste20 for activation to take place. This function is tied to proper recruitment of Ste5 through its conserved RING-H2 domain to Gβγ dimers at the plasma membrane, which also bind to Ste20 upon release from Gα after pheromone induction of the receptor. Formation of an active signaling complex requires oligomerization of Ste5; glycerol sedimentation is consistent with Ste5 functioning as a dimer.

The MKK Pbs2, of the yeast high osmolarity growth (HOG) pathway, also appears to serve a scaffold function based on pair-wise interactions between Pbs2 and the other two kinases in the MAPK module and separate binding sites for these kinase (reviewed in [12]). Pbs2 has a large regulatory domain in addition to its catalytic domain. A variety of evidence suggests that Pbs2 tethers the Hog1 MAPK and Ste11 MKKK to a transmembrane sensor called Sho1 that senses changes in osmolarity. This interaction is thought to occur through a proline-rich domain in Pbs2. Much like Ste5, Pbs2 is thought to recruit Ste11 to Ste20 while it is linked through its CRIB domain to Cdc42, which is asymmetrically enriched at the plasma membrane , with the end result of phosphorylation and activation of Ste11 by Ste20. Thus, the common theme of Ste5 and Pbs2 is that they link the cytosolic kinases to a plasma membrane-linked G protein or sensor and an activating GTPase-linked PAK. This arrangement allows a proximal sense of the status of the environment and provides a spatial arrangement that promotes phosphorylation of the uppermost kinase of each MAPK module.

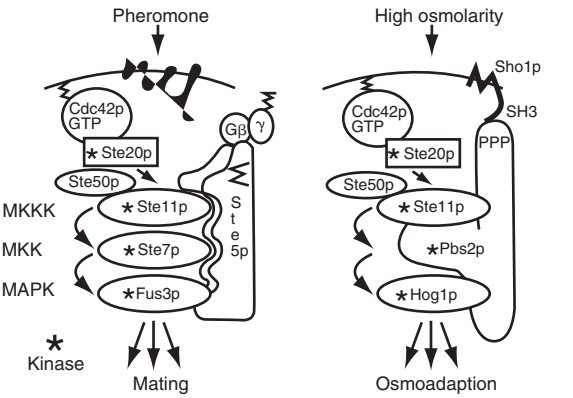


Figure 4 Ste5 and Pbs2 scaffold proteins regulate MAPK cascades of overlapping components.

It is interesting that a common regulatory scheme is used for the two pathways that share signaling components and respond to stimulus in a polarized manner—perhaps the scaffolds allow for a highly localized activation mechanism that facilitates a polarized response, such as by targeting the MAPK to substrates, and also prevent cross-activation.

MAPK cascade scaffolds are now being described in mammalian cells [17]. None bear homology to each other, suggesting that they have evolved independently for the individual requirements of individual subsets of kinases and upstream activating events. It is interesting, however, that the JIP and KSR scaffolds dimerize and are localized to the cell periphery through interactions with membrane-linked receptors or GTPases, thus suggesting that aspects of their function are similar to Ste5's. It has been proposed that a three-tier MAPK cascade has ultrasensitive switch-like responses [28]. The existence of MAPK cascade scaffolds raises the important question of how they affect signal propogation [11,17]. For example, co-localization of three tiers of kinases by a scaffold may prevent amplification by physical sequestration or provide a means to ensure feedback control and prevent cross-activation of other pathways that utilize the same kinases, localize the MAPK to targets, or protect it from phosphatases. Alternatively, a MAPK cascade scaffold could promote kinase-kinase phosphorylation and dissociation after activation and could enhance amplification.

Dynamic Localization of MAPK Cascades

Specificity is also regulated by proper localization of the MAPKs. MAPKs are stably associated with a variety of subcellular structures, including tubulin and centromeres (reviewed in [29,30]). MAPKs can shuttle between the nucleus and cytoplasm and be retained in the cytoplasm by a cognate MAPKK or undergo nuclear translocation as a result of activation and dimerization [30]. In *S. cerevisiae*, the MAPK Hog1 translocates to the nucleus in response to osmotic stress [18], then forms a stable complex with the transcription apparatus at specific promoters and activates

transcription through derepression of Sln1 ([32], Fig. 5). MAPKs Fus3 and Kss1 are predominantly nuclear; however, Fus3 shuttles continuously between the nucleus and cytoplasm and is translocated to the projection tip of pheromone treated cells along with MKK Ste7, possibly through Ste5 [19,20]. This localization event may serve to promote polarized growth and attenuation by co-localizing Fus3 with targets such as Far1 or other components of the morphogenesis apparatus in addition to upstream signaling components such as Ste5, Sst2, and Gα (Gpa1) (Fig. 6). Once activated, Fus3 dissociates rapidly from Ste5 and translocates to the nucleus, where it phosphorylates transcription factors. The Ste5 scaffold also shuttles through the nucleus, and this localization event positively regulates its recruitment and activation of MAPK Fus3 [19]. The Far1 and Cdc24 signaling proteins involved in morphogenesis are anchored together in the nucleus and exported to the cell cortex as a result of pheromone activation (Fig. 6, [3]). Thus, a majority of components in the mating MAPK cascade are dynamic. These findings may be generally applicable to other pathways.

Signal Strength

DOWNREGULATION

The duration and strength of activation of a given MAPK cascade may also affect the ultimate outcome of a response. For example, transient activation of the Ras/Raf/ERK pathway by epidermal growth factor in PC12 cells causes cell proliferation, whereas sustained activation of the same pathway by nerve growth factor (NGF) causes terminal differentiation as shown by neurite outgrowth [33]. Multiple factors can regulate the duration of activation of a MAPK cascade. The *S. cerevisiae* MAPKs are inactivated by several phosphatases that may form stable associations with their target MAPKs [30,34]. These include a dual-specificity phosphatase Msg5 that inactivates Fus3, protein tyrosine phosphatases Ptp2 and Ptp3 that inactivate Hog1, Fus3, and Mpk1 with different specificities, and a serine-threonine phosphatase Ptc1 that inactivates Hog1 MAPK (reviewed in [35]). Yeast MAPK phosphatases can be induced by their cognate MAPK either by direct phosphorylation or increased expression [34].

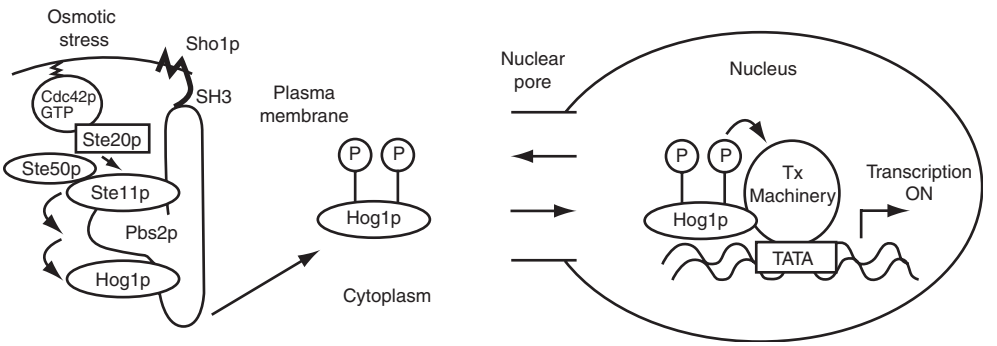


Figure 5 Hog1 forms a stable part of the transcriptional activation machinery.

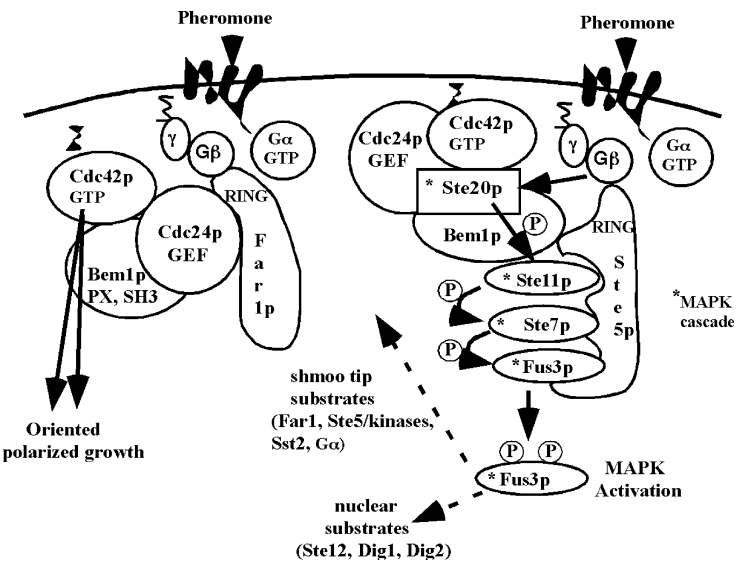


Figure 6 MAPK Fus3 at cell cortex may promote polarized growth fusion.

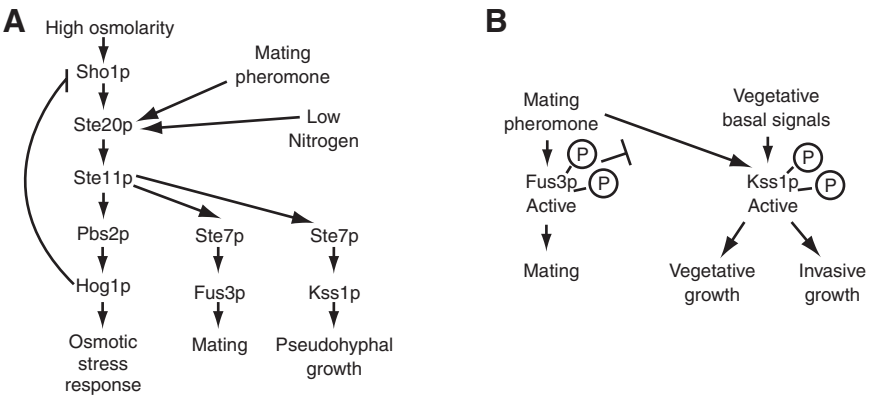


Figure 7 Yeast MAPKs crossregulate one another to maintain specificity.

Because there is considerable sharing of signal transduction components between the MAPK cascades, inputs into one MAPK cascade have the potential to affect multiple pathways. An interesting feature of the yeast MAPKs is their propensity to act as attenuation factors and prevent cross-talk with MAPKs within the same or other pathways. In haploid cells, the mating MAPK Fus3 inhibits the invasive growth and vegetative growth pathways and prevents their activation by mating pheromone (Fig. 4B, [7,13]). Activated Fus3 attenuates the activity of MAPK Kss1 [36], thereby reducing the strength and duration of its activation by pheromone, presumably preventing hyperactivation of the invasive growth pathway. The osmoregulatory pathway MAPK Hog1 inhibits the Fus3 and Kss1 MAPKs both in the absence and presence of hyperosmotic stress (Fig. 7, [37,38]). This cross-regulation requires Hog1 kinase activity and may involve attenuation of the transmembrane sensor Sho1 to prevent persistent activation of the MKKK Ste11. A variety of evidence suggests that MAPKs attenuate the activity of their own pathway through direct phosphorylation of upstream signaling components. For example, MAPK Fus3 phosphorylates Ste5, Ste7, and Ste11 *in vitro* and could directly regulate signal amplification. Fus3 also associates Gpa1-GTP (Gα) *in vivo,* and this interaction appears to attenuate pathway activity [22].

CELL ARCHITECTURE

The strength and nature of the MAPK cascade activation process is dependent upon proper cellular architecture. Regulators of the actin cytoskeleton, such as the Rho family member Cdc42 and an SH3-domain morphogenesis protein Bem1, have been demonstrated to modulate the activity of the *S. cerevisiae* mating and invasive growth and pseudohyphal development MAPK cascades [39]. A MAPK cascade can also be secondarily activated as a consequence of changes in cellular architecture that arise from activation of a primary MAPK cascade. For example, activation of the mating MAPK by pheromone induces polarized growth, which in turn activates the PKC1-regulated MAPK cascade [40].

Acknowledgments

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CHAPTER 184

x^{c,v} Mammalian MAP Kinases

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Introduction

MAP kinases are critical mediators of signal transduction in mammalian cells [17,43,54,58,95]. The MAP kinases are encoded by a group of genes that are related in sequence. These enzymes are also functionally related. For example, MAP kinases phosphorylate substrate proteins on conserved Ser-Pro and Thr-Pro motifs [22]. However, the substrate specificity of MAP kinases is different for individual MAP kinase isoforms. This substrate specificity is mediated, in part, by the selective docking of MAP kinases to substrate proteins [29]. A second functional similarity between MAP kinases is the mechanism of activation. MAP kinases are activated within conserved protein kinase signaling modules composed of a MAP kinase, a MAP kinase kinase, and a MAP kinase kinase kinase. Phosphorylation and activation of MAP kinases by MAP kinase kinases occurs on two residues within a tripeptide motif (Thr-Xaa-Tyr) located within the T loop that controls the conformation of the MAP kinase active site [18].

There are three major groups of mammalian MAP kinases, the extracellular signal regulated protein kinases (ERK), the p38 MAP kinases, and the c-Jun NH2-terminal kinases (JNK) (Table I). These groups of MAP kinases can be distinguished by the sequence of the dual phosphorylation motif that mediates MAP kinase activation: Thr-Glu-Tyr (ERK), Thr-Gly-Tyr (p38), and Thr-Pro-Tyr (JNK). In addition, there are several protein kinases that are related to the MAP kinases with similar dual phosphorylation motifs: Thr-Glu-Tyr (MAK and MOK), Thr-Asp-Tyr (ICK, KKIAMRE, KKIALRE), and Thr-His-Glu (NLK). Here I review the properties of these MAP kinases and MAP kinase-related protein kinases.

The ERK Group of MAP Kinases

ERKl and ERK2

The ERK1 and ERK2 protein kinases are activated by phosphorylation of the Thr-Glu-Tyr motif located in the T loop by the MAP kinase kinases MEK1 and MEK2 (Table II). Structural analysis of nonphosphorylated and inactive ERK2 [114] and phosphorylated and activated ERK2 [13] by X-ray crystallography reveals that the mechanism of activation involves conformational changes that remodel the T loop and the active site. The activated form of ERK2 has been identified as a homodimer [50] and it is likely that this dimeric form of ERK2 is critical for nuclear accumulation [19].

The ERK1 and ERK2 protein kinases are major targets of the Ras signaling pathway. Substrates of these MAP kinases include a wide array of proteins, including the epidermal growth factor receptor [22], cytoplasmic phospholipase A2 [59], and Ets family transcription factors [64]. The ERK1 and ERK2 protein kinases are implicated in proliferation [119], tumorigenesis [63], and differentiation [21]. It appears that the time course of ERK1 and ERK2 activation may be critical for specifying the biological outcome of signal transduction: transient activation correlates with growth and sustained activation correlates with growth arrest and differentiation [21].

Recent studies indicate that the targeted disruption of the *Erkl* gene in mice causes defects in thymocyte development [83] and causes increased synaptic plasticity associated with improved striatal-mediated learning and memory [67]. It is likely that these limited phenotypes reflect the partial complementation of ERK1-deficiency by ERK2. Further studies of ERK2-deficiency and compound mutations in ERK1 and ERK2 will be important to establish the function of these protein kinases *in vivo* (Table III).

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Table I Nomenclature of Human MAP Kinases

Name	Alternative name	Gene name	Chromosomal location	LocusID/accession number
ERK1	p44 MAPK	*MAPK3*	16pll.2	5595
ERK2	p42 MAPK	*MAPK1*	22qll.21	5594
ERK3-related	p63 MAPK	*MAPK4*	18ql2-q21	5596
ERK3	p97 MAPK	*MAPK6*	15q21	5597
ERK5	Big MAP kinase, BMK	*MAPK7*	17pll.2	5598
ERK7		*ERK7*		XM 139448
ERK8		*ERK8*	Chromosome 8	225689
p38α	CSBP, RK, SAPK2A	*MAPK14*	6p21.3-p21.2	1432
p38β	p38-2, p38β2, SAPK2B	*MAPK11*	22ql3.33	5600
p38γ	SAPK3, ERK6	*MAPK12*	22ql3.33	6300
p38δ	SAPK4	*MAPK13*	6p21.1	5603
JNK1	SAPKγ, SAPK1C	*MAPK8*	10q21.1	5599
JNK2	SAPKα, SAPK1A	*MAPK9*	5q35	5601
JNK3	SAPKβ, SAPK1B	*MAPK10*	4q22.1-q23	5602

Human MAP kinases corresponding to the ERK group (ERK1, ERK2, ERK3, ERK3-related, ERK5, ERK7, and ERK8), the p38 MAP kinase group (p38α, p38β, p38γ, p38δ), and the JNK group (JNK1, JNK2, and JNK3) are presented with their alternative names, the gene name, the chromosomal localization, and the LocusID or Accession Number.

Table II Nomenclature of Human MAP Kinase Kinases

Name	Alternative name	Gene name	Chromosomal location	LocusID
MEK1	MAPKK1,MKK1	*MAP2K1*	15q22.1-q22.33	5604
MEK2	MAPKK2, MKK2	*MAP2K2*	7q32	5605
MKK3	MEK3, SKK2	*MAP2K3*	17qll.2	5606
MKK4	MEK4, SEK1, JNKK1, SKK1	*MAP2K4*	17pll.2	6416
MEK5	MKK5	*MAP2K5*	15q22.1	5607
MKK6	MEK6, SKK3	*MAP2K6*	17q25.1	5608
MKK7	MEK7, SEK2, JNKK2, SKK4	*MAP2K7*	19pl3.3-pl3.2	5609

Human MAP kinase kinases are presented with their alternative names, the gene name, the chromosomal localization, and the LocusID.

ERK3

The ERK3 subgroup of MAP kinases is encoded by two genes [10,36]. These protein kinases are not true members of the ERK group because they contain the sequence Ser-Glu-Gly instead of the dual phosphorylation motif Thr-Glu-Tyr. A MAP kinase kinase activity for ERK3 has been described, but the regulation of the ERK3 protein kinases is not understood [17]. The physiological function of the ERK3 protein kinases is also unclear [17].

ERK5

The ERK5 protein kinase is activated by the MAP kinase kinase MEK5 by dual phosphorylation on the conserved T-loop motif Thr-Glu-Tyr [28,137]. Substrates of ERK5 include members of the MEF2 family of transcription factors [47]. The signaling pathways that lead to the activation of the ERK5/MEK5 module are poorly understood. However, ERK5 is critical for survival signal transduction by retrograde NGF receptors localized in endosomes [115] and for proliferation caused by epidermal growth factor [48]. Recent studies indicate that ERK5 is essential for viability during murine embryonic development because ERK5-deficiency causes defects in cardiovascular development [87].

ERK7 and ERK8

The ERK7 and ERK8 protein kinases form a subgroup of ERK-related protein kinases that contain the dual phosphorylation motif Thr-Glu-Tyr [2,3]. ERK7 and ERK8 are not activated by MEK1 and MEK2. Recent studies indicate that ERK7 is constitutively activated by autophosphorylation [1]. However, ERK8 appears to have a low basal activity that is increased in cells with activated Src or following

Table III Targeted Disruption of Genes in Mice

	Phenotype	Reference
ERK1	Viable. Defects in thymocyte maturation. Increased synaptic plasticity and improved striatal-mediated learning and memory.	[83,67]
ERK5	Lethal. Embryos die during mid-gestation. Defects in cardiovascular development.	[87]
p38α	Lethal. Defects in formation of the placenta and expression of erythropoietin.	[4,5,104]
JNK1	Viable. Defects in CD4 effector T cell differentiation and function. Defects in CD8 T cell activation.	[25,24,92,20]
JNK2	Viable. Defects in CD4 effector T cell differentiation and function. Increased CD8 T cell activation.	[90,24,92,20]
JNK1 + JNK2	Lethal. Embryos die during mid-gestation with neural tube closure defects and increased fore-brain apoptosis. Primary fibroblasts are resistant to stress-induced apoptosis.	[53,91,112]
JNK3	Viable. Defects in neuronal apoptosis in response to excitotoxic stress.	[130]
MEK1	Lethal. Defects in placental development *in vivo* and cell migration *in vitro.*	[35]
MKK3	Viable. Defects in cytokine secretion by CD4 T cells and macrophages. Defects in activation-induced cell death of peripheral CD4 T cells.	[60,125,105]
MKK4	Lethal. Embryos die during mid-gestation with liver apoptosis.	[79,80,128,33,81, 102,82,111]
MKK6	Viable. Defects in thymocyte apoptosis.	[105]
MKK7	Lethal. Embryos die during mid-gestation. Defects in TNF-stimulated JNK activation.	[24,93,111]
MKK4 + MKK7	Lethal. Embryos die during mid-gestation. JNK is not activated in cultured fibroblasts.	[111]

The effect of disruption of the murine genes that encode MAP kinases and MAP kinase kinases is summarized.

serum stimulation [3]. Interestingly, ERK7 appears to be associated with an intracellular chloride channel [85]. The physiological function of ERK7 and ERK8 is unclear and further studies of these MAP kinases are warranted.

The p38 Group of MAP Kinases

The regulation of the p38 group of MAP kinases differs from the ERK group of MAP kinases [95,54]. The ERK1 and ERK2 protein kinases are activated by many growth factors by a Ras-dependent mechanism. In contrast, the p38 MAP kinases are activated by inflammatory cytokines and by the exposure of cells to environmental stress. It is thought that members of the Rho family of small GTPases, rather than the Ras family of GTPases, are critical mediators of p38 MAP kinase activation. The p38 group of MAP kinases is activated by three different MAP kinase kinases [43]. Two of these MAP kinase kinases specifically activate the p38 MAP kinases (MKK3 and MKK6). In contrast, the third MAP kinase kinase (MKK4) activates both the JNK and p38 MAP kinases.

p38α and p38β MAP Kinases

The p38α and p38β MAP kinases are structurally related protein kinases that contain the dual phosphorylation motif Thr-Gly-Tyr [54,95]. The structure of p38α MAP kinase has been determined by x-ray crystallography [14,122]. This structure is similar to ERK2. However, there are significant differences that distinguish p38α MAP kinase from ERK2. Included among these differences is the structure of the active site of p38α MAP kinase. This difference has allowed the discovery of active site-directed inhibitors that are extremely selective for p38α and p38β MAP kinases [27]. These drugs are useful for functional dissection of the p38α and p38β MAP kinase signaling pathway. In addition, it is likely that such drugs may be useful for therapeutic intervention. In particular, a major role for p38α and p38β MAP kinases in inflammatory responses has been identified. The p38α and p38β MAP kinases appear to be critical for the expression of several inflammatory cytokines, including interleukin-1, interleukin-6, and tumor necrosis factor [56]. These p38 MAP kinases regulate several steps in cytokine expression including transcription, mRNA stability, and translation. The transcriptional effects of the p38α and p38β MAP kinases are mediated by phosphorylation of several transcription factors, including ATF2 and members of the MEF2 and Ets families [39,86,121]. The mechanisms that account for the effects of p38α and p38β MAP kinases on mRNA stability and translation have not been defined. However, recent studies have implicated proteins that bind to the 3' ARE element of regulated mRNAs and MAPKAP2, a protein kinase that is phosphorylated and activated by p38α and p38β MAP kinases [52,62,77,123].

Mice with targeted disruption of the p38α MAP kinase gene have been described [4,5,104]. These mice die during mid-gestation because of defects in the formation of the placenta. p38α MAP kinase-deficient cells derived from these embryos exhibit severe defects in the expression of inflammatory cytokines [5].

p38γ and p38δ MAP Kinases

The p38γ and p38δ MAP kinases are related enzymes that represent a separate subgroup of p38 MAP kinases [54,95].

Like other members of the p38 family, the p38γ and p385 MAP kinases contain a Thr-Gly-Tyr dual phosphorylation motif. However, the p38γ and p385 MAP kinases are not inhibited by drugs that selectively inhibit the p38α and p38β MAP kinases [27]. The regulation of the p38γ and p385 MAP kinases is similar to the p38α and p38β MAP kinases, and all four p38 MAP kinases are activated by inflammatory cytokines and exposure to environmental stress. Interestingly, p38γ MAP kinase interacts with the PDZ domain of od-syntrophin, although the functional significance of this interaction is unclear [40]. The physiological role of p38α MAP kinase is unclear. However, recent studies have demonstrated an important role for p38δ MAP kinase in the regulation of translation by eEF2 kinase [51].

The JNK Group of MAP Kinases

The JNK group of MAP kinases is activated by many of the same stimuli that cause activation of the p38 MAP kinases, including the exposure of cells to inflammatory cytokines and environmental stress [23]. This similarity in regulation indicates that the JNK and p38 MAP kinases may be functionally related. Indeed, both the JNK and p38 MAP kinases are collectively named stress-activated protein kinases (SAPK). However, the mechanism of JNK activation differs from the p38 MAP kinases. First, the dual phosphorylation motif located in the T loop of JNK is Thr-Pro-Tyr. Second, the signaling module that activates JNK includes the MAP kinase kinases MKK4 and MKK7 [23]. The protein kinase MKK7 is a specific activator of the JNK pathway, while MKK4 can activate both p38 MAP kinase and JNK [23]. Biochemical studies demonstrate that MKK4 and MKK7 can cooperate to activate JNK by selectively phosphorylating JNK on Tyr and Thr, respectively, and gene disruption experiments in mice demonstrate functional cooperation of MKK4 and MKK7 *in vivo* [30,55,111].

The JNK protein kinases are encoded by three genes. Two of the genes *(JNK1* and *JNK2)* are expressed ubiquitously and one gene *(JNK3)* is expressed in a limited number of tissues, including the brain, heart, and testis [23]. Alternative splicing creates ten different JNK protein kinases: four JNK1 isoforms, four JNK2 isoforms, and two JNK3 isoforms [38]. Analysis of JNK3 by x-ray crystallography indicates that this MAP kinase is structurally related to both ERK2 and p38α MAP kinase [127].

The physiological role of JNK appears to be complex. It is established that JNK is required for the normal regulation of AP-1 transcription activity [23]. This is mediated, in part, by the phosphorylation of the transcription factors ATF2, c-Jun, JunB, and JunD on two sites within the NH2-terminal activation domain [23]. JNK can also regulate other transcription factors by phosphorylation. Nevertheless, although it is known that JNK causes increased AP-1-dependent gene expression, the physiological consequence of JNK activation appears to be both cell-type and context dependent. JNK can cause apoptosis by a mechanism that involves the mitochondrial pathway [57,112]. JNK can also signal cell survival [41,84]. The mechanism that accounts for these markedly divergent cellular responses to JNK activation is unclear. However, it is likely that the cellular response to JNK activation reflects the activation state of other signal transduction pathways within the cell. A reasonable hypothesis is that the program of gene expression that is induced by JNK-stimulated AP-1 activity depends on the cooperation of AP-1 with other transcription factors bound to the promoters of relevant genes. Further studies will be required to test this hypothesis.

Gene disruption studies in mice demonstrate that JNK1, JNK2, and JNK3 are not essential for viability [20,25,90,92, 129,130]. However, these mice exhibit defects in apoptosis and immune responses. Compound mutations in JNK1 and JNK2 cause early embryonic lethality associated with neural tube defects and markedly increased apoptosis in the developing forebrain [53,91]. Studies of *Jnk1-/- Jnk2-/-* CD4 T cells isolated from *Rag1 -/-* chimeric mice indicate that JNK is required for effector CD4 T-cell function, but is not required for CD4 T-cell activation [24]. Primary embryo fibroblasts isolated from *Jnk1 -/- Jnk2 -/-* embryos exhibit severe growth defects with premature senescence associated with increased expression of ARF and p53. These JNK-deficient fibroblasts also exhibit marked resistance to stress-induced apoptosis [112]. This resistance to apoptosis was also observed in *Mkk4 -/- Mkk7 -/-* fibroblasts that contain JNK, but lack a mechanism to allow JNK activation [111]. Together, these data indicate that JNK contributes to multiple physiological processes, including differentiation, survival, and apoptosis.

MAP Kinase-Related Protein Kinases

There are three major groups of mammalian MAP kinases (ERK, p38, and JNK). Several additional human protein kinases have been identified that exhibit similarities with these MAP kinases (Table IV). Thus, two protein kinases (MAK and MOK) contain the same dual phosphorylation motif that is found in the ERK group of MAP kinases (Thr-Glu-Tyr). It is established that MOK is regulated by phosphorylation on this motif, but the mechanism that causes this phosphorylation is unclear [73]. Similar studies of MAK have not been reported [66]. The physiological role of MAK is unclear because MAK-deficient mice lack an obvious phenotype [100]. Three protein kinases have been identified (ICK, p42 KKIALRE, p56 KKIAMRE) that contain a related dual phosphorylation motif (Thr-Asp-Tyr). The role of the dual phosphorylation motif in these kinases is unclear. Mutational analysis indicates that the dual phosphorylation motif is required for the regulation of ICK [110], but it is not essential for the regulation of p42 KKIALRE and p56 KKIAMRE [103]. The protein kinase (NLK) contains the divergent motif Thr-His-Glu. Phosphorylation of this motif in response to the MAP kinase kinase kinase TAK1 is required for NLK activation [99]. Further studies are warranted to discover whether these protein kinases (and other related protein kinases) represent additional members of the MAP kinase family.

Table IV Human MAP Kinase-related Protein Kinases

Name	TXY motif	Gene name	Chromosomal location	LocusID
ICK	TDY	*ICK*	6pl2.3-pll.2	22858
p42 KKIALRE	TOY	*CDKL1*	14q21.3	8814
p56 KKIAMRE	TDY	*CDKL2*	4q21.1	8999
MAK	TEY	*MAK*	6q22	4117
MOK	TEY	*RAGE*	14q32	5891
NLK	THE	*NLK*	17qll.2	51701

Human protein kinases that are related to MAP kinases are presented with the sequence of the TXY dual phosphorylation motif, the gene name, the chromosomal location, and the LocusID.

MAP Kinase Docking Interactions

Although MAP kinases can phosphorylate Ser-Pro and Thr-Pro sites on substrate proteins, the substrate specificities of individual MAP kinases are distinct. One mechanism that can dictate the substrate specificity of a MAP kinase is the requirement for a docking site on the substrate [29]. The docking site is physically separate from the site of phosphorylation and is required for efficient substrate phosphorylation by MAP kinases. Mutational removal of the docking site prevents substrate phosphorylation by MAP kinases. Examples of docking sites include the δ domain on c-Jun that interacts with JNK [23], the D domain on the Elk-1 transcription factor that binds ERK and JNK [132,133], the D domain on MEF transcription factors that binds p38α MAP kinase [131], and the FXF motif on the SAP-1 transcription factor that binds ERK and p38δ MAP kinase [31,45]. The δ and D domains are similar and consist of a Leu-Xaa-Leu motif separated from several basic residues [29]. In contrast, the FXF domain contains the motif Phe-Xaa-Phe-(Pro) [29].

Interestingly, these docking domains are conserved in many proteins that interact with MAP kinases. Thus, the NH2-terminal region of MAP kinase kinases contains a D domain [7]. Disruption of this MAP kinase docking site on MAP kinase kinases is caused by the anthrax lethal factor protease and prevents MAP kinase activation [26]. Docking sites are also observed in MAP kinase phosphatases and scaffold proteins [29]. It therefore appears that many proteins that interact with MAP kinases contain conserved motifs that mediate this interaction [29]. Mutational analysis indicates that these motifs bind MAP kinases at a common site [106–108]. A recent study has provided structural insight into the mechanism of protein docking to MAP kinases. This study used x-ray crystallographic analysis to determine the structures of the complexes of p38α MAP kinase with the D domains of MKK3 and MEF2 [14]. This analysis demonstrated that the Leu-Xaa-Leu motif is directly involved in the protein-protein contact and that the basic residues in the D domain may interact with acidic residues in the common docking site [116]. The site of interaction on the surface of p38α MAP kinase is not located close to the T loop or the active site. Thus, the active site and the T loop of MAP kinases are available for interaction with docked proteins, including MAP kinase kinases, MAP kinase phosphatases, and substrates.

Scaffold Proteins

The protein kinases that form MAP kinase signaling modules can interact via a series of sequential binary interactions to create a protein kinase cascade. One example is represented by MKK4, which can dock to both an upstream kinase (MEKK1) and to a downstream MAP kinase (JNK) [126]. Alternatively, the protein kinases may simultaneously interact with a common component of the cascade. Thus, MEKK2 can synergistically interact with both MKK4 and JNK [16] and MEKK1 can bind c-Raf-1, MEK1, and ERK2 [46]. MEKK1 can also bind JNK in a phosphorylation-dependent manner [32]. These interactions may lead to the assembly of a functional signalling module [118].

Functional MAP kinase signaling modules can also be created by the interaction of the protein kinases with scaffold molecules that serve to assemble the protein kinases [118]. These putative scaffold proteins include KSR, MP1, the JIP proteins, β-arrestin-2, and SKRP1/MKPX (Table V). In addition, several other molecules have been proposed to function as MAP kinase scaffolds, including Filamin [65], Crk II [34], and IKAP [42].

KSR

Kinase suppressor of Ras (KSR) shares structural similarity with the MAP kinase kinase kinase c-Raf-1 [74,89]. One major difference between KSR and c-Raf-1 is that while KSR does have a protein kinase-like domain, KSR does not function as a protein kinase [70]. KSR binds to c-Raf-1, MEK1/2, and ERK1/2 and appears to function as a scaffold for the activation of the ERK1/2 signaling module that is activated by growth factors [74,89]. Following the activation of growth factor receptors, KSR is recruited to the cell surface by a phosphorylation-dependent mechanism that involves the

Table V Nomenclature of Human MAP Kinase Scaffold Proteins

Name	Alternative name	Gene name	Chromosomal location	LocusID
KSR-1	KSR	*KSR*	17qll.l	8844
KSR-2			Chromosome 12	125806
MP-1		*MAP2K1IP1*	4q22.3	8649
JIP-1	IB1	*MAPK8IP1*	Ilpl2-pll.2	9479
JIP-2	IB2	*MAPK8IP2*	22ql3.33	23542
JIP-3	JSAP1, SYD2	*MAPK8IP3*	16pl3.3	23162
β-Arrestin-2		*ARRB2*	17pl3	409
SKRP1	JKAP	*SKRP1*	2q32.1	142679
MKPX	VHX, JSP-1	*MKPX*	6p24.3	56940

Human MAP kinase scaffold proteins are presented with their alternative names, the gene name, the chromosomal localization, and the LocusID.

C-TAK1 protein kinase [75]. Gene disruption studies in mice have examined the requirement of KSR for growth-factor-stimulated ERK1/2 activation. KSR-deficient cells derived from these mice were found to exhibit partial defects in ERK activation [78]. This partial defect may reflect a specialized role for KSR under certain conditions. Alternatively, it is possible that the functions of KSR are redundant with the product of another gene that encodes a KSR-like protein (Table V). Further studies are required to define the role of these KSR proteins. Nevertheless, it is very likely that the mammalian KSR proteins do function as an essential scaffold for ERK1/2 activation because RNAi experiments have demonstrated an important role for KSR in the activation of ERK in *Drosophila* [88].

MP1

The MP1 scaffold protein binds to MEK1 and ERKl [94]. Transfection assays demonstrate that MP1 potentiates the activation of ERK1 caused by MEK1 [94]. Recent studies demonstrate that MP1 also binds to the late endosomal protein p!4 [124]. MP1 therefore localizes a MEK1/ERK1 signaling module on the cytoplasmic surface of late endosomes. It is possible that MP1 contributes to ERK activation following ligand-induced endocytosis of growth factor receptors. This role of MP1 on late endosomes serves to distinguish MP1 from the KSR scaffold proteins that appear to function at the cell surface. The possible functional interaction between the MP1 and KSR scaffold proteins warrants further study. In addition, gene disruption studies are required to establish the physiological function of MP1 in ERK activation *in vivo.*

JIP

Three genes encode the JIP group of scaffold proteins [23]. The JIP1 and JIP2 proteins are structurally similar and contain an SH3 domain and a PTB domain in the COOH-terminal region [9,76,117,134]. The PTB domain can interact with p190 RhoGEF and with members of the low density lipoprotein receptor family, including ApoER2 [37,69,101]. In addition, JIP2 has been reported to bind the Rac exchange factor Tiaml, Ras-GRF, and members of the fibroblast growth factor homologous protein family [12,96,97]. The JIP3 protein is structurally distinct and consists of an extended coiled-coil domain [44,49]. All of these JIP proteins share several common properties. Each JIP isoform binds to JNK, MKK7, and members of the mixed-lineage group of MAP kinase kinase kinases. An interaction of JIP3 with MEKK1 and MKK4 has also been described [44]. Transfection studies demonstrate that the JIP proteins potentiate the activation of JNK [44,49,117,134]. Some studies have demonstrated that JIP2 can also activate p38 MAP kinases under some circumstances [96,97].

The JIP1, JIP2, and JIP3 proteins bind to kinesin light chain and are transported by the microtubule motor protein kinesin [11,113,120]. This interaction with motor proteins accounts for the accumulation of the JIP proteins in the growth cones of developing neurons . In mature neurons, the JIP1 and JIP2 proteins accumulate at synapses and JIP3 is mostly localized to perinuclear vesicular structures. The JIP proteins may act as adapter molecules for the transport of cargo by the kinesin motor protein. In addition, the JIP proteins may act to locally regulate JNK activation in response to specific stimuli.

Two groups have reported the phenotype of mice with targeted disruption of the *Jip1* gene. One group reported that the JIP1-deficiency causes very early embryonic lethality prior to implantation [109]. In contrast, a second group reported that JIP1-deficient mice are viable [120]. It is possible that the difference in viability reflects an effect of the mouse strain background. The viable JIP1-deficient mice were found to exhibit defects in stress-induced JNK activation in hippocampal neurons following exposure to stress. Studies of JIP2- and JIP3 -deficient mice will be required to identify the redundant and nonredundant functions of these JIP scaffold proteins.

β-Arrestin

Ligand-induced activation of seven transmembrane-spanning receptors causes receptor phosphorylation, recruitment of arrestin molecules, and subsequent downregulation of heterotrimeric G protein signaling [71]. The arrestin molecules can also serve as a platform for the recruitment of additional molecules to the receptor [71]. Evidence has been presented that indicates that the ubiquitously expressed isoform β-arrestin-1 may serve as a scaffold for components of the ERK pathway [61]. More detailed studies have been performed on β-arrestin-2. This scaffold protein contains a D domain that selectively binds to JNK3 [68,72]. Interestingly, both β-arrestin-2 and JNK3 are selectively expressed in the brain and the heart. The β-arrestin-2 scaffold also binds the MAP kinase kinase kinase ASK1 [68]. The signaling module assembled by β-arrestin-2 also contains MKK4, which interacts with both JNK3 and ASK1, but does not directly contact β-arrestin-2 [68]. Biochemical assays demonstrate that β-arrestin-2 is essential for the activation of JNK3 caused by the angiotensin II receptor [68]. Interestingly, the activated receptor bound to the β-arrestin-2 scaffold complex is localized to endosomal structures. The β-arrestin-2 scaffold provides a mechanism for the activation of JNK by seven transmembrane-spanning receptors. In addition, the signaling module assembled by β-arrestin-2 provides a mechanism for the selective activation of the JNK3 isoform of JNK. Studies of β-arrestin-2-deficient mice have been reported [8]. Further studies of these mice to investigate defects in the activation of JNK3 are warranted.

SKRP1/MKPX

SKRP1 and MKPX are two related small phosphatases that belong to the MAP kinase phosphatase (MKP) family. Like other MKPs, these phosphatases can inactivate MAP kinases [6,135]. However, it appears that the normal function of these phosphatases is to activate JNK [15,98,136]. Interestingly, the phosphatase catalytic activity is required for these MKPs to activate JNK, but the physiologically relevant substrates have not been identified. It appears that these phosphatases interact with MKK7 [15,136] and may also interact with ASK1 [136]. Gene disruption studies in mice demonstrated that SKRP1 is not an essential gene, but SKRP1 is required for JNK activation caused by tumor necrosis factor-α and transforming growth factor-β, but not for JNK activation caused by ultraviolet radiation [15]. Together, these data suggest that these MKPs may act as scaffold proteins for the JNK signaling pathway.

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CHAPTER 185

Subcellular Targeting of PKA Through AKAPs: Conserved Anchoring and Unique Targeting Domains

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Introduction

Regulation of the opposing actions of adenylyl cyclases (AC) and phosphodiesterases (PDE) that control levels of the diffusible intracellular second messenger cAMP is central to many signaling responses. The major effector of cAMP in eukaryotic cells is the cAMP-dependent protein kinase (PKA). PKA is a heterotetrameric enzyme consisting of two regulatory subunits (R) that dimerize and each bind and inhibit a single catalytic subunit (C) to form an R_2C_2 holoenzyme (Fig. 1A) [1]. Activation of the inactive PKA holoenzyme by cAMP occurs when two molecules of cAMP bind to each R subunit, resulting in a conformational change that releases the active C-subunits from the inhibitory R_2 dimer (Fig. 1A). The released active C-subunits phosphorylate serine and threonine residues commonly found in sequence contexts of RRXS/T or KRXXS/T to regulate target protein function. The catalytic subunit (C) of PKA can phosphorylate target proteins rapidly near the site of release and in time diffuse to more distant location such as the nucleus, where additional targets are phosphorylated [2]. It is remarkable that this ubiquitous signaling pathway is used in different cell types to tranduce signals to myriad different yet very specific target proteins in a variety of cellular compartments. Thus, elucidating how this PKA signaling versatility is achieved without compromising specificity is fundamental to understanding cAMP signal transduction pathways.

Over the last decade we have learned that both diversity and specificity in cAMP signaling is in large part achieved by targeting the PKA holoenzyme to discrete subcellular locations such that, upon release of the C subunit, phosphorylation of co-localized target substrates is greatly enhanced (Fig. 1B) [2]. This subcellular targeting of PKA is mediated by a class of anchoring-scaffolding proteins called AKAPs (A-kinase anchoring proteins) [3–5]. AKAPs anchor PKA by binding the R-subunit dimer through a structurally conserved anchoring domain and then target the holoenzyme to specific locations within the cell through unique targeting domains (Fig. 1B). AKAPs frequently contain additional protein-protein interaction motifs that serve as tethering sites for the target substrates, as well as additional signaling proteins such as other kinases, phosphatases, scaffold, and adapter proteins. Thus, many AKAPs function as signal-integrating scaffolds that coordinate both subcellular localization and complex assembly of multiprotein signal tranduction machines. This chapter will focus on the structurally conserved R-subunit

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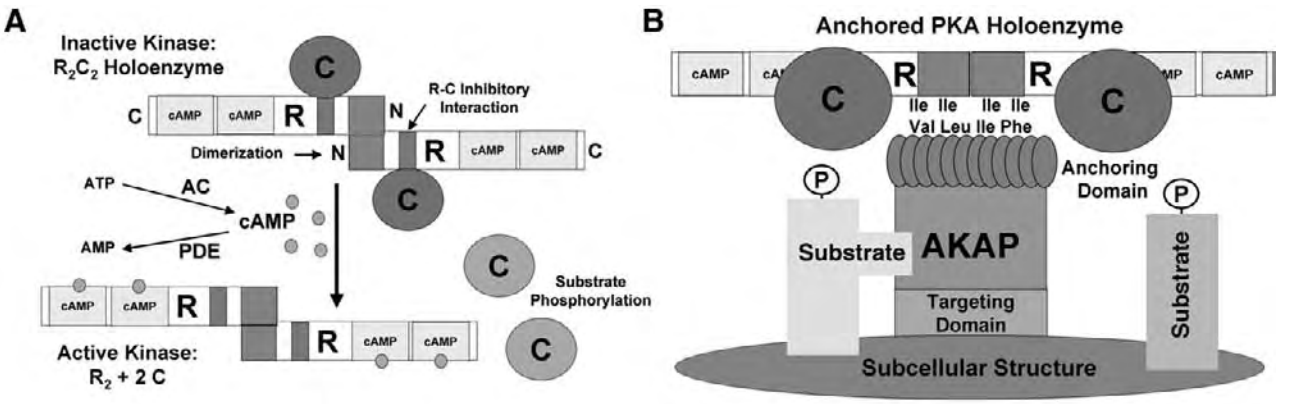


Figure 1 Anchoring and subcellular targeting of PKA by AKAPs. (A) Structure of the PKA holoenzyme and regulation of catalytic actvity by cAMP. (B) AKAP anchoring of the PKA holoenzyme near specific cellular substrates. A co-localized substrate and a tethered substrate (see Chapter 186 by Scott) are shown.

anchoring domain and examples of unique subcellular targeting domains that localize anchored-PKA for regulation of localized target substrates. The following chapter will deal more with the issue of multi-enzyme signaling complexes that are maintained by AKAPs.

Structurally Conserved PKA Anchoring Determinants

There are three PKA C subunit isoforms (Cα, Cβ, Cγ) and four R subunit isoforms (RIα, RIβ, RIIα, RIIβ) [1]. Although the C subunit isoforms share very similar properties, the RIα,β and RIIα,β subunit groups show significant differences in both cAMP-binding affinity [1] and AKAP anchoring [6]. Thus, due to these differences, PKA is divided into type I and type II holoenzymes based on the identity of the R subunit dimer present. It is clear that both type I and type II PKA holoenzymes can bind to AKAPs and thus be targeted to specific subcellular domains, but the majority of AKAP proteins bind RII dimers with 100 to 1000-fold higher affinity than RI dimers [6–10]. This difference in AKAP binding affinity is reflected in observations that in many cell types the type II PKA is more discretely localized to specific cellular structures whereas type I PKA is diffusely distributed in the cytoplasm. The differences in cAMP-binding affinity between RI and RII fit well with these differing distributions. Type I holoenzymes have higher cAMP-binding affinities and thus may be adapted to generate more prolonged responses to lower cAMP signals encountered in the bulk cytoplasm. In contrast, type II holoenzymes bind cAMP with lower affinity, a possible adaptation to being targeted by AKAPs to local environments where cAMP can be much higher due to compartmentalized regulation of either AC or PDE [11,12]. One important function of reduced cAMP sensitivity might be to maintain low activity of type II holoenzyme anchored in local environments, where even basal cAMP levels would fully activate the type I enzyme, thus leading to complete loss of C subunits.

A number of mutagenesis studies have shown that AKAPs bind hydrophobic residues in the N-terminus of the R subunits in a manner that depends on formation of the R-R dimer [13–15]. Indeed, recent NMR structural studies show that both the RI and RII N-terminal dimerization domains form anti-parallel four-helix bundles in which dimerization creates an extended hydrophobic surface that binds the AKAP (Fig. 2C) [16–18]. This hydrophobic surface is somewhat less extensive and more sterically hindered in RI compared to RII dimers, a result that possibly explains why in general most AKAPs bind RII with higher affinity than RI. Accordingly, the AKAPs all bind to the R-subunit N-terminal dimerization domain through hydrophobic interactions [18–21]. However, PKA-anchoring domains from different AKAPs have very little primary amino acid sequence similarity yet share a conserved hydrophobic character and secondary structure (Fig. 2A,B) [18,20,21]. Thus, AKAPs are a family of functionally related proteins arising from convergent evolution as opposed to diverging from a common ancestral AKAP protein.

The common secondary structure in AKAP PKA-anchoring domains is seen as a conserved spacing of hydrophobic residues that map to one side of an amphipathic alpha-helix of about 18 residues in length (Fig. 2A,B) [18,20]. One exception to this conservation of an amphipathic-helix anchoring domain is seen in Pericentrin, which anchors PKA-R subunits through a unique, more extended hydrophobic motif of unknown structure [22]. For the conserved anchoring motif found in all other AKAPs, mutagenesis studies have lent support to the amphipathic helix structural model by showing that substitution of hydrophobic residues with either hydrophilic residues to change polarity or proline residues to break helical structure causes inhibition of R-subunit binding [19,20]. Recent NMR structures have been obtained showing clearly that two AKAP-anchoring domain peptides, Ht31 (493-515) and AKAP79(392-413), with divergent primary sequences both bind to RII in similar helical conformations with extensive contacts made between the hydrophobic face of the helices and the large hydrophobic surface on the RII dimer (Fig. 2C) [18]. These extensive hydrophobic interactions explain why these two AKAPs bind PKA-RII with such high affinities. High-affinity R-subunit

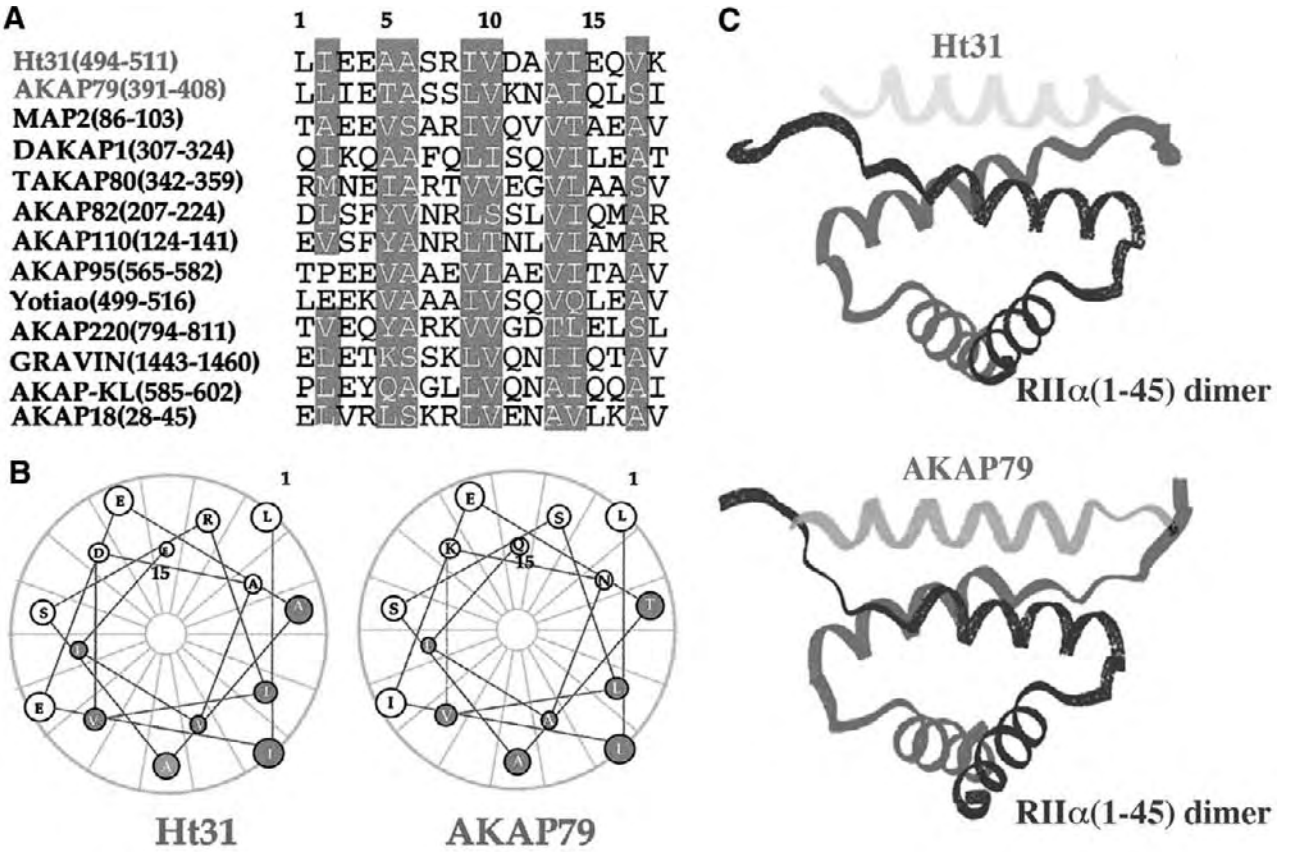


Figure 2 Structurally conserved AKAP-PKA anchoring domains. (A) Divergent primary structures for PKA-anchoring in AKAP family members. Hydrophobic residues are blocked. (B) Conserved amphipathic alpha-helical secondary structures for AKAP PKA-anchoring domains. Hydrophobic residues are shaded on helical wheel diagrams. (C) Conserved mechanisms of PKA anchoring revealed by NMR solution structures of AKAP-RII complexes. The structures show AKAP anchoring domain peptides from Ht31(493-515) and AKAP79 (392-413) in green bound to an RIIα(1-45) N-terminal domain dimer. This figure was adapted from Newlon *et al.* (2002) *EMBO J*. **20**, 1651–1662 (18) with permission from P. A. Jennings and Oxford University Press.

binding of isolated AKAP-anchoring domain peptides has allowed them to be used as competitive inhibitors of PKA-anchoring in cells to probe numerous cellular functions of AKAP-PKA complexes in cAMP signaling (discussed more below).

Unique Subcellular Targeting Domains

The variety and specificity that is made possible by AKAP-PKA anchoring is in large part a function of unique targeting domains in different AKAP molecules (Fig. 1B). AKAP molecules have been identified at many distinct subcellular locations (Fig. 3A,B), including the plasma membrane, intracellular vesicles [23,24], actin and microtubule cytoskeletons, mitochondria, endoplasmic reticulum (ER), Golgi, and centrosomes [25,26]. For example, MAP2 is targeted to dendritic microtubules in neurons by direct binding to tubulin [27–29]. Scar/Wave1, an AKAP that also anchors the abl-Tyrosine kinase, binds to actin both in focal adhesions (Fig. 3B) and membrane ruffles in fibroblasts, where it regulates the actin polymerization activity of the Arp2/3 complex (see next chapter) [30]. The skeletal and cardiac muscle-enriched mAKAP protein, which also anchors PDE4D3 (see next chapter) [11], is targeted by a series of spectrin repeats to perinuclear ER/SR membranes, including most prominently the nuclear membrane (Fig. 3B) [31,32]. D-AKAP1/sAKAP84 can be targeted either to the ER (in the liver) or mitochondria (in most other cells) by two different N-terminal targeting sequences produced by alternate mRNA splicing [33–35]. One AKAP, AKAP95, has even been shown to associated with the nuclear matrix and chromatin, even though PKA holoenzyme/R-subunits are excluded from the nucleus during interphase (Fig. 3A) [36,37]. However, interactions of AKAP95 with PKA and chromatin could have important functions in regulating chromosome condensation during mitosis when the nuclear envelope is absent [38,39].

In certain cell types precise localization to discrete plasma membrane domains is seen, such as localization to apical or basolateral membrane domains in polarized epithelial cells and synaptic membrane specializations in neurons. AKAP75/79/150, an AKAP scaffold protein that also binds protein kinase C and calcineurin-protein phosphatase 2B(CaN-PP2B) (see next chapter), is targeted to the plasma membrane/cortical cytoskeleton (Fig. 3A) by three N-terminal

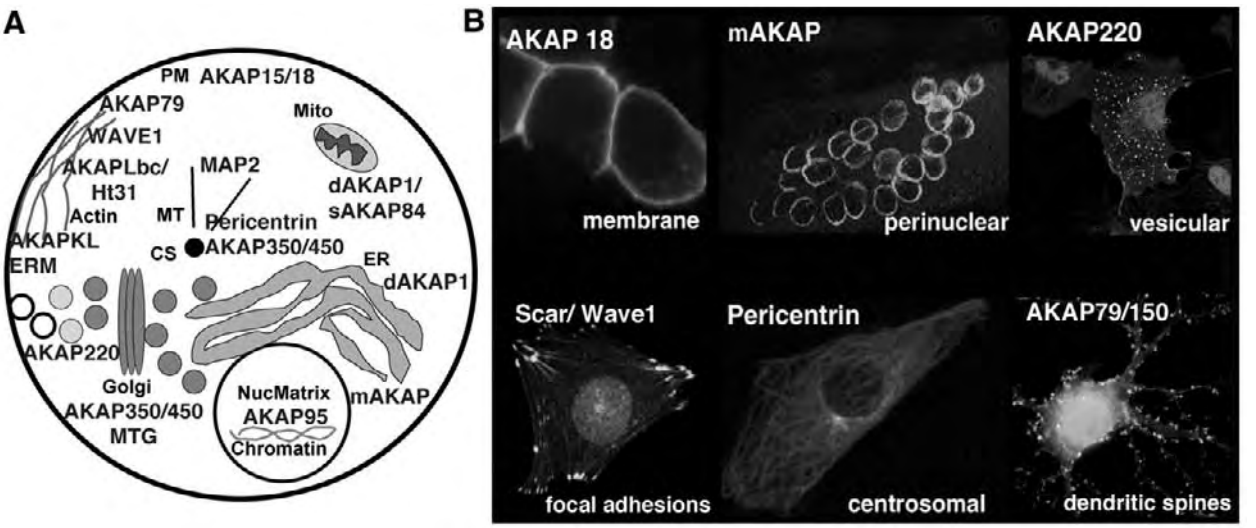


Figure 3 Unique AKAP targeting domains and subcellular localizations. (A) Subcellular compartmentalization of different AKAP family members. References for AKAPLbc/Ht31 [63] and MTG Localization [64]. For other references see the text. (B) Specific subcellular localizations observed for selected AKAPs.

polybasic domains that bind to acidic phospholipids, including phosphatidylinositol-4,5-bisphosphate, as well as F-actin [40–42]. This same N-terminal basic domain mediates targeting to excitatory postsynaptic membrane specializations located on actin-rich dendritic spines in neurons (Fig. 3B) [42]. The low-molecular-weight AKAP15/18α is targeted to the plasma membrane in HEK-293 cells by N-terminal lipid modifications of myristoylation of Gly-1 and dual palmitoylation of Cys-4, Cys-5 (Fig. 3B) [43]. In MDCK epithelial cells this AKAP15/18α isoform selectively targets to the basolateral membrane. However, an alternate splice variant, AKAP15/18β, which contains an additional exon coding for a 24 amino acid insert, localizes to the apical membrane [44]. It is interesting that two additional AKAP families, the AKAP-KL and ERM proteins (Ezrin/Radixin/Moesin), are also specifically targeted to apical membranes, where they bind to cortical actin (Fig. 3A) [45,46].

Probing Cellular Functions of AKAP-PKA Anchoring

The importance of compartmentalized pools of PKA has been implicated in numerous cellular responses, including transcription [47,48], secretion [43,49,50], and cell cycle-regulation [39,51,52]. However, functional roles for AKAP-PKA targeting have been studied in the greatest detail for cAMP regulation of membrane ion channels [53,54]. These studies of ion channel regulation have either used anchoring inhibitor peptides such as Ht31(493-515) to displace PKA from endogenous AKAPs or heterologous co-expression of an AKAP with the ion channel of interest. Some of the best examples of both of these approaches come from studies of PKA-regulation of neuronal ionotropic glutamate receptors and skeletal and cardiac muscle L-type calcium channels. In each case, use of anchoring inhibitor peptides to disrupt PKA-R-subunit anchoring was first shown to block cAMP regulation of endogenous plasma membrane channel activity similar to inhibition of PKA-C subunit catalytic activity [55–58]. Thus, these studies suggested that endogenous AKAPs were important for targeting PKA to the plasma membrane in close proximity to the regulated channels. Subsequent studies confirmed these results by showing that co-expression of these channels with an appropriate membrane-targeted AKAP partner, such as AKAP15/18α or AKAP79, could reconstitute cAMP-PKA regulation of channel activity in heterologous HEK-293 cell expression systems [57,59,60]. In contrast, heterologous expression of the channels by themselves was characterized by a complete lack of PKA regulation or abnormal PKA regulation relative to that seen for endogenous channels. Very recently it has been appreciated that AKAPs and their anchored pools of PKA can be even more directly targeted to membrane ion channel substrates through protein-protein interactions (see next chapter; Fig. 1B). In heterologous systems, AKAP79 and AKAP15/18α were shown to be able to substitute for each other in some aspects of PKA channel regulation [43,57,60]; however, more recent biochemical and electrophysiological studies clearly indicate that these AKAPs serve more specific functions *in vivo* as well as in heterologous systems. In particular, AKAP15/18α is very likely to serve specifically in PKA regulation of skeletal and cardiac L-type channels through forming a complex with channel proteins [61]. In contrast, AKAP79 seems to be adapted for PKA and CaN-PP2B regulation of postsynaptic AMPA-subtype ionotropic glutamate receptors linked through additional protein-protein interactions with PSD-95 family MAGUK scaffold proteins (see next chapter) [60,62].

Conclusions and Future Directions

In summary, subcellular targeting of PKA by AKAPs appears to be a very efficient mechanism for adapting the versatile cAMP signal transduction pathway for highly selective local regulatory phosphorylation events. The key factors in maintaining both the diversity and specificity of this system are the unique subcellular targeting domains present in different AKAP family members. Thus, it is these targeting interactions that might serve in the future as targets for the development of novel therapeutics. The attractiveness of this approach is already supported by studies described in the next chapter that show that selective disruption of interactions between AKAPs and ion channel substrates has the same functional effect as disrupting PKA anchoring to the AKAPs with the nonselective anchoring inhibitor peptides [61]. Furthermore, recent studies of AKAP79/150 targeting to excitatory synapses suggest that regulation of AKAP-targeting domains by cell signaling pathways may serve as important endogenous mechanisms that control PKA signaling [42]. Thus, further dissection of the mechanisms of AKAP targeting, as well as the substrate binding and scaffolding interactions discussed in the following chapter, will continue to be active and important areas of AKAP research in the next few years.

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CHAPTER 186

AKAP Signaling Complexes: The Combinatorial Assembly of Signal Transduction Units

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Understanding the molecular organization of intracellular signaling pathways is a topic of considerable research interest. Multiprotein signaling complexes create focal points of enzyme activity to disseminate the intracellular action of many hormones and neurotransmitters. The spatio-temporal activation of protein kinases and/or phosphatases is important in controlling where and when phosphorylaton events occur. Anchoring proteins and targeting subunits provide a molecular framework that orients protein kinases and phosphatases toward selected substrates. Prototypic examples of these "signal-directing molecules" are A-kinase anchoring proteins (AKAPs) that sustain multicomponent signaling complexes of the cAMP dependent protein kinase (PKA) and G proteins and other enzymes. These protein-protein interactions not only focus PKA toward certain substrates but also spatially segregate parallel signaling pathways.

Introduction

The efficient transmission of cellular signals often involves the positioning of signaling proteins in proximity to their upstream activators and downstream targets. In fact, the clustering of receptors, G proteins, and enzymes with their substrates is believed to contribute significantly to the specifcity of signaling. This sophisticated degree of organization may also help prevent the indiscriminate activation of related signaling complexes that are close by. This is of particular importance for second messenger dependant signaling pathways that lead to the activation of broad specificity enzymes such as the cAMP dependent protein kinase (PKA), protein kinase C (PKC), and a variety of calmodulin dependant kinases (CaM kinases). Compartmentalization of these enzymes is often achieved through their association with scaffolding proteins that simultaneously coordinate the location of several enzymes [1–4].

A-kinase anchoring proteins (AKAPs) are a growing family of scaffolding proteins that package PKA and other signaling enzymes into multiprotein complexes [5,6]. As discussed in the previous chapter, each AKAP contains both a conserved amphipathic helix that binds to the R subunit dimer with high affinity and a targeting domain that directs the PKA-AKAP complex to specific subcellular compartments [7,8]. Another important role for AKAPs is to place PKA in the proximity of enzymes such as phosphatases and phosphodiesterases that terminate cAMP signaling events [9–11]. The focus of this chapter is to highlight advances in our understanding of AKAP signaling complexes and their role in facilitating this bi-directional control of various signaling events.

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G-Protein Signaling Through AKAP Signaling Complexes

A shared property of several AKAPs is to position enzymes in microenvironments where they can respond to upstream signals. Clearly, there are potential advantages of anchoring PKA in close proximity to primary transduction elements such as G-protein coupled receptors and the cAMP synthesis machinery. In fact, two anchoring proteins, gravin/AKAP250 and AKAP79/150, maintain kinase complexes that bind to the β2-adrenergic (β2-AR) receptor [12,13]. The AKAP79 complex binds to regions within the third cytoplasmic loop and C-terminal tail of the β2-AR in an agonist-independent manner, whereas gravin/AKAP250 recruits PKA and PKC to the receptor in an agonist-dependent manner [13–15] (Fig. 1). These receptor based AKAP complexes also contribute to β2-AR phosphorylation, desensitization, and indirect activation of MAP kinase pathways that emanate from the receptor [16]. Furthermore, dephosphorylation of β2-AR and the receptor kinase GRK2 are likely to be important signal termination events in this process and could be mediated by an anchored pool of PP-2B that associates with AKAP79. Another anchoring protein MAP2 seems to nucleate a membrane associated signaling complex that includes β2-AR, adenylyl cyclase, PKA, PP-2B, and a substrate for the kinase of the class C L-type Ca^{2+} channel [17,18]. The identification of such a signaling complex emphasizes the notion that receptors, effectors, kinases, and their substrates are spatially coordinated. However, it also confirms the view put forward by a number of investigators that in some cases cAMP may not have to diffuse very far from its site of synthesis to activate the PKA holoenzyme.

Other classes of G proteins have been implicated in the channeling of signals through AKAP complexes, although not necessarily via cAMP dependant mechanisms [4]. Scar/WAVE-1 is a member of the Wiskott-Aldrich syndrome family of scaffolding proteins that binds PKA, the Abl tyrosine kinase, and the Arp2/3 complex, a group of seven proteins that control actin remodeling [19–21] (Fig. 1). The dynamic assembly of this complex at sites of lamellapodial extension occurs in response to growth factor signals that activate the low-molecular-weight GTPase Rac [19]. Consequently, Scar/WAVE may direct PKA and Abl toward cytoskeletal substrates and synchronize cell movement by ensuring efficient transmission of Rac-mediated signals to the actin remodeling machinery. Analogous AKAP signaling networks participate in the formation of actin stress fibers. AKAP-Lbc, a splice variant of the Lbc oncogene, encodes a chimeric molecule that anchors PKA and functions as a Rho-selective guanine nucleotide exchange factor [22]. Application of lysophosphatydic acid or selective expression of Gα12 enhances cellular AKAP-Lbc activation and leads to the formation of actin stress fibers in fibroblasts [22]. This provides an example where the spatial organization of heterotrimeric and small molecular weight G proteins may involve interactions with the same AKAP. Finally, certain unconventional modes of signaling to PKA may also be governed by G-protein recruitment to AKAP-signaling complexes. For example, the testis specific anchoring protein, AKAP110, has been reported to interact with the heterotrimeric

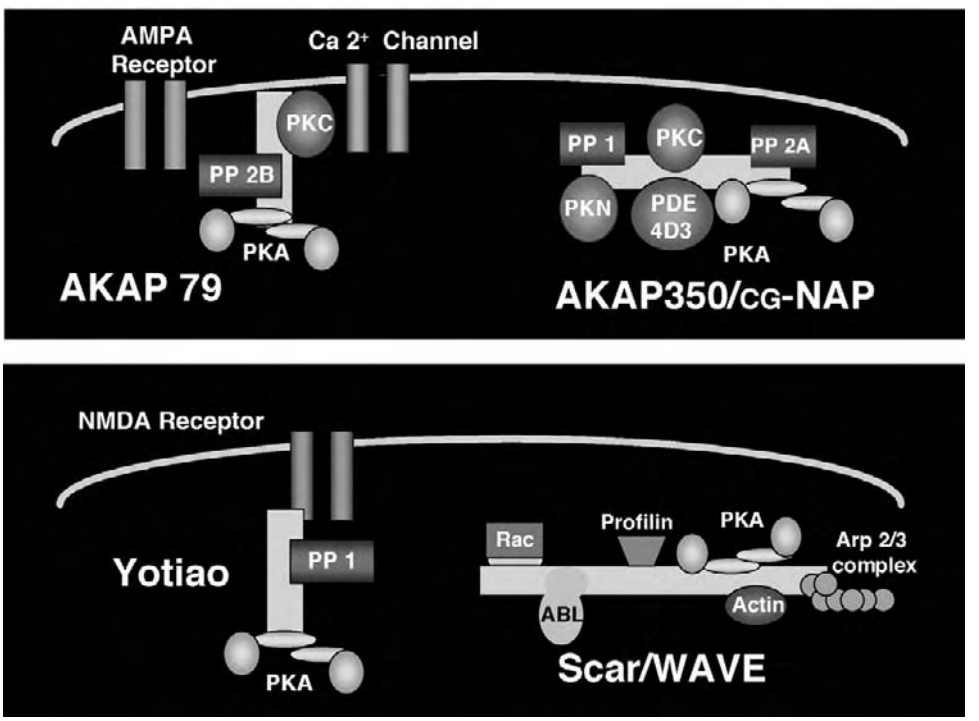


Figure 1 AKAP signaling complexes. A schematic representation of certain AKAP signaling complexes discussed in this chapter. Each interacting protein is labeled.

G-protein subunit Gα13 that activates AKAP110-associated PKA via a cAMP-independent mechanism [23]. Each of these examples underscores the notion that AKAP-signaling complexes can respond to G-protein signaling events in a variety of pathways.

Kinase/Phosphatase Signaling Complexes

Several AKAP signaling complexes include both signal transduction and signal termination enzymes. This generates a locus to regulate the forward and backward steps of a given signaling process. One example of this, mentioned in other chapters, is the clustering of second messenger regulated kinases and phosphatases at the excitatory synapses of neurons by the AKAP79/150 family of anchoring proteins [24].

The human form AKAP79 and its bovine and murine counterparts AKAP75 and AKAP150, respectively, are enriched in the synaptosomal and postsynaptic densitiy fractions of neuronal lysates and are present in dendritic spines [25,26]. In 1995 the A subunit of the calcium/calmodulin dependent phosphatase PP2B was identified in a two-hybrid screen with AKAP79 as bait [27]. Subsequent biochemical and cellular analyses defined the phosphatase-binding site and demonstrated that both enzymes simultaneously associate with AKAP79/150 in neurons [28]. A year later it was demonstrated that PKC is also a component of the AKAP79 signaling complex [29]. At that time it was postulated that the simultaneous anchoring of these three signaling enzymes generated a locus for the integration of distinct second messenger signals at the postsynaptic membranes [30]. Functional studies have largely confirmed this notion by showing that the AKAP79/150-signaling complex controls the phosphorylation status and facilitates the regulation of a variety of ion channels, including L-type calcium channels, KCNQ potassium channels, aquiporin water channels, and AMPA type glutamate receptor ion channels [30–33] (Fig. 1).

The most detailed studies have dissected the phosphorylation events that occur on the cytoplasmic tail of AMPA type glutamate receptors. This channel is present at the terminals of excitatory synapses and is gated by the release of glutamate across the synaptic cleft [34,35]. A series of reports have shown that the AKAP79 signaling complex is recruited into a larger transduction unit with the GluR1 subunit of the AMPA type glutamate receptor (reviewed by Dodge and Scott [24]). Simultaneous association with the membrane-associated guanylate kinase bridging protein SAP97 brings the channel and the signaling complex together [36]. Functional studies indicate that AKAP79-bound PKA enhances GluR1 phosphorylation on serine 845 in the cytoplasmic tail of the channel subunit, an important site for the regulation of channel function during the induction of long-term synaptic depression (LTD) [37–39]. These findings extend an earlier report showing that perfusion of anchoring inhibitor peptides into cultured hippocampal neurons antagonizes PKA anchoring and causes rundown of synaptic AMPA-type glutamate receptor activity [40]. Since this phenomenon occurs with a time-course that is similar to the inhibition of the kinase, it was initially assumed that disruption of PKA anchoring displaced the kinase from the proximity of the AMPA receptor. However, more recent studies indicate that the phosphatase PP2B may play a prominent role in the downregulation of channel activity. Electrophysiological recordings suggest the proximity of the AKAP79-bound phosphatase to sites of calcium entry ensures that the enzyme is rapidly activated upon synaptic elevation of intracellular calcium and is responsible for the dephosphorylation of serine 845. It is interesting that serine 845 is phosphorylated by PKA upon elevation of synaptic cAMP levels [41,42]. Thus AKAP79/150 maintains a kinases and a phosphatase in close proximity to the channel in a manner that allows second messenger dependant changes in the phosphorylation status and activity of GluR1 (Fig. 1).

Other synaptic AKAPs also maintain kinase/phosphatase complexes. For example, yotiao interacts with the NR1a subunit of synaptic NMDA glutamate receptor ion channels and anchors PKA and protein phosphatase 1 (PP-1) [10,43–46]. The modulation of NMDA receptors containing NR1a requires interactions with the scaffolding protein as peptide-mediated displacement of either PKA or PP-1 causes changes in the modulation of channel activity [10]. Thus yotiao maintains a signaling complex that is directly attached to the substrate, the NMDA receptor (Fig. 1). Although this example certainly highlights the role of AKAPs to ensure the rapid preferential phosphorylation of substrates, the compartmentalization of enzymes in the yotiao complex may also contribute to the segregation of signals at excitatory synapses, where the GluR1/AKAP79 complex is also in the immediate vicinity.

Two AKAP additional signaling complexes are found at the centrosome. AKAP350/CG-NAP, a large centrosomal AKAP of unknown function, has been reported to bind three kinases (PKA, PKC, and PKN) and two phosphatases (PP-1 and PP2A; Fig. 1) [20,47–49]. Likewise, pericentrin, an integral component of the centreolar machinery, anchors PKA and other enzymes, presumably for a role in the coordination of centrosomal phosphorylation events. An interesting finding is that both AKAP350. CG-NAP and pericentrin contain a c-terminal PACT domain that is responsible for targeting each anchoring protein to the centrosome. Expression of this 100 amino acid region alone is sufficient to promote centrosomal targeting of GFP [50]. This raises the intriguing possibility that the PACT domains of these anchoring proteins might interact with the same structure in the centrosome in a mutually exclusive manner. This could represent one mechanism to generate greater diversity, as distinct signaling complexes could be tethered to the same cellular locus. Thus, the possibilities for coordinated phosphorylation and dephosphorylation events mediated by association with AKAPs are increased.

cAMP Signaling Units

Another way to exert tight control of PKA phosphorylation events is to compartmentalize the kinase with enzymes that

control the intracellular concentrations of its activator, cAMP. In fact, two recent reports have demonstrated that phosphodiesterases, the enzymes that catalyze cAMP degradation, are components of AKAP/PKA signaling complexes [51,52]. These findings add to the complexity of cAMP signaling as they point toward a role for anchored pools of phosphodiesterase in the tight control of local second messenger concentrations. This in turn controls where and when PKA becomes active. For example, a muscle-selective anchoring protein mAKAP directly binds PKA and a splice variant of the cAMP-specific, type 4 phosphodiesterase PDE4D3 and targets them to the perinuclear membranes of cardiomyocytes [51]. Yet in Sertoli cells the PDE4D3 interacts with AKAP350/CG-NAP, one of the large centrosomal AKAPs discussed above (Fig. 1) [52].

Two important regulatory factors that are built into these cAMP-signaling modules favor the signal termination process. First, the tethered PDE is constitutively active and will rapidly restore basal cAMP levels when the flow of second messenger is turned off from its site of synthesis at the plasma membrane. Second, elegant experiments have demonstrated that PKA phosphorylation of PDE4D3 on serine 54 increases the Vmax of the enzyme two- to threefold over basal conditions [53–57]. Phosphorylation of PDE4D3 increases cAMP degradation to favor reformation of the PKA holoenzyme. PKA anchoring is a unique and critical element in this PKA-PDE4D3 feedback loop, as displacement of the kinase with the anchoring inhibitor peptide Ht31 prevents cAMP-dependent stimulation of the mAKAP associated PDE4D activity [51]. This finding emphasizes the importance of PDE localization to maintain the balance of intracellular cAMP levels. This notion is also supported by recent imaging studies using intermolecular FRET that have shown that micro-gradients of cAMP emanate from sites of synthesis at the plasma membrane. Hormonal stimulation of cardiomyocytes induced changes in the rate and magnitude of local cAMP gradients with the concomitant effect on the activation of anchored PKA pools [58]. Thus multiple regulatory processes are involved in controlling where and when cellular PKA activation occurs.

Although PDE4D3 is a substrate for the kinase, it is clear that there are other PKA substrates associated with the mAKAP scaffold. For example, the regulation of ryanodine receptor (RyR) phosphorylation is important for maintaining contractility in response to β-adrenergic signaling and increases in intracellular Ca^{2+} concentration in the heart. Hyperphosphorylation of sarcoplasmic reticulum RyR leads to increased Ca^{2+} sensitivity of the channel and decreased sensitivity to β-adrenergic stimulation [59–61]. These changes are manifest in human heart tissue undergoing heart failure where changes in RyR phosphorylation are also detected [59]. Atypical regulation of RyR function may be due to several factors that regulate cAMP/PKA signaling in heart, including loss of phosphatase activity from the RyR complex [59] and defects in regulation of cAMP levels by PDE activity associated with the complex [51]. It is interesting that two groups have detected PP1 and PP2A phosaphatase subunits in the mAKAP signaling complex. Given the myriad binding partners for mAKAP, it is plausible to suggest that the composition of this signaling network may be altered in response to different intracellular stimuli and in disease states.

Conclusions and Perspectives

AKAPs provide the platforms for the assembly of multiprotein signaling complexes in a variety of cellular compartments. Two factors contribute to the diversity of these signaling units. First, individual AKAP complexes may control distinct signaling events within the same subcellular compartment. This may be best exemplified by WAVE-1 and AKAP-Lbc that nucleate the formation intracellular signaling cascades to catalyze distinct forms of cytoskeletal reorganization. In both cases receptor occupancy at the plasma membrane triggers the assembly of signaling complexes that transmit distinct signals to the actin cytoskeleton [19,22]. Likewise, AKAP350 and pericentrin may synchronize different phosphorylation events at the centrosome, and three anchoring proteins, D-AKAP-1/sAKAP82, D-AKAP-2, and Rab32, are involved in mitochondrial signaling processes [62–64]. Second, the recruitment and release of individual enzymes from the signaling complex provides a dynamic component to the composition of a given protein network. For example, Ca^{2+}/calmodulin antagonizes PKC anchoring by competing for binding to AKAP79. The calcium influx from ion channels within the synaptic membrane releases PKC from its anchor, changing the activity status of this kinase. Presumably the soluble enzyme is more available to propagate calcium/phospholipid signaling events, as it has a less restricted access to its substrates. An additional level of complexity may be present, as biochemical and proteomic experiments have detected each PKC isoform in AKAP79/150 complexes isolated from rat brain [65,66]. This implies that at these synapses a variety of AKAP79/150 signaling complexes exist that contain conventional, novel, or atypical PKC isozymes. Again this adds to the diversity of AKAP signaling, as each PKC class responds to different combinations of phospholipid activator. These examples not only highlight the sophisticated degree of spatial organization achieved by anchoring proteins but also emphasize the degree of specificity that can be generated through the combinatorial assembly of unique AKAP signaling complexes.

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CHAPTER 187

Protein Kinase C Protein Interactions

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Introduction

Protein kinase C (PKC) was initially identified in screens for broad specificity kinases that could, like protein kinase A (PKA), respond to second messengers and so integrate agonist-induced responses (see [1]). There is no doubt that the PKC family, comprising the classical (α,β,γ), novel (δ,ε,η,θ), and atypical (ζ,ι/λ) isoforms, fulfil both elements of this definition, that is, they are responsive to second messengers and they are of broad specificity (at least *in vitro*). This latter property has triggered the question of how specificity is imposed on this rather promiscuous group of proteins. In part such considerations have led to the identification of binding partners and substrates that provide specificity to the system. However, this is not all that is achieved by such PKC-binding partners; interactions are seen to operate at a number of levels that can be related to the "life cycle" of the kinase. These can be summarized as follows (see Fig. 1):

PKC priming
PKC activation
PKC substrate engagement
PKC inactivation

Although a number of reviews on this subject have described classes of binding proteins or specific examples (see [2] and references therein), here we discuss examples of PKC-binding proteins with reference to the above properties conferred on PKC proteins.

Priming

Although considered to be under acute allosteric control by diacylglycerol (DAG) and possibly other bioactive lipids [3], PKC proteins all appear to require multisite phosphorylation for their optimum function (see [4]). In some respects this can be viewed as a priming device for subsequent DAG-dependent (or other) activation.

The dephosphorylated form of PKCα is intrinsically insoluble [5], and it is likely that the unphosphorylated primary translation product is associated with an as yet unidentified chaperone. This is suggested by the observation that the newly synthesized protein is soluble, unlike its phosphorylated and then dephosphorylated counterpart [5]. There is to date no evidence that PKCα is a client protein for known chaperones such as Hsp90, and the identity of its "chaperone" remains to be determined. However, for PKCε, which undergoes a similar array of phosphorylation events, the anchoring protein CG-NAP (centrosome and Golgi localized PKN-associated protein) binds the hypophosphorylated form of the protein. It has been proposed that CG-NAP acts as a scaffold to promote phosphorylation of PKCε [6], taking the chaperone role for this member of the family.

The subsequent steps in this priming process are the phosphorylations themselves. There is general agreement that a key step in this process involves the action of the upstream kinase PDK1 ([7,8] and see Newton, this volume). This PtdIns3,4,5P_3-activated kinase can bind directly to all

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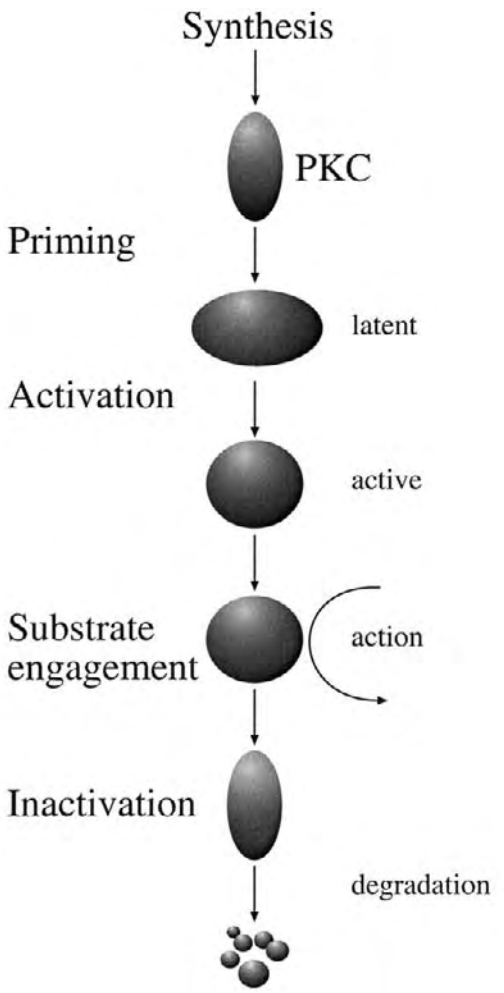


Figure 1 The life and death of PKC. Various states along the pathway of PKC synthesis, modification, activation, and proteolysis are indicated. The generic events associated with the different phases of existence are denoted on the left and referred to in the text in respect of PKC-binding proteins. These events take place in distinct compartments in part defined by binding partners (see text).

members of the PKC superfamily (α, β, γ, δ, ε, ζ, η, θ, ι /λ) [7]. PDK1-PKC interaction appears to be mediated through the PKC kinase domain [7], and based upon the PRK2-PDK1 interaction studies it would appear to involve the conserved phosphorylated C-terminal hydrophobic motif FXXF{S(P),T(P),E}F/Y [9]. Thus PDK1 can engage these PKC substrates at a site distal to its target site of phosphorylation, which resides in the activation loop of the kinases at the conserved TFCGTP motif. It is unclear whether these PKC-PDK1 interactions confer specificity, efficiency, or some other property to the system. In principle this may reflect an ordering of events at least for those PKCs requiring hydrophobic motif phosphorylation.

14-3-3 proteins act as dimers and engage phosphorylated proteins with a preference for RXXSXP or RXXXSXP motifs [10]. It is known that PKCs can bind 14-3-3 proteins *in vitro* and also *in vivo*, although the role of the interaction is not clear. However, the precedents set by the c-Raf1 protein kinase and its functional interaction with 14-3-3 (as established genetically [11]) indicate that this is likely to be important also in PKC function. Perhaps as for c-Raf1 [12], 14-3-3 facilitates stabilization of phosphorylated conformers of PKCs, thereby contributing to the priming process.

Though not a priming device, tyrosine phosphorylation of PKC, in particular PKCδ, has critical effects on cellular responses (see for example [13]). The distinct functions and reported properties for different phosphorylated forms of PKCδ (reviewed [14]) indicate that some of these tyrosine phosphorylations may be protein autonomous; however, there remains the probability that some involve direct recognition, with the likely recruitment of partners via SH2 domain (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=smart00252) interactions.

Activation

An intrinsic property of the classical and novel PKC allosteric activator DAG is that it is membrane-limited. It is the ability of PKCs to "sample" the membrane environment that provides the opportunity for activation. This feature of PKC activation lends itself to the compartmental targeting of PKC to provide for selectivity of activation.

Targeting of PKC prior to activation is clearly exemplified in the *Drosophila* compound eye, where the scaffolding protein InaD is responsible for assembling the eye-specific phospholipase C NorpA, the eye-specific PKC InaC, and the ion channel protein TRPL (recently reviewed [15]). This assembly contributes to the efficiency with which photoreception is relayed and by which it is terminated. In this instance the interaction of the scaffolding protein InaD with the downstream effectors occurs via PDZ domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=smart00228), with the different proteins binding to distinct PDZ domains. A similar PDZ-dependent interaction is seen for PKCα and the binding partner PICK1 [16]; PKCα has a canonical PDZ binding motif (..SAV) at its C-terminus. The physiological role of this partnership is not understood.

AKAPs (A-kinase associated proteins) represent a distinct class of scaffolds that were first identified as protein kinase A (PKA) binding proteins [17]. Elegant studies by Scott and colleagues have provided evidence for the ability of AKAPs to direct PKA to sites of activation and action (see Chapters 62, 63, this volume). Two of these proteins, AKAP79 and Gravin (AKAP250), independently bind PKC as well as PKA and other proteins [18,19]. In particular, Gravin, which has been isolated in a number of different guises, scaffolds a broad spectrum of signal transducers and cell-cycle regulators (reviewed [20]). One other AKAP (AKAP78) has been identified as ezrin [21], which has been shown recently also to bind PKCα [22] (see further below). Although the specific roles for this group of PKC-binding proteins remain largely unknown, the precedents set by the studies on PKA provide a clear paradigm, and it is anticipated that these scaffolds serve to place PKCs at sites where their action is required. The ability of this class of scaffolds to assemble multiple

transducers also provides for integration (as for InaD) and duplication of signal outputs (for example, a common target for distinct upstream pathways, such as PKA and PKC).

For the atypical PKCs (ι/λ, ζ), activation is not DAG-dependent but can be effected by the assembly of a cdc42/PAR6/PAR3/aPKC complex (recently reviewed [23]). The importance of such an assembly was demonstrated in *C. elegans*, where it was shown that PKC3 (the only aPKC in *C. elegans*) ablation produced a polarity phenotype related to that documented for PAR3 and PAR6 mutants [24]. Subsequent studies have shown that the mammalian homologues of these proteins are involved in establishing the polarity of epithelial cells, where they assemble at and control the formation of tight junctions (see [23]). The sites of interaction for these proteins have been mapped (see Fig. 2). For aPKC the binding site for PAR6 resides in the aminoterminal domain whereas that for PAR3 is in the carboxyterminal, catalytic domain. It also remains possible that subcomplexes form, for example between the brain-specific PKMζ protein (catalytic domain protein from the PKCζ gene involved in memory; see [25]) and PAR3; however, this possibility requires further investigation.

Transmembrane proteins (including receptors) also interact with PKCs, targeting them to the resident compartment. These include β_1-integrin [26], syndecan 4 [27], and tetraspanins [28]. In the case of β_1-integrin, the interaction with PKCα occurs between the cytoplasmic C-terminal integrin tail and the central variable region (V3) of PKCα [29]. This interaction has been reported to be involved in β_1-integrin migratory responses by controlling the traffic of β_1-integrin [26]. There is a complex two-way relationship between β_1-integrin and PKCα, a part of which represents an integrin-scaffolding role in the local assembly with, and PKC phosphorylation of the protein ezrin (see below).

A broad class of transmembrane proteins long-associated with PKC function are ion channels (see [30]). Although it has become apparent that in fact DAG can directly control the TrpL proteins [31], DAG also acts via PKC in other contexts. For example, PKC forms a complex with the GABA(A) receptor β_1 and β_3 subunits [32]. This association, like that conferred by InaD, predisposes the receptor to localized and efficient control by PKC—in this instance through phosphorylation of sites within a conserved motif shared by β1, β2, and β3 subunits [33,34].

The interaction of PKCα with syndecan is distinctive in being promoted by PtdIns4,5,P_2 [35], and the association is negatively regulated by phosphorylation of syndecan on S183, possibly by PKCδ [36]. In the syndecan 4/PtdIns4,5P_2 complex, PKCα is in an active conformation resembling the interaction with RACKs (see below). Syndecan 4 is implicated in responses to FGF2, and the association and activation of PKCα via syndecan 4 is thought to contribute to FGF2-induced endothelial cell migration and proliferation [37].

There is a well-documented class of PKC-binding proteins that interact selectively with the activated conformer. These are typified by the RACKs (receptors for activated c-kinases) first documented by Mochly-Rosen and colleagues [38]. The two characterized RACKs are RACK1 [39] and β-COP (RACK2) [40]. Although the specific roles for these RACKs in the context of PKC action remain incompletely understood, their interactions with PKCs have proved informative. In particular, two features are notable here. First, interaction of PKC with RACK1 has been shown to lock the protein in an active conformation, providing a mechanism for DAG-triggered but DAG-independent sustained activation. Second, the site of interaction of RACKs with PKCs appears to be through the PKC C2/C2-like domain [41]. The latter property has been exploited to demonstrate that short

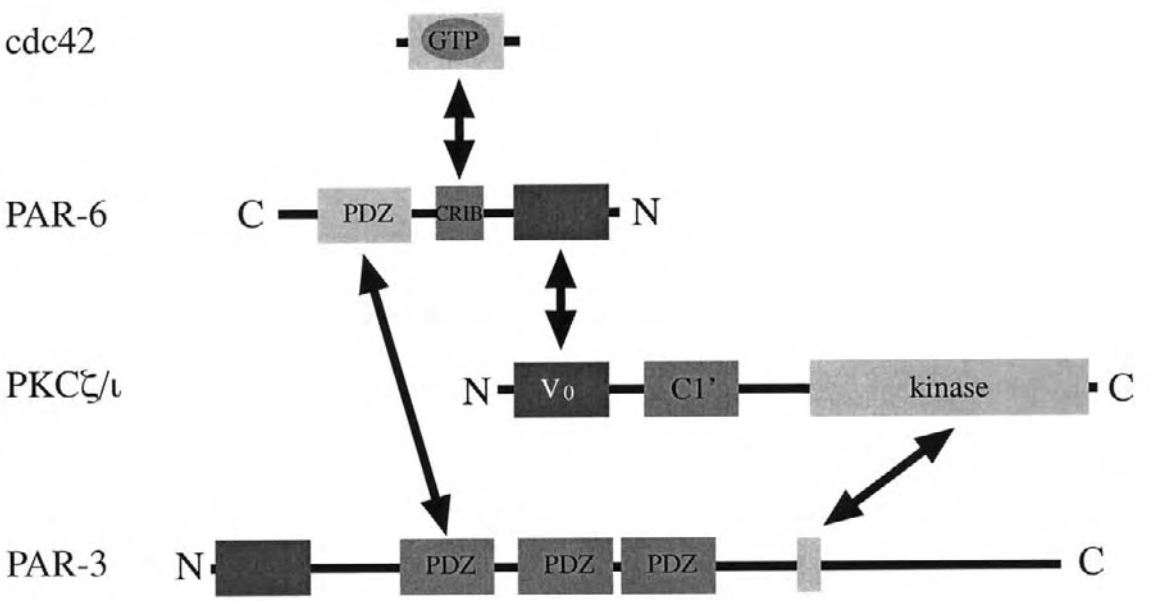


Figure 2 PAR-aPKC associations. The various interactions that compose the aPKC-PAR complex are illustrated (adapted from [23]). The defined domains are as indicated. The PKCζ C1 domain is a C1′-related domain that does not bind DAG/phorbol esters. The association of the small GTP-binding protein cdc42 with PAR6 requires its GTP-bound state.

oligopeptides corresponding to the sites of interaction can block PKC translocation and PKC effects (see [41]). Thus, interactions through these regions of PKC (whether with RACKs or other proteins) appear to be important in PKC action. Corroboration of this has come from studies showing the inhibition of preconditioning in cardiac myocytes with a PKCε inhibitory peptide [42] and the subsequent demonstration in knockout mice that PKCε is essential for one component of preconditioning *in vivo* [43].

Annexin VI is another example of a PKC-binding protein that interacts with PKC (PKCα and β) but is not itself a substrate for the kinase [44]. This interaction is of interest because both proteins bind phospholipids in a Ca^{2+}-dependent fashion and their interaction is dependent upon lipid binding. Nevertheless, there is evidence for direct protein-protein contact in this association [44].

Substrates and Pathways

The distinctions between scaffolds and substrates are somewhat blurred where the scaffolds themselves are substrates. However, conceptually there is a clear distinction between those proteins that bind PKC in its inactive conformation predisposing it to localized responses and those that are recruited postactivation. This latter category is exemplified by the STICK proteins(substrates that interact with c-kinase) [45]. These proteins have been identified by far-western analysis and include the PKC substrates clone72/SSecKS and γ-adducin (see [2]).

The STICK adducin is associated with actin and forms specific heterotetramers of the types α–β or α–γ (reviewed [46]). The PKC-dependent phosphorylation of γ-adducin (at serine 660) is associated with its release from this cytoskeletal location [47]. SSecKS binds a number of other signaling proteins (see above). It is interesting that like the PKC substrates MARCKS and GAP-43, the phosphorylation of SSeCKS by PKC occurs at a calmodulin-binding site, interfering with calmodulin interaction [48]. SSeCKS also undergoes an interaction with cyclinD, which is attenuated by PKC phosphorylation of SSeCKS [48]. Phosphorylated SSeCKS is associated with membrane protrusions and ruffles, thus implying an involvement in the reorganization of cortical actin. How this relates to the nonbinding/release of calmodulin and cyclin D is as yet unclear. The cortical actin association and its control by phosphorylation is also observed for another PKCα-interacting protein, ezrin [22]. In this latter case, there is evidence for the phosphorylation of ezrin by PKCα playing an essential role in β1-integrin dependent directional movement of cells [Ng, 2001 #6991]. As noted above, this also involves integrins (see Fig. 3).

In the context of substrates and pathways, phospholipase D (PLD) is a very interesting example of a PKC-binding protein. PKCα will activate PLD1 *in vitro*, and, as first evidenced in membrane reconstitutions [49], the complex formed between PKCα and PLD1 is sufficient for activation in the presence of other factors; phosphorylation is not required.

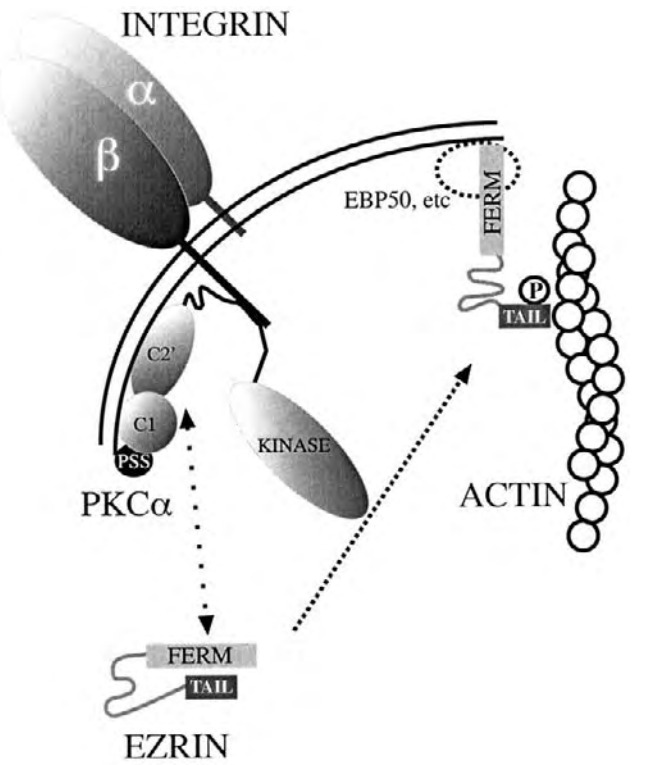


Figure 3 Integrin-PKCα-ezrin associations. PKCα and integrinβ1 form a complex through the V3 domain of PKCα and the C-terminal tail of integrinβ1 [29]. Ezrin can be recruited into this complex, and this leads to ezrin phosphorylation on T567. This phosphorylation alters the conformation by reducing the FERM-tail domain interaction and so permitting distinct contacts to be made through the FERM domain (for example with EBP50) and through the tail domain with actin (see [74]). The domains of PKCα and ezrin are indicated. PSS refers to the PKC pseudosubstrate site, which, along with the C1 and C2 domains, can interact with membranes.

This interaction occurs through the regulatory domain of PKCα and requires the open/active conformer [50]; how the activity of PKC relates to the downstream pathways in this context remains to be determined.

Mammalian genetic analysis of PKC functions has provided a number of insights. Of note here, the mouse knockout of the PKCβ gene leads to a B-cell phenotype reminiscent of Btk loss-of-function [51]. This implies that there is a functional relationship between these two kinases, and indeed they have been shown to interact [52]. This interaction occurs through the PH domain of Btk [52]. Other PH domain-PKC interactions have been documented, including that for PKCη -$PH^{PKCμ/PKD}$ [53]; PKCμ/PKD, like Btk, is also a downstream target for PKC [54].

PKC Inactivation

A characteristic property of classical and novel forms of PKC is that their chronic activation frequently leads to their inactivation and/or degradation. This feature is often observed on phorbol ester (or functionally related pharmacological agonist) stimulation of cells. For PKCα this

process of activation-induced degradation has been documented to proceed via caveolae/raft-dependent traffic, dephosphorylation, and then degradation [55]. PKCα can interact with caveolin 1 through its scaffold domain [56]. This interaction may well contribute to its traffic through the caveolae compartment, although the serum deprivation protein, sdr, has also been proposed as a caveolae targeting device for PKCα [57]. It is possible that a related role is played by p62/ZIP, a PKCζ-interacting protein [58,59] that has been reported to recruit PKCζ and traffic through endosomal fractions in response to EGF [59] and NGF [60].

Dephosphorylation of PKC appears to be effected through protein phosphatase 2A (PP2A) (for example [61]) and there is evidence that PP2A can associate with certain PKC isoforms [62], as has been described for other AGC kinase family proteins [63]. As noted above, dephosphorylated PKC is often found to be associated with the neutral detergent-insoluble fraction. Although this may reflect an intrinsic property, it is of note that the p32 protein recovered from the detergent-insoluble fraction of hepatocytes has been shown to interact with certain conformers of PKC isoforms and hence may contribute to this behavior [64].

The process of degradation of PKC is a ubiquitin-dependent one [65,66], implying that E3 component(s) recognize and interact with PKCs to facilitate ubiquitination. To date the only such component identified that binds PKC isoforms is the VHL tumor suppressor gene product [67]. Whether this is the only interacting E3 protein remains to be determined.

Perspectives

There is an increasing need to be able to monitor at a subcellular level the interactions of PKCs and their binding partners. It will be important to be able to follow such events in real time and in the context of catalytic activity, for example by using antisera that monitor phosphorylation events [68,69]. The elucidation of the spatio-temporal behavior of complexes promises to be very informative in defining how individual ones contribute to responses. Beyond this, application of accumulating molecular knowledge to the development of improved health care is increasingly tractable. Indeed, our understanding of PKC targets has already started to have an impact on clinical trials [70].

In a broader context, it is of interest that comprehensive interaction maps have been compiled for yeast (for example [71]) and other organisms (see web sites in [71]). To date these generic activities have had limited novel input into our appreciation of PKC and its partners in higher eukaryotes; in yeast the defined partner for PKC1, a Rho family member, in fact reflects a property of the PKC-related kinases (PRKs/PKNs) [72]. However such mapping approaches promise much for the future.

It will be evident to those in the field that this review has really only touched on what is a substantial collection of PKC-interacting proteins. Indeed, the diversity of these PKC-associated proteins means that we must acknowledge that the majority have not been discussed in this commentary, and we hope colleagues will accept our apologies if their favorite PKC partner is missing.

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CHAPTER 188

Dendritic Protein Phosphatase Complexes

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AKAP	A-kinase (PKA) anchoring protein
AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AMPAR	AMPA-type glutamate receptor
CaM	calmodulin
CaMKII	Ca^{2+}/CaM-dependent protein kinase II
CREB	cyclic AMP-response element binding protein
DARPP-32	dopamine and cyclic AMP-regulated phosphoprotein of 32 kDa
I1	inhibitor 1
LTD	long-term depression
LTP	long-term potentiation
NMDA	*N*-methyl-D-aspartate
NMDAR	NMDA-type glutamate receptor
PKA	protein kinase A, A-kinase, or cyclic AMP-dependent protein kinase
PP1	protein phosphatase 1
PP2B	protein phosphatase 2B or calcineurin
PSD	postsynaptic density

Introduction

Fast excitatory glutamatergic synaptic transmission is a primary contributor to many normal behaviors such as learning and memory and is often disrupted in complex neural disorders. The primary mediators of excitatory transmission are dendritic ionotropic AMPA-type and NMDA-type glutamate receptors, which are phosphorylated by many protein kinases. The modulatory actions of these kinases are antagonized by dendritic protein phosphatases, although there are incomplete, often contradictory, data implicating specific enzymes in modulating synaptic function, because only a relatively limited number of these enzymes often display promiscuous *in vitro* activity (see chapter 91, Volume 1 by Cohen). This review focuses on the nature and roles of dendritic protein phosphatase (PP1) and calcineurin (PP2B), the two phosphatases that have been best implicated in postsynaptic signaling.

The Importance of Dendritic Localization

The glutamate receptors involved in excitatory transmission are localized to the synapse by association with other proteins in a cytoskeletal structure called the postsynaptic density (PSD), which is often located at the tip of dendritic spines (see Chapter 55 by Kennedy). "Extra-synaptic" glutamate receptors are found in the membrane of dendritic shafts and the cell body. Synaptic and extra-synaptic receptors may be differentially regulated depending on the localization of enzymes involved in their regulation. Although specific receptors may be *in vitro* substrates for kinases and phosphatases or be modulated in whole-cell electrophysiological studies, this may not reflect synaptic regulation. Thus, understanding mechanisms that localize the relevant kinases and phosphatases to PSDs is a critical part of this puzzle.

Protein Phosphatase 1

Three mammalian genes encode four distinct but highly homologous PP1 catalytic subunits (PP1α, PP1β, PP1γ_1, and PP1γ_2), which interact with about 50 divergent proteins. Most PP1-binding proteins contain a binding motif

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defined by a consensus sequence, R/K-I/V-X-F. The interacting proteins modulate the activity or subcellular location of $PP1_C$ (see chapters 103 and 105 by DePaoli-Roach and Shenolikar in Volume 1, and [1]).

All four PP1 isoforms are expressed in mammalian brain, but with variable cellular distribution [2–5]: PP1β and $PP1\gamma_1$ also exhibit differential subcellular distribution. For example, PP1β is enriched in microtubule fractions whereas $PP1\gamma_1$ is selectively abundant in F-actin enriched extracts [3]. In addition, $PP1\gamma_1$, but not PP1β, is highly enriched in PSDs [6,7], which also contain F-actin. Consistent with these observations, immunohistochemistry shows that $PP1\gamma_1$ is enriched in synaptic layers and dendritic spines of neurons in brain slices [2,3], whereas PP1β is enriched in cell body layers [3]. Moreover, $PP1\gamma_1$ is localized to synapses in cultured cortical neurons, whereas PP1β colocalizes with microtubules in the cell body [3]. PP1α exhibits similar distribution to $PP1\gamma_1$ [2,6,7], but the subcellular localization of $PP1\gamma_2$ is unknown. Thus, PP1 isoforms are differentially targeted, suggesting that they have at least partially distinct neuronal functions. Furthermore, targeting appears to be dynamically regulated by synaptic NMDAR activation, which recruits additional unidentified PP1 isoforms to synapses [8]. Understanding the isoform selectivity and modulation of PP1 localization, and thus PP1 functions, demands a thorough understanding of the mechanisms involved. Several dendritic PP1-binding proteins (see Fig. 1) may play critical roles, as discussed below.

Inhibitor Proteins

PP1 interacts with I1 and DARPP-32, primarily soluble proteins that both inhibit PP1 activity when phosphorylated by PKA at homologous Thr residues (Thr35 in I1 and Thr34 in DARPP-32). Inhibition is mediated by interactions involving an R/K-I/V-X-F consensus motif (approximately residues 6–12) and residues surrounding Thr34/Thr35 [9,10]. Phospho-Thr34/35 in I1/DARPP-32 are effective substrates for calcineurin, the Ca^{2+}/CaM-dependent phosphatase [11]. Thus, cAMP and Ca^{2+} signaling modulate PP1 activity by antagonistic regulation of Thr34/35 phosphorylation [12]. In addition, regulation of PP1 by both DARPP-32 and I1 is modulated by phosphorylation at distinct sites by other kinases (reviewed in [13] and chapter 105 in Volume 1 by Shenolikar). Although there is no evidence indicating that I1 or DARPP32 distinguish between PP1 isoforms, their differential but overlapping expression suggests that they have different roles. For example, each protein appears to regulate dendritic PP1, but DARPP-32 is more abundant in the striatum, where as I1 is prevalent in hippocampus [14].

Targeting Proteins

Four major PP1-binding proteins (216,175,134, and 75 kDa) were initially detected in isolated PSDs [15]. The 175 and 134 kDa proteins selectively bound $PP1\gamma_1$ over PP1β, and were identified as *neurabin and spinophilin* [16], proteins previously identified as F-actin- and PP1α-binding proteins [17,18], respectively. Spinophilin also was isolated as an F-actin binding protein and termed neurabin II [19]. Both spinophilin and neurabin are selectively associated with PP1α and $PP1\gamma_1$ in brain extracts, but not PP1β [16,20], and may contribute to the differential localization of PP1β and $PP1\gamma_1$ in neurons (see above). An R/K-I/V-X-F motif is critical for binding and inhibition of PP1 by both proteins *in vitro*, but additional residues are also important

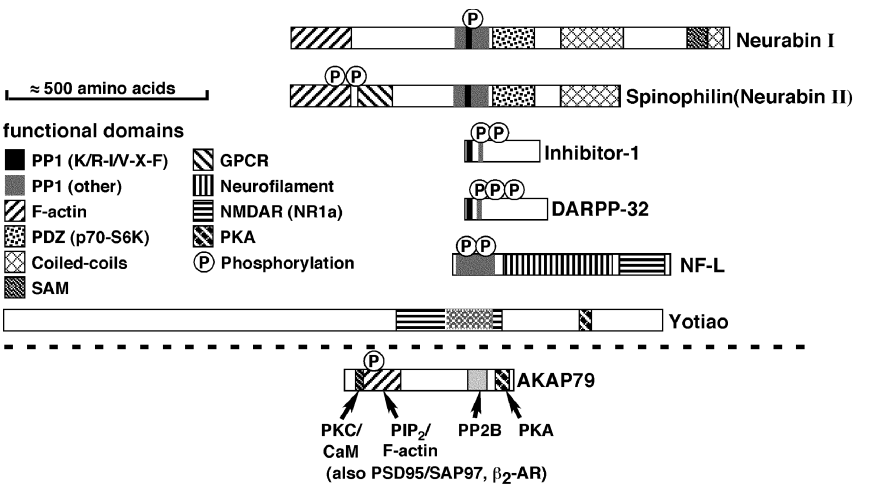


Figure 1 Dendritic PP1- and calcineurin-associated proteins. Amino acid sequences of the selected proteins are represented by open bars drawn to approximate scale. Many interact with PP1 via K/R-I/V-X-F motifs (black boxes) or other domains (gray boxes) (above dashed line), whereas AKAP79 interacts with calcineurin (PP2B). Most of these proteins also interact with protein kinases, cytoskeletal proteins, or other signaling proteins, probably serving as signaling scaffolds, and are phosphorylated, as indicated by additional shaded boxes (see legend). GPCR, G-protein coupled receptor; PDZ, PSD95/Dlg/ZO-1 domain; SAM, sterile alpha motif.

for inhibition [21,22]. However, it should be noted that PP1 activity is detected in neurabin complexes immunoprecipitated from brain extracts [21]. Although the activity of these complexes was not compared to that of free PP1, it seems that endogenous PP1 complexes with spinophilin or neurabin may possess phosphatase activity.

Subsequently, neurabin and spinophilin were shown to interact with many additional proteins that play critical roles in signal transduction, subcellular trafficking, and cytoskeletal dynamics (Fig. 1). Despite functional homology (e.g., interaction with PP1, F-actin, p70 S6 kinase), spinophilin and neurabin are likely to serve different cellular functions. For example, only spinophilin binds D2 dopamine and $\alpha 2$ adrenergic receptors [23,24], although it is not clear whether these interactions occur in neurons. In addition, reduction of neurabin expression in cultured neurons blocks neurite outgrowth [17], whereas neurons cultured from mice lacking spinophilin prematurely develop processes and the neurons from mature animals have an overabundance of spines [25].

The 216 and 75 kDa binding proteins in PSDs [15] may be yotiao and the neurofilament-L protein (NF-L), respectively. *Yotiao*, a protein first identified by binding NR1a subunits of NMDARs, was shown to be a PP1-targeting protein in addition to an AKAP [26]. It is interesting that small PP1-binding fragments of yotiao lack a recognizable K/R-I/V-X-F motif, although peptides containing the consensus motif compete for binding. Unusually for a PP1-binding protein, yotiao did not significantly inhibit PP1. High constitutive phosphatase activity in the NR1a-yotiao-PP1 complex may maintain the NMDAR in a dephosphorylated state under basal conditions [26]. PP1 also binds to the head domain of NF-L, resulting in inhibition of PP1, although this domain also lacks a K/R-I/V-X-F consensus motif. NF-L was identified in isolated PSDs [7] and also binds to the NMDAR NR1a subunit [27]. Thus, both yotiao and NF-L may contribute to PP1 targeting to NMDARs, but their isoform selectivity and specific contributions to subcellular localization remain unknown.

Calcineurin (Protein Phosphatase 2B)

Calcineurin was originally identified as an abundant Ca^{2+}/CaM-binding protein in brain extracts and was later shown to possess Ca^{2+}/CaM-dependent phosphatase activity identical to PP2B. The holoenzyme is a heterodimer of a Ca^{2+}/CaM-binding catalytic A subunit with a Ca^{2+}-binding regulatory B subunit (see chapter 106 in Volume 1 by Klee & Yang). Calcineurin is largely soluble in brain extracts, but it is also present in isolated PSDs [6] and colocalizes with F-actin in dendritic spines [28]. Calcineurin activity is inhibited by AKAP79, a PSD- and actin-associated dendritic spine protein that also interacts with many other synaptic proteins ([29–31] and see Fig. 1). Ca^{2+} influx via NMDA receptors stimulates calcineurin-dependent remodeling of actin in dendritic spines [28] and also the re-distribution of AKAP79, calcineurin, and PKA [29].

Dendritic Phosphatase Substrates

NMDARs and AMPARs

Phosphorylation may regulate the conductance, open probability, or trafficking of AMPARs and NMDARs. Initially, relatively nonselective inhibitors implicated PP1, PP2A, and PP2B in the regulation of AMPARs [32,33] and NMDARs [34]. Later studies in mice with a DARPP-32 knockout or overexpressing a constitutively active I1 mutant specifically implicated PP1. PKA-mediated regulation of extrasynaptic AMPAR and NMDAR is disrupted in striatal neurons from DARPP-32 knockout mice [35–37]. Although PP1 activities in extracts from wild-type and DARPP-32 knockout mice were not directly compared, these data suggest that PP1 inhibition by PKA-phosphorylated DARPP-32 is essential for phosphorylation of other PKA substrates. In contrast, expression of constitutively active I1 reduced hippocampal PP1 activity by 68 percent and enhanced phosphorylation of hippocampal AMPARs, as well as that of CaMKII and CREB [38]. In combination these data implicate I1/DARPP-32 modulation of PP1 activity in normal AMPAR and NMDAR regulation.

PP1 targeting has also been implicated in modulation of glutamate receptors. Intracellular perfusion of peptides containing the R/K-I/V-X-F PP1-binding motif disrupts dopamine D1 receptor regulation of extrasynaptic AMPARs in striatal neurons [39] but does not affect the basal activities of synaptic AMPARs and NMDARs in hippocampal neurons [8]. However, interpretation of these data is complicated because similar peptides disrupt PP1 interactions with spinophilin, neurabin, yotiao, NF-L, DARPP-32, and I1 *in vitro*. Subsequently, it was shown that striatal AMPARs and hippocampal NMDARs were abnormally regulated in spinophilin knockout mice [25]. These studies suggest that in wild-type animals spinophilin targets PP1 to appropriate subcellular locations to permit efficient regulation (dephosphorylation) of extrasynaptic AMPARs and NMDARs. However, the role of spinophilin in regulation of synaptic receptors is not as clearly defined.

Yotiao and NF-L also may play a role in PP1 modulation of NMDARs. PP1-binding to yotiao and NF-L is disrupted by R/K-I/V-X-F peptides; thus, disruption of these complexes may account for some of the effects of similar peptides in cells (see above). However, disruption of PP1 complexes with yotiao and NF-L would not be expected in spinophilin knockout animals. Thus, although ternary complexes containing NMDAR subunits, PP1, and either spinophilin, yotiao, or NF-L have not been reported, it seems that the mechanism of PP1 targeting to NMDARs may depend on the cell type, developmental stage, or other factors.

Calcineurin is also strongly implicated in NMDAR regulation [40–42] and mediates Ca^{2+}-dependent rundown of AMPARs in hippocampal neurons [43]. Association of calcineurin with AMPARs via AKAP79 and SAP97 promotes Ca^{2+}-stimulated rundown of AMPAR currents in HEK293 cells, an effect requiring an intact PKA site (Ser845) in the AMPAR [43]. However, despite evidence for PSD targeting

of calcineurin by AKAP79, direct actions of calcineurin on synaptic AMPARs or NMDARs have not been clearly established. Calcineurin-dependent regulation of PP1 via I1 or DARPP-32 may contribute to some of the observed effects.

Ca^{2+}/CaM-Dependent Protein Kinase II (CaMKII)

Autophosphorylation at Thr286 generates a Ca^{2+}/calmodulin-independent form of CaMKII [44–46] and is critical for stable association of CaMKII with PSDs *in vitro* and in brain slices [47], as well as in cultured neurons [48]. Some forms of synaptic plasticity, learning, and memory require an intact Thr286 autophosphorylation site in CaMKII [49]. Thus, protein phosphatases have a potentially important role in regulating CaMKII.

Initially, PP1 was identified as the major CaMKII phosphatase in PSDs, with a minor role for PP2A and no significant direct role for calcineurin or PP2C [50,51]. Subsequently, PSD-associated CaMKII was shown to be primarily dephosphorylated by PP1, but PP2A was the major activity toward soluble CaMKII [6,47]. Thus, CaMKII translocation to PSDs may modulate its availability to cellular phosphatases. Although mechanism(s) accounting for this effect are unclear, it could play a role in determining the half-life of autophosphorylated CaMKII in cells. Addition of okadaic acid to cells enhances CaMKII autophosphorylation [52], consistent with a dominant role for PP1 and PP2A in intact cells. Moreover, inhibition of hippocampal PP1 by induced overexpression of constitutively active I1 results in enhanced autophosphorylation of CaMKII at Thr286 [38]. Thus, calcineurin may play a role in CaMKII regulation by modulating PP1 (see above) and PP2C-related phosphatases may be involved in some cells [53].

Role of Phosphatases in Synaptic Plasticity

Synaptic plasticity describes the long-lasting adaptations of synaptic function that are thought to underlie certain forms of memory. The best-studied forms of long-term potentiation (LTP) and long-term depression (LTD) in hippocampal CA1 neurons require dendritic Ca^{2+} influx via the NMDARs, and modulate the phosphorylation, activity, and subcellular trafficking of AMPARs. LTP requires Thr286 autophosphorylation of CaMKII and the activation of several other kinases (reviewed in [54–56]). The actions of protein phosphatases oppose the kinases, thus depressing or depotentiating synaptic transmission (see Fig. 2).

Initially, calcineurin and PP1 were implicated in LTD induction [57,58], in part because LTD required Ca^{2+} influx and the Ca^{2+}/CaM-dependent calcineurin regulates PP1 via I1 or DARPP-32 or both. One relevant substrate appears to be GluR1 subunits of AMPARs. LTD of naïve cells correlates with dephosphorylation of Ser845 (a PKA site), whereas depotentiation (LTP reversal) induces dephosphorylation of Ser831 (a CaMKII site) [59]. In contrast, LTP of naïve cells enhances Ser831 phosphorylation, and potentiation of "previously depressed" synapses enhances Ser845 phosphorylation [59]. However, these changes occur in the total AMPAR pool, not just in synaptic receptors, and the identity of the relevant phosphatases remains unclear.

Genetic manipulation of calcineurin activities in mice provided important insight into mechanisms of synaptic plasticity. A surprising finding was that mice expressing either constitutively active calcineurin or a calcineurin inhibitory peptide exhibited no detectable defect in LTD induction [60,61]. Rather, both transgenic models implicate calcineurin as exerting a negative effect on LTP [60,61], consistent with some prior pharmacological data [57,62]. It is interesting that mice lacking the calcineurin α gene, but retaining the minor calcineurin β gene, exhibit normal LTD and LTP but are defective in depotentiation [63]. Given the known changes in AMPAR phosphorylation during LTP, LTD, and depotentiation (see above), these data implicate calcineurin in the dephosphorylation of Ser831 but not Ser845 in synaptic AMPARs. However, this could be due to direct dephosphorylation of Ser831 by calcineurin or, indirectly, to PP1-mediated dephosphorylation/inactivation of CaMKII. Although this model is inconsistent with data implicating calcineurin in Ser845 dephosphorylation ([43]; see above), the synaptic AMPARs relevant to synaptic plasticity may be regulated differently.

Synaptic plasticity may involve modulation of PP1 activity by DARPP-32 or I1 or targeting of PP1 to AMPARs by spinophilin or other PP1-binding proteins. R/K-I/V-X-F motif peptides disrupt PP1 interactions with I1, DARPP-32, spinophilin, neurabin, and yotiao *in vitro* and block induction of some forms of LTD [8]. More specifically, spinophilin knockout mice are deficient in the induction of LTD but not LTP [25]. Moreover, PP1 plays a role in selective dephosphorylation of AMPARs at Ser845 but not Ser831 in striatal neurons [35]. Thus, AMPAR dephosphorylation at Ser845 associated with LTD may be due to activation of PP1 associated with spinophilin. Dephosphorylation of I1 by calcineurin may liberate PP1 to interact with spinophilin [8] and dephosphorylate Ser845, whereas dephosphorylation of Ser831 associated with depotentiation may be due to a direct action of calcineurin (Fig. 2).

In addition to a role for PP1 activation in LTD induction, inhibition of hippocampal PP1 appears important for LTP induction. LTP induces PKA-mediated phosphorylation of I1 and inhibition of PP1, thereby "gating" Thr286 autophosphorylation of CaMKII and promoting phosphorylation of AMPARs (Ser831) and other substrates [64,65]. Consistent with this model, Thr286 phosphorylation of CaMKII and Ser831 phosphorylation of AMPARs were enhanced in transgenic mice induced to overexpress a constitutively active mutant I1, correlating with enhanced learning and memory [38]. Thus, at least some forms of LTP require PKA-dependent inhibition of PP1 activity via the phosphorylation of Thr35 in I1. This contrasts with data obtained from I1 and spinophilin knockout mice in which LTP at the hippocampal CA3-CA1 synapses is normal [25,66]. However, these observations may reflect adaptations to chronic protein deficiency

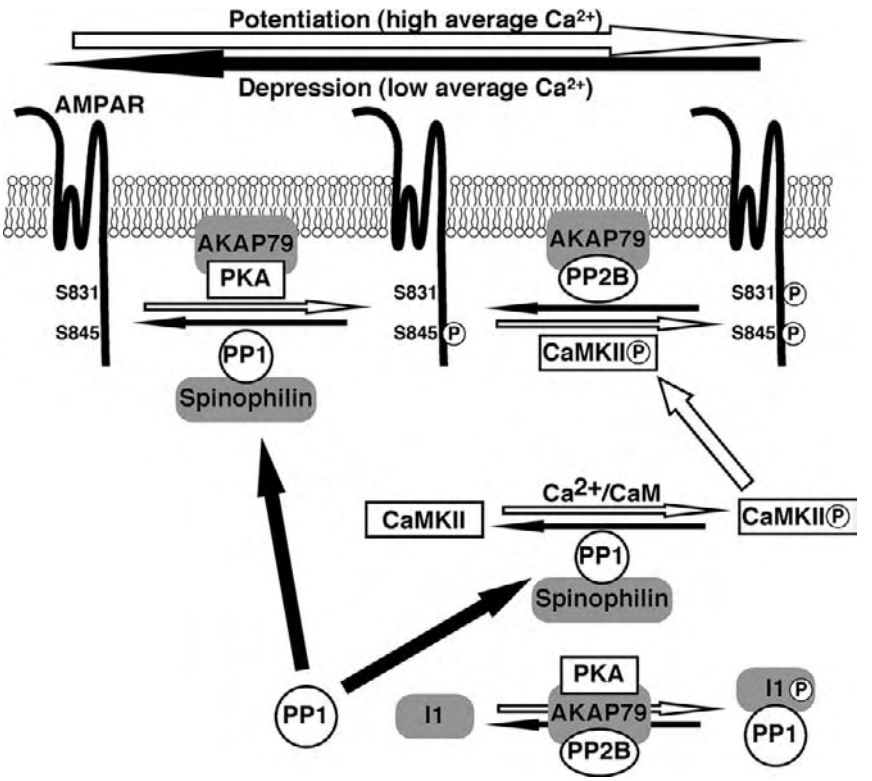


Figure 2 Working model implicating dendritic protein phosphatase complexes in hippocampal CA1 synaptic plasticity. Phosphorylation-dephosphorylation of AMPARs (black line) in synaptic membranes and other locations is associated with synaptic plasticity. *Unfilled arrows* indicate reactions and promoted by intense synaptic stimulation (e.g., high-frequency stimulation) that potentiate synaptic transmission. Activation of NMDARs results in high-average Ca^{2+} concentrations that favor activation of protein kinases (open rectangles), such as CaMKII and PKA (via Ca^{2+}/CaM-dependent adenylyl cyclases). PKA substrates include AMPARs (Ser845) and inhibitor 1 (I1). Phosphorylated I1 directly inhibits PP1 activity and may compete PP1 away from spinophilin. PP1 inhibition promotes the accumulation of Ser845 phosphorylated AMPARs and autophosphorylated (activated) CaMKII, which phosphorylates AMPARs at Ser831. In contrast, *solid arrows* indicate reactions promoted by low-intensity synaptic stimulation (e.g., prolonged low-frequency stimulation). NMDAR activation again provides the primary signal, but lower average Ca^{2+} concentrations favor phosphatase (open circles) activation, since calcineurin (PP2B) has a higher affinity for Ca^{2+}/CaM than CaMKII or adenylyl cyclases. Dephosphorylation of I1 by calcineurin liberates free PP1 that may associate with spinophilin and dephosphorylate (inactivate) CaMKII. Calcineurin and PP1 may dephosphorylate Ser831 and Ser845 in AMPARs, respectively. Assembly of phosphatases and kinase in complexes with anchoring targeting or regulatory proteins (gray) plays a critical role in dictating signaling specificity and fidelity.

in these knockout animals. Thus, the precise role of specific PP1 complexes in CaMKII regulation and LTP induction remains unclear.

Summary

There is compelling evidence that multiprotein complexes containing PP1 and calcineurin are critical for synaptic regulation, but specific roles of their molecular components still need to be determined. Roles for other serine/threonine and tyrosine phosphatases are also likely to be better described. Apparently contradictory data in the cited literature may be due to cell-specific issues related to differences in cellular functions, prior synaptic activity or developmental stage analyzed, and variations in experimental conditions. The challenge is to carefully control all the variables and use emerging animal models and specific molecular tools to more precisely understand the roles of these phosphatase complexes in synaptic regulation.

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CHAPTER 189

Protein Phosphatase 2A

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Introduction

Serine/threonine phosphatases are integral components of many signal transduction pathways. There are eight classes of serine/threonine phosphatases in vertebrates. Protein serine/threonine phosphatases 1, 2A, 2B/calcineurin, 4, 5, 6, and 7 are members of the PPP gene family that contain a conserved serine/threonine phosphatase domain. Protein phosphatase 2A (PP2A) is a ubiquitously expressed member of the PPP gene family that accounts for a substantial portion of the total serine/threonine phosphatase activity in many cell types. PP2A is an essential enzyme that functions in fundamental cellular processes, including metabolism and the cell cycle. Like the other signaling molecules discussed in this chapter, proximity interactions play a primary role in regulating PP2A.

Once thought of as a single, broad-specificity phosphatase, PP2A is actually many different enzymes composed of complexes between catalytic subunits, scaffold subunits, regulatory subunits, and interacting proteins [1–3]. The catalytic and scaffold subunits bind tightly to form a core dimer that is the common component of most, but not all, forms of PP2A. The core dimer interacts with an array of regulatory subunits to generate multiple heterotrimeric holoenzymes. Additional interactions between PP2A and a variety of interacting proteins generate additional diversity. The regulatory subunits and interacting proteins target PP2A to specific substrates and intracellular locations. The existence of many different forms of PP2A accounts for the ability of the enzyme to regulate a wide variety of biological processes.

Interaction of the core dimer with regulatory subunits is critical for PP2A function. The regulatory subunits bind to the core dimer through interactions with both the scaffold and the catalytic subunits. The scaffold subunit is composed entirely of 15 copies of a conserved motif termed the HEAT repeat [4]. HEAT repeats 1–10 mediate interactions with regulatory subunits whereas repeats 11–15 mediate interaction with the C subunit [5]. The regulatory subunits must form contacts with both the scaffold and the catalytic subunits to generate stable heterotrimers [5,6]. The regulatory subunits bind to the core dimer in a mutually exclusive manner. Although some sites of interaction are conserved, there are unique amino acids within the scaffold subunit that are involved in the interaction with individual regulatory subunits [7] (Fig. 1).

PP2A Regulatory Subunits Mediate Proximity Interactions

Regulatory subunits play a primary role in specifying the proximity interactions of PP2A. Three families of PP2A regulatory subunits have been identified in vertebrates by biochemical and genetic methods. A list of PP2A subunits is presented in Table I. In order to avoid confusion, we have used a nomenclature for the PP2A subunits derived from their official human gene symbols. In contrast to the scaffold and catalytic subunits, which are ubiquitously expressed, the PP2A regulatory subunits are expressed in a cell- and tissue-specific manner. PP2A regulatory subunits are also differentially expressed during development and have distinct subcellular localizations. Neither the structural basis for interaction of regulatory subunits with the PP2A core dimer nor the biochemical effects of these interactions have been clearly elucidated. The PP2A regulatory subunit families have little overall amino acid sequence similarity. Several regulatory

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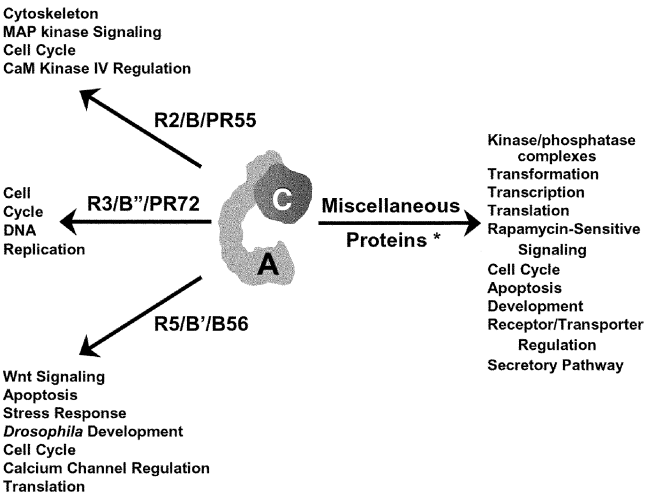


Figure 1 PP2A is a family of enzymes with multiple cellular functions. The PP2A holoenzyme consists of a common core dimer (AC) that complexes with a wide variety of regulatory molecules to generate a diversity of enzyme forms. These regulatory molecules include three regulatory subunit families (R2, R3, and R5) and a variety of miscellaneous proteins that interact with the core dimer or the free catalytic subunit. The regulatory molecules target PP2A to distinct substrates and intracellular locations, allowing the enzyme to participate in numerous cellular functions. The types of functions targeted by the individual regulatory subunits and miscellaneous proteins are listed.

subunits contain WD domains, which have been proposed as a conserved motif responsible for the interaction with the core dimer [3,8–10]. Recently a loosely conserved A-subunit binding domain has been identified in each of the regulatory subunit families [11]. The regulatory subunits have effects on the kinetics of dephosphorylation that are consistent with a role in controlling the binding of substrates to PP2A [12–15]. This model is consistent with the notion that regulatory subunit-mediated proximity interactions play a role in targeting PP2A to phosphoprotein substrates. In contrast to simple enzyme-substrate interactions, the interaction of PP2A with many substrates involves a stable interaction involving regions of the enzyme removed from the active site. These stable interactions serve to maintain a high effective concentration of PP2A in the vicinity of the substrate.

Consistent with roles in defining PP2A specificity, different families of PP2A regulatory subunits have non-overlapping functions. The stress-induced growth arrest caused by mutations in the R5 subunit gene (RTS1) in yeast can be rescued by introduction of wild-type versions of either the yeast R5 gene or the human R5γ gene [16]. In contrast, wild-type R5 cannot rescue the cold-sensitive phenotype resulting from mutations in the yeast R2 subunit gene (CDC55). Knockdown of individual PP2A regulatory subunits in *Drosophila* S2 cells by RNA interference causes distinct defects. Loss of the R2 subunit

Table I Nomenclature of Mammalian PP2A Subunits

Name	Gene symbol	Aliases	Chromosomal location	LocusID/accession number[a]
Catalytic subunits				
α isoform	PPP2CA	Cα, $PP2A_{Cα}$	5q23–31	*5515*
β isoform	PPP2CB	Cβ, $PP2A_{Cβ}$	8p21–12	*5516*
Scaffold subunits				
α isoform	PPP2R1A	Aα, PR65α	19	*5518*
β isoform	PPP2R1B	Aβ, PR65β	11q23	*5519*
R2 subunits				
α isoform	PPP2R2A	Bα, PR55α	8	*5520*
β isoform	PPP2R2B	Bβ, PR55β	5q31–5q33	*5521*
γ isoform	PPP2R2C	Bγ	4p16	*5522*
δ isoform		Bδ		*AF180350*
R3 subunits				
α isoform	PPP2R3	PR72, B″	3	*5523*
β isoform		PR59		*AF050165*
γ isoform		PR48		*28227*
R5 subunits				
α isoform	PPP2R5A	B′α, B56α	1q41	*5525*
β isoform	PPP2R5B	B′β, B56β	11q12	*5526*
γ isoform	PPP2R5C	B′γ, B56γ	3p21	*5527*
δ isoform	PPP2R5D	B′δ, B56δ	6p21.1	*5528*
ε isoform	PPP2R5E	B′ε, B56ε	7p11.1–12	*5529*

[a]Entries in this column include the LocusID, when available, for the *NCBI LocusLink* entry for the corresponding the protein, or the GenBank/EMBL Accession number.

causes an increase in insulin-dependent MAP kinase signaling, whereas loss of both R5 isoforms induces apoptosis [17]. These data suggest that PP2A holoenzymes containing the R2 subunit play a negative regulatory role in MAP kinase signaling whereas holoenzymes containing the R5 subunit function in cell survival.

The R2 Family

The R2 family comprises a set of proteins present in a form of PP2A originally designated $PP2A_1$ [18]. This family currently contains four known isoforms (Table I) that are 79–87 percent identical. R2α mRNA is ubiquitously expressed and is the most abundant PP2A regulatory subunit in many cells and tissues. The R2β and R2γ isoforms are only expressed at high levels in brain and testis. Although R2α and R2β are both expressed in the brain, they are present at different levels in different types of neurons [19]. R2α is distributed mainly in neuronal cell bodies and is localized in both the cytosol and nucleus. In contrast, the β isoform is excluded from the nucleus and is localized in axons and dendrites in addition to the cell body. Expression of R2 subunit mRNA is also differentially regulated during development. The differential expression and localization of R2 subunits support the idea that different members of this family play distinct roles in regulating PP2A functions.

Information about the functions of PP2A regulatory subunits has been derived from genetic analysis in yeast, *Drosophila*, and *C. elegans*. The pleiotropic phenotypes of mutant alleles of the R2 subunit in yeast and its numerous genetic interactions indicate that the R2 (cdc55p) protein plays multiple roles during mitosis, including the bud morphogenetic checkpoint and the mitotic spindle-assembly checkpoint [20–22]. The genetic results suggest that R2/cdc55p is involved in promoting activation of the yeast cell cycle regulatory kinase CDC2 (cyclin B/Cdc28 in *S. cerevisiae*) via dephosphorylation of the inhibitory tyrosine 19 phosphorylation site. Since PP2A does not directly dephosphorylate tyrosine, a likely target of R2 action is the cdc25 dual-specificity phosphatase, which is responsible for dephosphorylating tyrosine 19 in *S. cerevisiae* cdc28p.

Reduced levels of the R2 subunit in *Drosophila* result in varied phenotypes depending on the severity of the alleles. The *aar1* allele (for abnormal anaphase resolution) contains a P-element insertion in the R2 gene [23]. Mutant *aar1* flies die as larvae or early adults with overcondensed chromosomes and abnormal anaphase figures in larval brain cells. These defects can be rescued by reintroduction of the wild-type R2 gene. The *aarl* phenotype is reminiscent of the mitotic spindle-assembly checkpoint defects seen in the yeast R2 mutants. Another P-element mutant allele of *Drosophila* R2 ($twins^P$) causes death at an early pupal stage and shows pattern duplication of wing imaginal discs [24]. Flies harboring a weaker allele, $twins^{55}$, survive but have duplicated bristles in sensory neurons [25]. The effects of the *twins* mutation are consistent with a role for the R2 subunit in *Drosophila* embryonic cell fate determination. Both the *aar1* and $twins^p$ mutant larvae have a specific reduction in phosphatase activity toward substrates of cyclin-dependent kinases, suggesting the R2 subunit directs PP2A toward these substrates.

The R2 subunit targets PP2A to pathways that regulate MAP kinase activity. Overexpression of the small-t antigen of SV40 virus disrupts endogenous PP2A complexes containing the R2 subunit. This leads to enhanced activation of MAP kinase in response to growth factors in some but not all cell types [26,27]. The small-t antigen effects may involve protein kinase C and the PI-3 kinase pathway [28]. Depletion of the R2 subunit in *Drosophila* S2 cells via RNA interference also leads to a prolonged activation of MAP kinase in response to insulin [17]. These studies indicate that the R2 subunit plays a negative role in regulating MAP kinase activity, presumably by targeting PP2A to a component that is activated by phosphorylation. The *C. elegans* R2 subunit (*sur-6*) was isolated as a suppressor of the multivulval phenotype caused by an activated ras mutation [29]. Sur-6 mutations do not cause defects in vulval development by themselves but enhance the effects of weak mutant alleles of the *C. elegans* Raf protein kinase. These genetic interactions indicate that *sur-6* mutations reduce signaling through the Ras pathway and may act with the kinase suppressor of raf (KSR) protein in a common pathway to positively regulate signaling through the Ras-Raf-MAP kinase pathway. The PP2A core dimer can associate with the Raf-1 protein kinase (Table II). This interaction appears to mediate the dephosphorylation of inhibitory phosphorylation sites and enhance activation of Raf-1 during mitogenic stimulation [30]. This interaction does not appear to be mediated by the R2 subunit, since neither R2α nor R2β were detected in Raf-1 complexes. These studies suggest that PP2A is targeted to components of signaling pathways that regulate MAP kinase in both positive and negative ways. At least some of this targeting is mediated by R2 subunits. Multiple roles in MAP kinase signaling are consistent with genetic studies showing that mutations in the PP2A catalytic subunit have both positive and negative effects on MAP kinase activation in *Drosophila* [31]. The multiple actions in MAP kinase signaling are likely to be due to different forms of PP2A acting at distinct sites in this regulatory network.

Another R2-mediated proximity interaction regulates the microtubule cytoskeleton. A population of PP2A is associated with microtubules in neuronal and non-neuronal cells [32]. The association of PP2A with microtubules in brain is specific for R2α- and R2β-containing isoforms, and can be enhanced by a heat-labile anchoring factor [15]. PP2A holoenzymes containing R2α or R2β also interact with the neuronal microtubule-associated protein tau (Table II) and act as potent tau phosphatases [33]. The microtubule-binding and organizing activity of tau is regulated by phosphorylation. Hypophosphorylated forms of tau bind to microtubules, leading to increased microtubule stability. In contrast, hyperphosphorylated tau dissociates from microtubules, leading to a decrease in microtubule stability. Tau-dependent stabilization of microtubules is important for formation and maintenance

Table II PP2A Interacting Proteins

Protein	Comments	Refs
Signaling proteins/transcription factors		
Adenomatous polyposis coli (APC)	APC binds to R5 subunits in yeast two-hybrid assays. This interaction may target PP2A to the Wnt signaling pathway, but physical complexes between PP2A and APC have not been demonstrated. Overexpression of R5 subunits decrease β-catenin levels and suppress Wnt signaling.	[49]
Axin	Axin forms complexes with the C and R5 subunits. The interaction targets PP2A to a complex of axin, APC, GSK3, and β-catenin and plays a role in regulating Wnt signaling.	[50, 51]
Cas (p130 Crk-associated substrate)	Cas is a Src substrate that has increased association with PP2A when Src is activated. PP2A dephosphorylates serine residues on Cas *in vitro*.	[60]
E-cadherin/β-catenin	The C_α but not the C_β subunit is required for stabilization of E-cadherin/β-catenin complexes at the Plasma membrane.	[61]
Heat shock transcription factor 2 (HSF2)	HSF2 interacts with the A-subunit in two-hybrid and co-immunoprecipitation assays. HSF2 may displace the catalytic subunit from PP2A holoenzymes.	[62, 63]
HOX11	HOX 11 is homeobox transcription factor that controls development of the spleen. HOX11 binds to the PP2A catalytic subunit and inhibits phosphatase activity. HOX 11 also interacts with protein phosphatase 1.	[64]
HRX	HRX binds to PP2A through the SET/I_2^{PP2A} inhibitor protein. HRX is commonly mutated in acute leukemias.	[65]
Sex combs reduced (SCR)	SCR is a *Drosophila* homeobox transcription factor that interacts with the *Drosophila* R5 subunit in two-hybrid assays. SCR is homologous to human HOX5 and HOX6. PP2A may control phosphorylation and DNA binding activity of SCR.	[57]
RelA	RelA interacts with the scaffold subunit *in vitro*. The association may be transient since cross-linking is required to isolate a PP2A/RelA complex. RelA is dephosphorylated by PP2A *in vitro*.	[66]
Shc	PP2A associates with the PTB domain of Shc in the basal state and dissociates in response to insulin- and EGF-induced tyrosine phosphorylation. Expression of SV40 small-t antigen also causes dissociation of this complex.	[67]
Sp1	The Sp1 transcription factor interacts with the catalytic subunit in dividing T lymphocytes.	[68, 69]
Stat5	Stat5 associates with PP2A in an IL-3-dependent manner in the cytoplasm but not the nucleus.	[70]
Cell cycle related proteins		
Anaphase-promoting complex/cyclosome (APC/C)	APC/C binds to the adenovirus E4orf4-PP2A complex. E4orf4 may target PP2A to APC/C, leading to its inactivation. This interaction may play a role in E4orf4-mediated cell cycle arrest and apoptosis.	[71]
Cdc6	Cdc6 binds to the R3γ/PR48 subunit and interacts with the AC-R3γ heterotrimer. The interaction may regulate Cdc6 phosphorylation and DNA replication. Overexpression of R3γ causes G1 arrest.	[43]
Cdc25c dual-specificity phosphatase	Cdc25c co-immunoprecipitates with PP2A following cross-linking of cell lysates. The interaction requires the R2 subunit and results in dephosphorylation of cdc25c. The interaction is enhanced by the HIV-1 Vpr protein, suggesting that dephosphorylation and inactivation of cdc25c is involved in Vpr-mediated G2 arrest.	[72]
Cyclin G2	The association of cyclin G2 with PP2A catalytic and R5 subunits correlates with its ability to inhibit cell cycle progression.	[54]
DNA polymerase α-primase	PP2A is recovered with the hypophosphorylated form of DNA polymerase α-primase in G1. PP2A dephosphorylates DNA polymerase α-primase and restores its origin-dependent initiation activity *in vito*.	[73]
p107	p107 (a retinoblastoma-related protein) binds the R3β/PR59 subunit-containing holoenzyme. Overexpression of R3β/PR59 causes p107 dephosphorylation and G1 arrest.	[41]
Membrane receptors/transporters		
$Beta_2$-adrenergic receptor	The association of PP2A with this G-protein coupled receptor is dependent upon agonist stimulation, receptor internalization, and acidification of endosomes. PP2A dephosphorylation is important for receptor resensitization and recycling to plasma membrane.	[74]
Biogenic amine transporters	Dopamine, norepinephrine, and serotonin transporters associate with PP2A. Transporter phosphorylation results in disruption of the PP2A association. The interaction may be involved in the regulation of the surface expression of transporters.	[75]
Class C L-type calcium channel ($Ca_v1.2$)	PP2A binds to the pore-forming α_{1C} subunit of this channel and reverses PKA-catalyzed serine phosphorylation. The interaction is selective for R5γ-containing PP2A complexes.	[58]
CXCR2 chemokine receptor	The chemokine receptor CXCR2 is a G-protein coupled receptor involved in chemotaxis. CXCR2 interacts with the AC core dimer. The interaction is dependent on internalization of the receptor following agonist stimulation.	[76]
NMDA receptor	PP2A forms a stable complex with NR3A subunit of the NMDA receptor. The association increases phosphatase activity and dephosphorylation of the NR1 subunit. Stimulation of the receptor leads to dissociation of PP2A and a reduction in phosphatase activity.	[77]

Table II *continued*

Protein	Comments	Refs
Protein kinases		
CaM kinase IV (CaMKIV)	CaMKIV binds to the AC-R2 form and is dephosphorylated by PP2A.	[78]
Casein kinase II (CK2)	CK2 binds to the AC core dimer. CK2 can phosphorylate and stimulate PP2A activity *in vitro*.	[79]
JAK2	There is a transient association of JAK2 and PP2A upon interleukin-11 stimulation of adipocytes.	[80]
p21-Activated kinase (PAK1)	PAK1 interacts with and is a substrate of PP2A.	[81]
p70 S6 kinase	p70 S6 kinase is a PP2A substrate.	[81]
PKCα	The PP2A catalytic subunit co-immunoprecipitates with PKCα. PKCα is dephosphorylated by PP2A. This association may be involved in the regulation of mast cell IL-6 production.	[82]
PKCδ	PKCδ is a substrate for PP2A.	[83]
PKR (Double-stranded RNA-dependent protein kinase)	PKR binds to and phosphorylates the R5α regulatory subunit. Phosphorylation of R5α enhances PP2A activity and may alter the activity of the translation initiation factor eIF4.	[84]
RAF-1	RAF-1 interacts with the AC core dimer. PP2A dephosphorylates inhibitory sites on RAF-1.	[30]
Src	PP2A binds to the SH2, SH3, and catalytic domains of Src. This interaction decreases Src tyrosine kinase activity.	[85]
Apoptotic proteins		
Cyclin G1	Cyclin G1 binds to R5 subunits and the association is dependent on the induction of p53. Cyclin G1 plays a role in enhancing apoptosis.	[53, 55]
Bcl-2	Bcl-2 interacts with the PP2A isoform containing the R5α subunit. PP2A dephosphorylates Bcl-2 and regulates the function of Bcl-2 in apoptosis.	[86–88]
Cytoskeletal proteins		
CG-NAP (AKAP 350/450/CG-NAP)	This 450-kDa centrosome and Golgi localized PKN-associated protein coimmunoprecipitates with PP2A in R3α-130 expressing cells. CG-NAP is involved in regulation of centrosome dynamics during the cell cycle.	[40]
Mid-1	Mid-1 binds to the PP2A interacting protein alpha 4 at a site independent from the C-subunit binding site. This interaction may regulate mid-1 binding to microtubules and formation of the midline during embryonic development.	[89]
Myosin	PP2A associates with myosin following mast cell activation. This interaction may play a role in regulating cytoskeletal remodeling and mast cell secretion.	[90]
Neurofilament proteins (NFs)	The AC-R2 complex associates with NF proteins. PP2A dephosphorylates sites in all three NF proteins (NF-L, NF-M, and NF-H). Dephosphorylation by PP2A promotes assembly of NF-L into filaments.	[91, 92]
Paxillin	Paxillin interacts with C-subunit and R5γ regulatory subunit. R5γ1 co-localizes with paxillin at focal adhesions and may target PP2A to paxillin.	[59]
Tau	Tau specifically interacts with R2-containing trimers. AC-R2 trimers dephosphorylate tau, promote microtubule binding, and stabilize microtubules.	[33, 93]
Vimentin	The AC-R2 complex associates with and dephosphorylates vimentin in an interaction mediated by the R2 subunit. Depletion of R2 by antisense RNA causes hyperphosphorylation of vimentin and reorganization of intermediate filaments.	[94]
Secretory pathway proteins		
Carboxypeptidase D (CPD)	PP2A binds to and dephosphorylates the cytoplasmic tail of this secretory pathway protein. PP2A may play a role in the intracellular trafficking of CPD between the cell surface and the trans-Golgi network.	[95]
Mannose-6-phosphate receptor (cation-independent)	PP2A binds to the cytoplasmic tail of this secretory pathway protein.	[95]
Peptidylglycine-a-amidating mono-oxygenase (PAM)	PP2A binds to the cytoplasmic tail of this secretory pathway protein.	[95]
TGN38	PP2A binds to the cytoplasmic tail of this secretory pathway protein.	[95]
Translation		
Eukaryotic termination factor-1 (eRF1)	eRF1 binds to the AC core dimer through C subunit. This interaction may target PP2A to ribosomes.	[96]
α4/Tap42 (IGBP1)	Alpha 4 interacts directly with the C subunit and decreases phosphatase acitivity toward eIF4E-BP1 that has been phosphorylated by the mTOR kinase.	[97–100]

continues

Table II *continued*

Protein	Comments	Refs
Viral proteins		
Adenovirus E4orf4 protein	E4orf4 binds to the AC-R2 and AC-R5 complexes. Formation of a complex with AC-R2 is required for E4orf4-mediated apoptosis.	[56, 101, 102]
HIV Vpr protein	Vpr binds to AC-R2 complex and mediates Vpr-induced G2 arrest. This interaction regulates the Cdc25 dual-specificity phosphatase and Wee1 kinase.	[68, 72, 103]
Polyomavirus middle tumor antigen	Middle-T antigen binds to the AC core dimer and targets PP2A to the signaling complex assembled by middle-T antigen. The role of this interaction in middle-T mediated transformation is not clear.	[104–106]
Polyomavirus small tumor antigen	Similar to SV40 small-t antigen. Binds to the AC core dimer.	[104–106]
SV40 small tumor antigen	Binds to AC core dimer, displacing the R2 subunits and inhibiting PP2A acitivity toward some substrates. This interaction enhances MAP kinase signaling and viral transformation.	[104, 105]
Other cellular proteins		
I_1^{PP2A} (PHAP1, mapmodulin)	I_1^{PP2A} can inhibit PP2A activity *in vitro*, but its physiological function is unknown.	[107]
I_2^{PP2A} (SET)	I_2^{PP2A} can inhibit PP2A *in vitro*, but its function is unknown.	[108]
Phosphotyrosyl phosphatase activator (PTPA)	PTPA displays a weak interaction with PP2A and can enhance the low activity of the AC core dimer toward phosphotyrosine.	[3]
Protein phosphatase 5 (PP5)	PP5 interacts with the scaffold subunit of PP2A and may replace the catalytic subunit. The interaction appears to involve the R3α subunit, which co-immunoprecipitates with PP5.	[39]
Protein phosphatase methylesterase (PME-1)	Associates with catalytically inactive C-subunit point mutants. Demethylates the catalytic subunit *in vitro*.	[109]
SG2NA	SG2NA binds to the AC core dimer. The protein is localized in nucleus. SG2NA contains WD repeats, such as R2 subunits and striatin, and binds calmodulin. The function of SG2NA is currently unknown.	[10]
Striatin	Striatin binds to the AC core dimer. The protein contains WD repeats, such as R2 subunits and SG2NA, and binds to calmodulin. The function of Striatin is currently unknown.	[10]

of axons in the central nervous system [34]. Disruption of the PP2A-tau interaction by expression of SV40 small-t antigen (which disrupts interaction of R2 subunits with the core dimer) causes hyperphosphorylation of tau and its dissociation from microtubules [33]. These observations suggest that proximity interactions among R2-containing forms of PP2A, microtubules, and tau play important roles in maintaining tau in a hypophosphorylated state. The targeted dephosphorylation of tau is important for axonal integrity, since inhibition of PP2A leads to tau hyperphosphorylation, loss of organized microtubules, and axonal degeneration in cultured neuronal cells [35]. The R2-mediated interactions of PP2A with microtubules and tau may have implications in neurodegenerative diseases, including Alzheimer's disease, where tau becomes hyperphosphorylated.

Expansion of a novel CAG trinucleotide repeat within the human R2β gene (PPPR2B) is associated with a form of autosomal dominant spinocerebellar ataxia termed SCA12 [36]. SCA12 is caused by neurodegeneration with atrophy of the cortex and cerebellum. The CAG expansion lies near the transcription start site of the R2β gene and could alter expression of this brain-specific isoform. The presence of the CAG expansion in affected individuals and its absence in non-affected family members suggest that altered expression of R2β may cause this disease. Although the mechanism of R2β loss in SCA12 is unknown, these data suggest that R2β may play a role in maintenance of neuronal viability.

The R3 Family

The second family of regulatory subunits identified by molecular cloning was the R3 family (Table I). The R3 subunit was first identified as a 74 kDa protein present in a PP2A holoenzyme termed PCS_M [37]. Current evidence indicates that this family plays a role in targeting PP2A to proteins involved in cell cycle regulation, including Cdc6, p107, and CG-NAP (Table II). The gene encoding the R3 subunit (designated R3α in Table I) produces two alternatively spliced transcripts encoding proteins of 72 and 130 kDa [38]. R3α-72 and R3α-130 contain the same C-terminal protein sequence, but PR130 contains a 665-amino-acid N-terminal extension. Both the 72 and 130 kDa variants are selectively but not exclusively expressed in skeletal muscle and heart. *In vitro*, the R3α subunit suppresses the activity of the AC dimer toward exogenous substrates and increases sensitivity of the enzyme to polycations [37]. The functions of R3α-72 or R3α-130 subunits have not been identified. Protein phosphatase 5 (another member of the PPP gene family) can interact with PP2A. Immunoprecipitated PP5 is associated with R3α-72 but not other regulatory subunits [39]. Although the significance of this interaction is not known, the data suggest that PP5 can be present in a PP2A oligomer containing the scaffold and R3α-72 subunits and that PP5 might act as the catalytic subunit in this heterocomplex. R3α-130 interacts with the giant scaffolding protein CG-NAP (centrosome and

Golgi localized PKN-associated protein). CG-NAP anchors a signaling complex containing protein kinase-A, protein kinase-N, protein kinase-Cε, PP2A (R3α-130), and protein phosphatase 1 to the centrosome and Golgi apparatus in a cell-cycle-dependent manner [40]. The CG-NAP signaling complex may mediate some of the complex phosphorylation-based regulation of the centrosome that occurs during the cell cycle. One potential substrate for PP2A in this complex is protein kinase-N.

The R3 family contains additional isoforms that function in cell cycle regulation through unique proximity interactions. The R3β (PR59) protein was discovered in a yeast two-hybrid screen via the retinoblastoma-related protein p107 as bait [41]. R3β forms complexes with the PP2A core dimer when expressed in cells. Although R3β shares 56 percent identity with R3α-72, the interaction with p107 is specific. Furthermore, although R3β binds to p107, it fails to interact with the retinoblastoma protein. Forced overexpression of R3β results in dephosphorylation of p107 and cell cycle arrest in the G1 phase. R3β-mediated cell cycle arrest may be the result of hypophosphorylation of p107 (due to increased PP2A targeting) and its association with the E2F transcription factor. Binding of p107 to E2F would repress expression of genes required for entry into S phase. R3β may be targeted to dephosphorylate p107 in response to UV irradiation [42].

The R3γ regulatory subunit (PR48) was discovered in a yeast two-hybrid screen with the Cdc6 protein as bait [43]. Cdc6 is required for formation of pre-replication complexes during DNA replication. Phosphorylation of Cdc6 by S-phase cyclin-dependent kinases is the rate-limiting step for initiation of DNA replication. In mammalian cells, phosphorylation of Cdc6 at the beginning of S phase causes its dissociation from chromatin and triggers replication. In addition, Cdc6 phosphorylation induces its nuclear export and ubiquitin-dependent degradation. R3γ shares 50 and 68 percent sequence identity with R3α and R3β, respectively. R3γ localizes to the nucleus in mammalian cells and, like PR59, forced overexpression of PR48 results in cell cycle arrest at G1.

The R5 Family

The R5 regulatory subunits are a complex family of proteins that are components of a PP2A holoenzyme originally termed $PP2A_0$ [18,44]. There are at least five isoforms (Table I) that have distinct patterns of expression [45–47]. The α and γ isoforms are expressed predominantly in muscle, the β and δ isoforms in brain, and the ε isoform in brain and testis. In cardiac muscle, nearly all of the PP2A holoenzyme is composed of the R5α subunit [44]. *In vitro*, the R5 subunits suppress phosphatase activity toward multiple substrates [14]. This implies that the R5 subunits target PP2A by disfavoring interactions with some substrates while favoring interactions with others. The R5 family has been subdivided into cytosolic and nuclear types based on localization of transiently expressed proteins [46,48]. The R5α, R5β, and R5ε isoforms are cytoplasmic whereas R5γ and R5δ are present in both the cytoplasm and nucleus. Ectopically expressed R5 subunits are also phosphorylated in intact cells. Thus, the regulation of PP2A or interaction with other proteins may be modulated by covalent modification of R5 family members.

The R5 subunits mediate interactions between PP2A and components of the Wnt signaling pathway involved in cell growth and transformation. Members of the R5 family were identified in a yeast two-hybrid screen by using the adenomatous polyposis coli (APC) protein as bait [49]. APC forms a signaling complex with axin and glycogen synthase kinase 3β that mediates the phosphorylation and proteasome-dependent degradation of β-catenin. A basal level of β-catenin degradation normally prevents transcription of β-catenin target genes involved in cell growth and transformation. Stimulation of the Wnt pathway causes inhibition of β-catenin phosphorylation and degradation, leading to increased transcription of β-catenin target genes. Ectopic expression of R5 subunits in mammalian cells causes a reduction in β-catenin levels and a decrease in expression of β-catenin target genes. Further supporting a role for PP2A in the Wnt/β-catenin pathway, the catalytic subunit of PP2A interacts with axin in two-hybrid assays and can be co-immunoprecipitated with axin [50]. Subsequent studies have shown that the scaffold subunit, the catalytic subunit, and R5 subunits can be immunoprecipitated with axin from *Xenopus* embryos [51,52]. Ectopic expression of the PP2A scaffold subunit, the catalytic subunit, or R5 subunits all have ventralizing activity in *Xenopus* embryos, consistent with a negative role in Wnt/β-catenin signaling. The R5 subunits appear to interact directly with axin at a site that is distinct from the sites that interact with APC, GSK-3β, and β-catenin [51]. The data are all consistent with an important role for R5 subunits in targeting PP2A to the axin/GSK-3/APC complex and regulating the Wnt signaling pathway.

The R5 subunits are also linked to cell survival and apoptosis. Cyclin G1, cyclin G2, and cyclin I are members of a unique family of cyclin-related proteins that are expressed in brain and muscle. R5 subunits interact with both cyclin G1 [53] and cyclin G2 [54]. Cyclin G1 and R5 subunits can be co-immunoprecipitated from neurons whereas cyclin G2-R5-catalytic subunit complexes can be isolated from cultured cells [54]. Although the function of the cyclin G1 is not known, the p53 tumor suppressor protein regulates its transcription. Ectopic expression of cyclin G1 enhances apoptosis in response to multiple stimuli in cultured cells [55]. Similarly, forced overexpression of cyclin G2 causes formation of aberrant nuclei and cell cycle arrest [54]. These observations raise the possibility that the cyclin G1-PP2A interaction could be involved in cell cycle arrest and apoptosis. The interaction of R5 subunits with the adenovirus E4orf4 protein is essential for E4orf4-mediated apoptosis [56]. Finally, the use of RNA interference in *Drosophila* cells has shown that loss of both of the *Drosophila* R5 subunits results in apoptosis [17].

R5 subunits interact with a variety of other proteins, thus indicating roles for this family in other signaling pathways (Table II). A *Drosophila* homolog of R5 interacts with a homeodomain-containing transcription factor called Sex Combs Reduced. This interaction positively modulates transcriptional activity [57]. The R5γ subunit is associated with L-type calcium channels, where it appears to target PP2A to regulatory sites phosphorylated by protein kinase A [58]. R5α interacts with the double-stranded RNA-dependent protein kinase PKR. PKR phosphorylates R5α, leading to an increase in PP2A phosphatase activity. PKR-enhanced PP2A activity may lead to decreased phosphorylation of eIF4E and altered protein synthesis. R5-containing PP2A may also be targeted to focal adhesions through interaction with paxillin [59].

PP2A-Interacting Proteins

Proximity interactions are the most important mechanism for regulating the activity of PP2A. Association with interacting proteins mediates many proximity interactions of PP2A, and allows targeting of this phosphatase to a wide variety of signaling pathways. PP2A interacting proteins include phosphoproteins that are PP2A substrates, scaffold proteins, and components of the cytoskeleton. As discussed above, many of these interactions occur with PP2A holoenzymes and are mediated by specific regulatory subunits. However, interacting proteins have been identified that interact directly with the PP2A core dimer and the catalytic subunit. PP2A-interacting proteins include virally encoded proteins and a host of cellular proteins that participate in interesting aspects of signal transduction. A compilation of the currently identified PP2A-interacting proteins is presented in Table II. Although many of the proteins listed in the table are substrates for PP2A, others act to target PP2A to specific signaling complexes, and some alter signaling by disrupting endogenous PP2A complexes. These proteins have been grouped into categories based on functional similarities. Brief descriptions of individual proteins and their interaction with PP2A are presented in the table.

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SECTION F

Cyclic Nucleotides

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CHAPTER 190

Adenylyl Cyclases

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Introduction

The second messenger cyclic AMP is a key component in intracellular signaling pathways in both prokaryotes and eukaryotes[2]. The enzyme responsible for its synthesis, adenylyl cylcase, is found in these organisms as either membrane-bound or soluble forms. Ten genes have been identified in mammals that encode either membrane-bound (AC1 to AC9) or soluble forms (sAC) of AC, and may be regulated by various factors [3–5]. Soluble and membrane-bound forms of AC encoded by genes from various genera have also been identified, although their modes of regulation are not fully appreciated [6,7].

In eukaryotes the primary role cAMP plays is to activate protein kinase A (PKA), however, cAMP also directly activates exchange factors of small molecular weight G proteins (RaplA)[8,9], activates cyclic nucleotide-gated channels, and regulates the activity of some cGMP-specific phosphodiesterases [10]. Through protein phosphorylation activated PKA can regulate a plethora of enzymes, secondary kinases, transcription factors, receptors, and channels [11]. The actions of PKA may support mechanisms of feed-forward (activation) or feedback (desensitization and downregulation). In bacteria, cAMP binds directly to transcription factors and is responsible for repression of expression of genes involved in metabolism, also serving as a feedback mechanism [12].

In higher eukaryotes such as mammals, receptor-activated G proteins, Ca^{2+}-activated calmodulin (CaM), protein kinases, and bicarbonate ions appear to be the native modulators of AC activity [3,4,13,14]. AC function may also be affected by cellular stress [15,16], as well as by exogenous small molecules such as adenosine analogs and the diterpene, forskolin. The responses to these regulators are exquisitely AC-subtype specific. Although several AC isoforms may be expressed together in the same cell, each isoform may be selectively regulated by specific factors. Even though this complicates studies evaluating the physiological role of ACs, overexpression studies, gene knockouts, and gene mutations have been developed to elucidate these roles. Furthermore, aberrant AC is implicated in several human diseases, making this a very important molecule to study.

In recent years the biochemical characterization of ACs has been the subject of intense research. Through structural and functional approaches, researchers have gained a firm understanding of how ACs are regulated and even elucidated the mechanism of catalysis [17–19]. Moreover, improvements in biochemical approaches have allowed scientists to study the function of AC at cellular and even atomic detail. The following few pages will place emphasis on the mammalian forms of AC but highlight some differences in the ACs from other genera. We will summarize our current understanding of the mechanism of regulation of ACs, summarize the catalytic mechanism, and discuss the physiological roles this regulation plays in the function of AC.

Structure-Function

Ten AC isoforms, nine membrane-bound and one soluble, have been identified and cloned in mammals [3,4,6,13]. The membrane-bound forms share the same topology in that they are composed of 12 transmembrane (TM) segments and 2 large cytoplasmic domains (Cl and C2). These proteins exist in the membrane as tandem repeats of 6-TM regions followed by a large cytoplasmic loop (Fig. 1A and Fig. 2A). The sequence similarity between the different ACs is about 60% with the most conserved residues residing in the cytoplasmic domains. These two loops also share considerable sequence similarity with guanylyl cyclases (GC), to the degree that as few as two point mutations may be introduced into AC to convert it to a functional GC.

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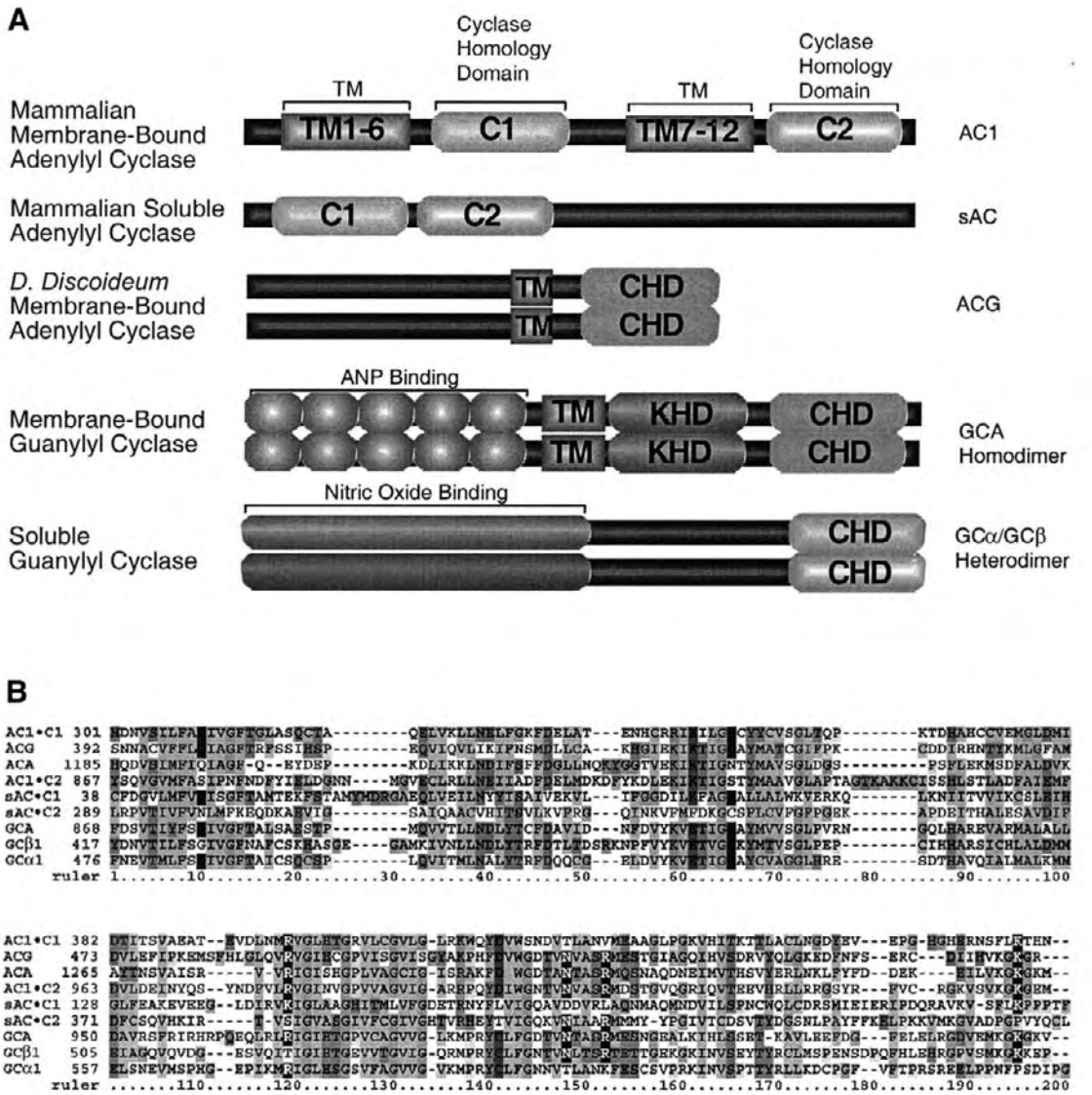


Figure 1 (**A**) This is the alignment of the domain structure of adenylyl and guanylyl cyclases. The putative domain structures of the cyclase homology domain (CHD), green and light blue; transmembrane domain (TM), yellow; ligand binding domain for atrial natriuretic peptide (ANP), pink; heme binding domain where nitric oxide binds, silver; and the kinase homology domain (KHD), purple. Note that the functional enzymes are organized as homo- or heterodimers of the catalytic domains. Membrane-bound and soluble ACs have both domains contained within one polypeptide, whereas the other cyclases require two proteins to have activity. (**B**) This is the alignment of the amino acid sequences of the adenylyl and guanylyl cyclases. Amino acid sequences bovine AC1 (GI: 162612), rat sAC (GI: 11067412), *Dictyostelium discoideum* ACG (GI: 167661) and ACA (GI: 457431), rat GC-A (GI: 204265), rat soluble GCα1 (GI: 1655846), and rat soluble GCβl (GI: 6980995). The mammalian ACs (AC1 and sAC) are divided into the Cl and C2 domains. Only the C2 domain from the *D. discoideum* ACA was included. The selected cyclase sequences were chosen as representative of eukaryotic nucleotide cyclase and were not singled out based on regulatory or mechanistic attributes. Residues are color coded to outline residue conservation. Residues that are important to enzyme function are also color coded as follows: lysine (K), glutamate (E), aspartate, (D) and cysteine (C) residues which contribute toward substrate specificity are boxed in red; conserved aspartate (D) residues which coordinate the two magnesium ions are boxed in royal blue; residues (arginine, R; asparagine, N; and lysine, K) which contribute toward stabilizing the transition state and that coordinate polyphosphate binding are boxed in black.

Several other unique forms of AC have been found in invertebrates as well as pathogenic bacteria. In the slime mold *Dictyostelium discoideum,* two diverse ACs containing a single TM region (ACA and ACR) have been identified, in addition to the canonical 12-TM form [20,21]. The bacteria *Bordella pertussis, Bacillus anthracis,* and *Pseudomonas aeruginosa* each excrete exotoxins, which possess AC activity [22–24]. These soluble ACs are taken up by host cells where they are then activated and begin producing very high levels of cAMP, thereby disrupting intracellular signaling pathways.

The X-ray crystal structure of the cytoplasmic Cl and C2 domains has recently been solved and has provided much information about the relevant active sites as well as the

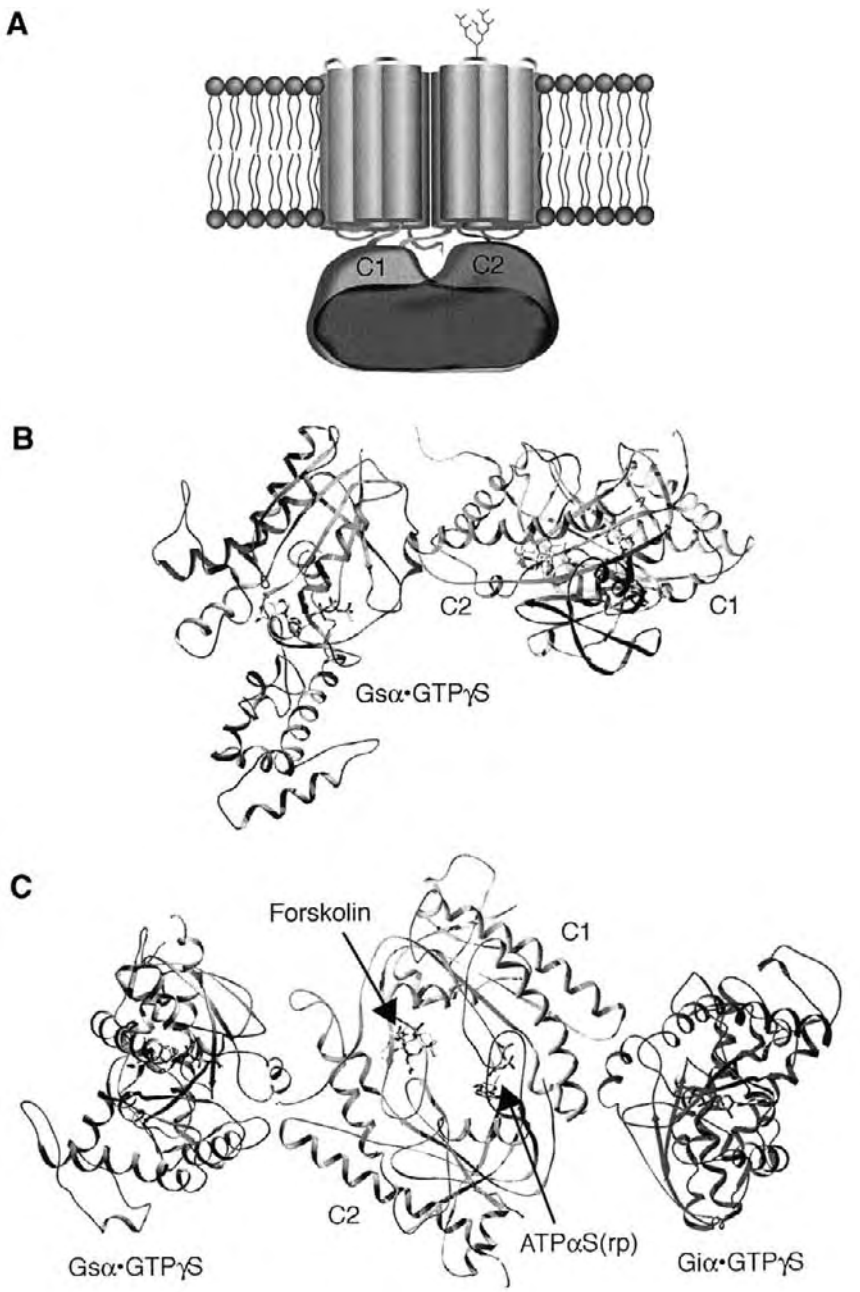


Figure 2 (A) This is an illustration of the membrane topology of a typical mammalian membrane-bound adenylyl cyclase. The 12-TM domain polypeptide also contains two large and homologous cytoplasmic domains each approximately 40 kDa in size. The Cl domain (lime green) and the C2 domain (sky blue) are represented by pseudosymmetrically related globular proteins. An asparagine-linked glycosylation site is also depicted in green. (B) This is an X-ray crystal structure of the stimulatory G protein, Gsα, bound to the catalytic core of adenylyl cyclase. GTPγS-activated Gsα (gray steel) is shown bound to the Cl(lime green) and C2 domain (sky blue) of adenylyl cyclase in the presence of forskolin and substrate inhibitor, ATPαS(rp). Illustrations were generated using SwissPDBViewer [73] and rendered with POV-RAY™ using the coordinates for the Gsα-GTPγ·Cl·C2·Fsk complex with ATPαS(rp) in the presence of Mn^{2+} and Mg^{2+} (PDB id: 1CJK)[26]. (C) Rotated view of the Gsα-GTPγS·Cl·C2·Fsk structure in (B) and with Giα-GTPγS (PDB id:lGIA) modeled into the pseudosymmetrically related Gsα-binding site. Visible also is the diterpene activator forskolin and the substrate inhibitor, ATPαS(rp). Note the twofold pseudosymmetry in the C1·C2 complex.

mechanism of catalysis (Fig. 2C) [17,18]. The catalytic core of AC is composed of a heterodimer of the Cl and C2 domains, which are related to each other by a twofold pseudosymmetry (Fig. 2). Forskolin binds to a hydrophobic pocket at the interface of the two domains, while G proteins bind on the surface, contacting both domains. The active site of catalysis, where nucleotides bind, is also located at the interface of the Cl and C2 domains and is pseudosymmetrically related to the forskolin binding site. The residues in this active site that are responsible for coordinating the binding of the nucleotide as well as two magnesium ions are highly conserved across all isoforms of both AC and GC [25,26]. In a manner similar to DNA and RNA polymerases, RNA spliceosomes, and reverse transcriptases, ACs utilize the metals to both stabilize the transition state of the reaction and also to deprotonate the 3' hydroxyl moiety of the ribose ring of ATP [19]. This is a key step that is necessary for the nucleophilic attack on the alpha phosphate by the newly formed oxyanion. The products are cAMP and the leaving group in the reaction, pyrophosphate (PPi).

The structure of AC from *Trypanosoma brucie* was recently solved and shares a similar protein-fold to the mammalian forms [27]. In sharp contrast, the structure of the catalytic AC domain of the exotoxin from *B. anthracis* recently delineated by the Tang laboratory, portrays a highly divergent protein-fold and a completely different catalytic mechanism [28]. AC from *B. anthraxis* utilizes the traditional catalytic triad consisting of histidine, serine, and aspartic acid residues to stabilize the transition state and deprotonate the 3'-OH.

Regulation

In invertebrates or vertebrates, neurotransmitter and hormonal regulation of ACs occurs primarily through heterotrimeric G proteins [29] (Table I and Fig. 3 for summary). G-protein-coupled receptor (GPCR) activation by these extracellular stimuli in turn leads to activation of bound G proteins by initiating the exchange of GDP for GTP. The α subunit of the stimulatory G protein (Gsα) activates all nine membrane-bound isoforms of AC in a nucleotide-dependent fashion, preferring the GTP-bound form to the GDP-bound form by a factor of 10 [4,13,30,31]. Gsα activation of ACs is terminated by GTP hydrolysis to GDP, a reaction that is accelerated by RGS proteins [32,33]. The α subunits of the inhibitory family of G proteins, $Gi_{1,2,3}$, Go, and Gz [34–36], inhibit AC activity, as the name would indicate, in an isoform-dependent manner [3,4,13,30]. Giocl-3, Goα, and Gzα inhibit AC5 and AC6, and Goα also inhibits AC1 and possibly AC8. For AC5 and AC6 Giα-inhibition does not occur by competition with Gsα; in fact, mutagenesis experiments suggest that Giα binds to a site pseudosymmetrically related to the Gsα site, on the opposite side of AC (see Fig. 2C) [37]. Gβγ subunits are also important modulators of AC activity. They can potently stimulate the activity of AC2, AC4, and AC7, but in a manner that is dependent on co-activation by Gsα [34,38]. Gβγ subunits are also potent inhibitors of AC1 and AC8 [34].

CaM is a ubiquitous Ca^{2+} sensor protein and is a potent activator of several mammalian membrane-bound AC isoforms: AC1 [39], AC8 [40], and perhaps AC3 [41]. The primary source of calcium ions is thought to be derived from capacitative entry through Ca^{2+} channels, rather than the G-protein-regulated and inositol triphosphate (IP3) sensitive release of Ca^{2+} from intracellular stores [42,43]. CaM is also implicated in the pathology of the bacterial exotoxins mentioned above, as it is the principal AC activator [44,45]. CaM activation of edema factor (EF; the exotoxin from *B. anthraxis*) yields a catalytic rate 1000-fold higher than that of CaM-activated mammalian ACs.

CaM also inhibits AC1 and AC3 indirectly through the activity of CaM-dependent protein kinase II and IV (CaMKII, IV)[46,47]. Phosphorylation of AC1 and AC3 by CaM kinases inhibits cyclase activity by blocking the binding of activators. In this sense, posttranslational modification of ACs by phosphorylation is generally inhibitory and can also be caused by the PKA as well as protein kinase C (PKC). PKA supports a negative-feedback mechanism whereby the more cAMP that is produced by ACs, the more PKA is activated, and thus the more ACs that are phosphorylated and inhibited.

The effects of Ca^{2+} have also been shown to be quite inhibitory on AC5 and anthrax [48,49]. Low micromolar concentrations of Ca^{2+}, well below the toxic levels and certainly within the physiological dynamic range found in a cell, effectively and specifically inhibit these two isoforms. While all isoforms of AC are inhibited by higher concentrations (mM), the effect on these isoforms are consistent with levels derived from capacitative entry, similar to the CaM-dependent stimulatory effect.

The small molecule forskolin (isolated from the plant *Choleus forskohlii)* is a potent activator of all mammalian membrane-bound isoforms of AC except for AC9, which is weakly activated [50]. AC isoform-specific forskolin analogs have been discovered using structural-based drug design, and it has been hypothesized that endogenous forskolin-like molecules may exist [51]. While the binding site of forskolin is within the conserved catalytic domain, the stimulatory actions appear to be selective for the membrane-bound vertebrate and invertebrate forms [50].

In contrast, ACs are inhibited by a class of adenosine analogs known as P site inhibitors [52]. These small molecules act by binding to a conformation of the enzyme that closely resembles the product-bound state or the posttransition state [53,54]. Inhibition is enhanced with the presence of PPi. The potency of P-site inhibitors is therefore increased by higher levels of AC activity [53]. Several adenosine analogs have been developed that appear to display some isoform preference [51,55]. In addition, polyphosphorylated acyclic nucleosides (such as 9-(2-triphosphonylmethoxyethyl) adenine, PMEApp) and foscarnet (phosphonoformic acid), also inhibit ACs [55,56]. Both drugs are used clinically as antiviral and antifungal agents and share a similar proposed mechanism of action as the P-site inhibitors on AC. It should be noted

Table I Summary of the Regulatory Properties of the Mammalian Adenylyl Cyclases*

AC isoform	Tissue distribution	Gαs	Gβγ	Gαi	Protein kinases	Calcium	Forskolin	Notes
AC1	Brain, adrenal (medulla)	↑	↓	↓(Gαo)	↑ PKC ↓ CaMKIV	↑ CaM	↑	
AC2	Brain, skeletal muscle, Lung (heart)	↑	↑*		↑ PKC		↑	
AC3	Brain, olfactory epithelium	↑			↑ PKC ↓ CaMKII	↑ CaM	↑	
AC4	Brain (heart, kidney, liver, lung, BAT, uterus)	↑	↑*		↑ PKC		↑	
AC5	Heart, brain, kidney, liver, lung, Uterus, adrenal, BAT	↑	↓	↓	↑ PKCα,ζ ↓ PKA	↓	↑	
AC6	Ubiquitous	↑	↓	↓	↑ PKC ↓ PKA	↓	↑	
AC7	Ubiquitous, High in brain	↑	↑*		↑ PKC		↑	
AC8	Brain, lung (testis, adrenal, uterus, heart)	↑				↑ CaM	↑	
AC9	Brain, skeletal muscle	↑						
sAC	Testis							↑ Bicarbonate

*Gβγ stimulation of AC isoforms is conditional upon Gsα co-activation.

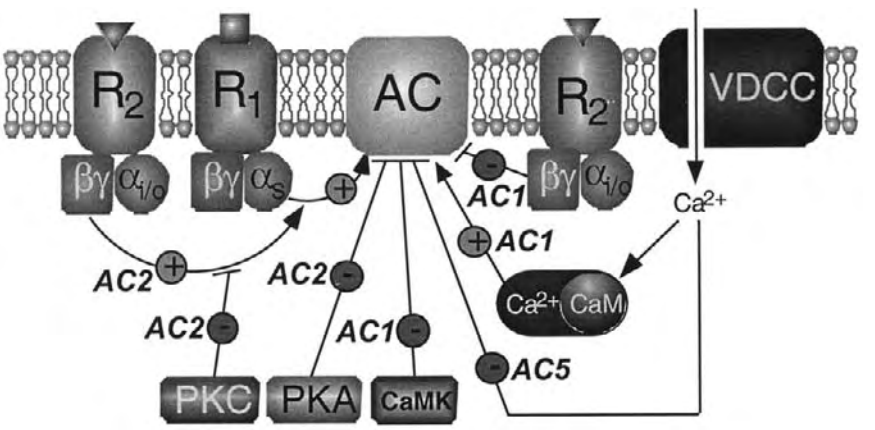


Figure 3 This is an illustration of the complex modes of regulation of membrane-bound mammalian adenylyl cyclase. Summarized are the stimulatory (green circles with pluses) or inhibitory (red circles with minuses) influences of hormone-receptor-mediated G-protein regulation, protein kinase regulation, and Ca^{2+} and/or CaM effects on AC activity. AC isoform-specific effects are demarcated in italics beside the stimulatory or inhibitory signs. For example, the Gβγ effects are inhibitory on the AC1 family of cyclases (AC1, AC8, and presumably AC3) and stimulatory on the AC2 family of cyclases (AC2, AC4, and AC7). The stimulatory effects of Gβγ are dependent on prior activation by Gsα. Ca^{2+}-CaM directly and potently activates the AC1 family of cyclases, whereas Ca^{2+} alone effectively inhibits the AC5 family of cyclase (AC5 and AC6). Not illustrated is forskolin, which activates all membrane-bound isoforms except AC9.

that the primary therapeutic target of these compounds is the mechanistically similar viral polymerases and transcriptases.

The lone soluble mammalian AC isoform (sAC) is as unique in overall structure as it is unique in regulation [6,7,57]. sAC activity is not affected at all by the classic AC modulators: G proteins, CaM, or forskolin [57]. Instead, it is activated *in vivo* as well as *in vitro* by bicarbonate ions [14]. Soluble forms of AC in prokaryotes are also sensitive to bicarbonate, suggesting that sAC is an evolutionarily conserved bicarbonate sensor [15]. The single TM domain AC in *Dictyostelium* is unique in the same way. It is regulated by osmotic stress; however, it is not yet clear if it actually has intrinsic osmosensing activity [16]. Although the mechanisms of regulation of these isoforms are quite different, the sequence of the catalytic domains are similar and the putative catalytic residues are conserved.

Physiology

Adenylyl cyclases are studied in many systems and have been implicated in numerous physiological roles. At the least,

it is known that all mammalian AC isoforms are expressed in the central nervous system and in excitable tissues; but, for the most part, AC is expressed in nearly every tissue (see Table I). More precise patterns of expression have been difficult to obtain due to relatively low levels of expression as well as a general lack of highly specific antibodies. Exceptions in expression patterns do exist, most notably in sAC, which is most highly expressed in the testis [6].

The precise roles of specific AC isoforms have been difficult to assess because most cells express multiple isoforms. The specific contributions of these ACs have only recently been segregated from the remaining isoforms. Researchers have taken advantage of genetic mutations in AC or gene disruption using homologous recombination in mice. Several studies have investigated the function of AC in the *Drosophila* mutant *rutabaga* [58]. These mutants are deficient in a calcium-activated AC, which is quite similar to the mammalian AC1. Deficiency of this AC causes these flies to avoid a trained odor, indicating that AC1 is important in memory and learning [59]. Likewise, specific disruption of the AC1 gene in mutant mice or the spontaneous mutation of AC1 in the *barrelless* mouse, have a negative effect on long-term potentiation (LTP) [60,61]. AC1 and AC8 are both necessary for both late-LTP (L-LTP) as well as long-term memory (LTM) [62]. Knockouts of either AC gene by itself yields normal L-LTP and LTM; however, double knockout mice exhibit no L-LTP or LTM. This effect can be reversed by infusion of forskolin into the hippocampus, which may compensate the null AC1 and AC8 by producing cAMP through other AC isoforms.

AC3 has been demonstrated to be involved in transmitting olfactory responses in mice [63]. AC isoforms 2,3, and 4 are all present in olfactory cilia; however, it is interesting that a knockout of just AC3 is sufficient to completely ablate responses to odorants. It has also been shown that ACs are important in developing drug dependencies. Following chronic opiate treatment, several ACs are upregulated and become supersensitized to additional stimulation by either Gsα or forskolin. Specifically, AC1, AC5, AC6, and AC8 are sensitized, while AC2, AC3, AC4, and AC7 are not [64–66]. Depending on the system, upregulation of AC may or may not involve a transcriptional step.

Upregulation of AC isoforms is also important for cell differentiation. AC2, AC5, and AC6 are upregulated in differentiation of pluripotent PI9 cells. Additionally, upregulation of AC2, AC5, and AC8 accompany neuronal differentiation [67]. It is also interesting that ectopic expression of AC2 in NIH3T3 cells inhibits cell cycle progression [68]. One resulting hypothesis is that for cell differentiation to occur, upregulation of AC2 is necessary to induce a temporary arrest of cell proliferation.

Summary

Adenylyl cyclase is clearly an incredibly important molecule as it is intimately involved in the very complex signaling pathways that regulate the numerous facets of life itself. The identification and characterization of ACs has come a long way since the initial discovery of cAMP nearly forty years ago, especially with recent advances applying molecular genetic and structural biology approaches. Nevertheless, many important questions still remain unanswered. For instance, what is the function of the 12-TM domain structure, other than localizing ACs to the membrane? Can the 12-TM structure support transport of molecules across the plasma membrane, as originally proposed when the first AC cDNA [39] was reported? Membrane-bound isoforms of ACs have recently been shown to homodimerize [69]. Although the relevance of AC dimerization is unknown, it is particularly intriguing with regard to the specter of heterodimerization. Heterodimers between different AC isoforms would add a new dimension to the already complex network of AC regulation. In any case, much more research is needed to more fully understand the precise regulation and physiological roles of ACs.

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CHAPTER 191

Guanylyl Cyclases

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Receptor guanylyl cyclases and their ligands together with guanosine-3′,5′-monophosphate (cGMP), and its effectors compose signal transduction pathways regulating essential tissue and cell functions. For example, cGMP is the documented second messenger for NO- and atrial natriuretic peptide (ANP)-induced vascular smooth muscle relaxation, and in the kidney cGMP mediates ANP-induced natriuresis and diuresis. Likewise, pathologic elevations of cGMP in intestinal mucosal cells result in severe diarrhea in response to secretion of a heat-stable peptide (Sta) from pathogenic strains of *Escherichia coli*; Sta is a potent agonist of a guanylyl cyclase in these cells. And disruption of the murine C-type natriuretic peptide (CNP) gene, a ligand for a chondrocyte guanylyl cyclase, results in dwarfism and early death, while disruption of a photoreceptor guanylyl cyclase results in cone-specific dystrophy in the mouse. Therefore, the various guanylyl cyclase/cGMP signaling pathways are physiologically important, but the relevance of the interactions with other signaling pathways has been less clear. Recently, cGMP signaling pathways have been shown to impact on signaling systems that regulate cell proliferation and differentiation.

Historic Perspectives

Guanylyl cyclases catalyzing the formation of cGMP and pyrophosphate (P~P) from MgGTP or MnGTP were identified in crude extracts of mammalian tissues shortly after the discovery of cGMP in rat urine [1]; importantly those studies established that the guanylyl cyclases were distinct from adenylyl cyclases implying different functions for each of the families of cyclases. Differences in subcellular distribution and kinetics of guanylyl cyclases in mammalian tissue homogenates prompted the suggestion that different forms of the cyclase exist rather than there being a differential distribution of a single form of the enzyme. Several decades later through the use of recombinant DNA technology, the early speculations were confirmed, and two categories of guanylyl cyclases were identified through cloning: single-pass plasma membrane or particulate guanylyl cyclases (pGC) and cytosolic or soluble guanylyl cyclases (sGC). P~P has not been shown to signal, and thus cGMP is considered the second messenger following ligand activation and no initial signaling pathway other than the generation of cGMP has yet been firmly documented for any of the cyclases (Fig. 1).

Guanylyl Cyclases

Seven single-pass plasma membrane (pGC) and four cytosolic or soluble (sGC) guanylyl cyclase subunits have been identified in mammals. Many more (23 putative guanylyl cyclase genes) have been identified in *Caenorhabditis elegans*, and multiple-pass plasma membrane cyclases, similar to the mammalian membrane forms of adenylyl cyclase, have been reported in *Dictyostelium discoideum, Plasmodium falciparum, Paramecium tetraurelia,* and *Tetrahymena pyriformis* [1].

The seven mammalian pGCs (GCA through GCG) are expressed in many different tissues and cultured cells (Table I). The structurally similar 120- to 140-kDa proteins contain an amino terminal extracellular domain (BCD; the apparent ligand-binding domain is the least similar within the family), a single-pass transmembrane domain (TMD), a protein kinase homology domain (KHD; 30% homologous to protein kinase catalytic domains [2], and a carboxyl-terminal cyclase catalytic domain (CCD; the most conserved and the most highly similar to adenylyl cyclases [l]). Homodimeric plasma membrane cyclases appear to be preferentially expressed *in vivo* even though more than one cyclase is expressed

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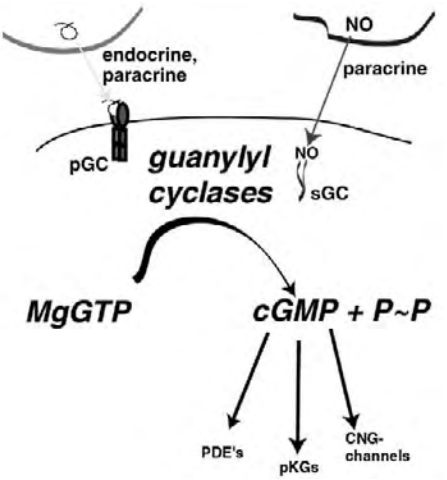


Figure 1 The two general forms of guanylyl cyclase found in mammals. The plasma membrane (pGC) forms exist as minimal homodimers and the soluble (sGC) forms exist as minimal heterodimers to display catalytic activity. The increased concentrations of cGMP found as a result of stimulation of these forms then acts on phosphodiesterases (PDEs) that are stimulated or inhibited by cGMP, ion channels (CNG) directly gated by cGMP, or on cGMP-dependent protein kinases (PKGs) to elicit a cell behavioral response.

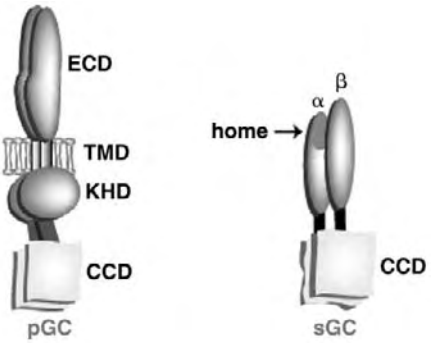


Figure 2 The general domain structure of the membrane forms (pGC) or the soluble forms (sGC) of guanylyl cyclase. BCD represents the extracellular ligand binding domain, TMD the transmembrane segment, KHD the protein kinase homology domain, and CCD the cyclase catalytic domain. The KHD appears to bind ATP as a regulatory molecule. For sGC, the subunits of the heterodimer have been arbitrarily named a and p. Heme binds to the amino terminal region, probably to the p subunit.

simultaneously by a single cell. The minimum catalytic unit of the pGCs appears to be that of a homodimer [3] (Fig. 2).

The sGCs are heterodimeric proteins composed of subunit isoforms a (cti, *0.2,* both 82 kDa) and P (pi,70 kDa; 02,76 kDa) and are expressed in many of the same cells and tissues as the pGCs (Table II). A heme moiety noncovalently bound to the p subunit amino terminus confers ligand (NO) sensitivity to the cyclase. Two human subunits, named 0:3 and ps are orthologs of rat oti and pi. The aipi heterodimer is more commonly found *in vivo* but aapi has been detected as well. The *$2* subunit also contains a potential geranylgeranylation site [4] raising the possibility of plasma membrane localization of this form and the possibility therefore of NO-stimulated cGMP elevations at the level of the membrane. The minimum active mammalian soluble cyclase appears to be a heterodimer as expression of a or p subunits alone results in no detectable guanylyl cyclase activity while co-expression of various subunits produces high-soluble guanylyl cyclase activity. The studies of Sunhara *et al*. [5] and of Liu *et al.* [3] also support the existence of a dimer as the minimal unit for catalytic activity.

Guanylyl Cyclase Ligands

Ligands have been identified for some but not all mammalian GCs (Table II). Human ANP (28 amino acids), brain natriuretic peptide (BNP, 32 amino acids), and CNP(22 or 53 amino acids) compose a family of distinct and structurally similar oligopeptides having a highly conserved 17-member ring required for biological activity. ANP and BNP are endocrine ligands released from the heart that promote natriuresis, diuresis, and vasorelaxation by direct activation of GCA in the kidney, in vascular smooth muscle, or the adrenal gland. CNP, classified as a natriuretic peptide by virtue of its structure and the only known ligand for GCB, has no known tissue depots and only marginally mimics the vascular and renal actions of ANP and BNP. CNP is generally considered a paracrine ligand involved in regulation of cell proliferation and differentiation [6,7].

ANP, BNP, and CNP bind with near equal avidity to a third cell surface receptor, the natriuretic peptide clearance receptor (NPR-C). NPR-C has a BCD similar to GCA and GCB, a short cytosolic domain devoid of the KHD and CCD, and is expressed by most mammalian cells. Internalization of the peptide-receptor complex serves to remove or "clear" natriuretic peptides from the extracellular space thus buffering their effects on cell function. There is some evidence suggesting that NPR-C may also signal via a pertussis-toxin-sensitive pathway to inhibit adenylyl cyclase [8].

GCC was identified initially as the receptor for the enterotoxin, Sta. Subsequently three mammalian ligands for GCC, guanylin, uroguanylin, and lymphoguanylin were isolated. All are small peptides with structural homology and sequence identity and expressed in the intestine and other tissues.

NO, the ligand for sGCs, has diverse actions on cardiovascular, renal, and immune cell function and is expressed by many cell types [1]. NO has effects similar to the natriuretic peptide signaling pathways in those cells where GCA, GCB, and sGC are expressed.

Extracellular ligands for GCD, GCE, GCF, and GCD have not been identified and remain orphan receptors. GCE and GCF (human RetGC-1 and RetGC-2), expressed in photoreceptors, are activated intracellularly by Ca^{2+}-free forms of guanylyl cyclase activating proteins (GCAPs) which bind to the coiled-coil region linking the KHD and CCD [9,10]. Although GCE and GCF may not require extracellular ligands based on these observations, the conservation of Cys within the BCD compared to the guanylyl cyclases with known ligands, and the conservation of the BCD across all vertebrates that have been studied suggests heavy evolutionary pressure

is being exerted to retain the BCD structure. One source for this pressure would be the need to recognize a ligand.

cGMP Effectors

At least three classes of cGMP-binding proteins amplify and mediate changes in intracellular cGMP levels in mammalian tissues: cGMP-dependent protein kinases (PKG), cyclic nucleotide-gated (CNG) ion channels, and cyclic nucleotide phosphodiesterases (PDE). The serine/threonine protein kinases PKG1 and PKG2 mediate most of the known effects of cGMP. The cytosolic PKG1 is the more widely distributed form, highly expressed in vascular smooth muscle, cerebellum, and platelets. Gene disruption of this kinase in the mouse results in vascular, intestinal, and erectile dysfunctions [1]. PKG2, abundant in intestine, bone, lung, and brain, contains a myristolated site and is localized to the plasma membrane, and gene disruption in the mouse results in resistance to Sta-induced diarrhea, intestinal secretory defects, and dwarfism [11].

The 11-gene PDE family functions to decrease signaling levels of both cGMP and cAMP and also provides a point at which both cGMP and cAMP signaling pathways can intersect. PDE5, -6, and -9 are cGMP-specific [12], cGMP-stimulated PDE-2 hydrolyzes both cGMP and cAMP, and PDE3A is a cGMP-stimulated, cAMP-specific family member.

Cyclic nucleotide-gated channels are ubiquitously expressed, the prototypical CNG channel being the photoreceptor, relatively nonselective cation channel.

Guanylyl Cyclases and Cell Growth Regulation

Although NO, ANP, and BNP appear to be important counterbalances to the renin/angiotensin/aldosterone axis in the cardiovascular system, there is considerable and convincing evidence that various guanylyl cyclases also regulate cell proliferation and differentiation. The molecular basis of such regulation remains principally at the descriptive level in that a number of growth factors, including serum, act rapidly to desensitize either GCA or GCB [1,13] and likewise, cGMP, possibly in the same manner as cAMP, inhibits growth factor activation of the MAP kinase pathway. Interestingly, this apparent adversarial relationship between various mitogens and the guanylyl cyclases appears to primarily involve the membrane forms of the enzyme [13]. hi fibroblasts, whereas serum, basic fibroblast growth factor, or platelet-derived growth factor decrease CNP-stimulated GCB activity, they fail to alter the activity of NO-stimulated guanylyl cyclase [13]. Identification of the pathway by which the various mitogens, including serum, communicate with the membrane forms of guanylyl cyclase, in particular GCA and GCB, remains unknown.

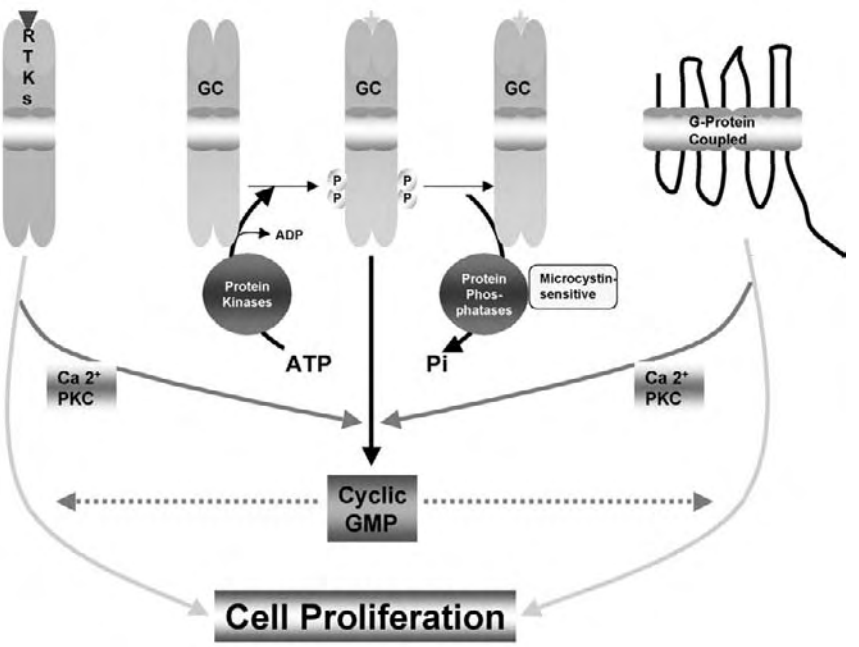


Figure 3 Schematic showing the adversarial relationship between various growth factor receptor signaling pathways and guanylyl cyclases A and B. By mechanisms not yet understood, various growth factors including serum act on receptor tyrosine kinase receptors or G-protein-coupled receptors to rapidly desensitize the ligand-stimulated forms of GCA or GCB. Both Ca^{2+} and protein kinase C(PKC) have been suggested as important components of the desensitization pathway. The dephosphorylation of the guanylyl cyclase receptor, leading in part or totally to desensitization, is mediated by a microcystin-sensitive protein phosphatase. Likewise, cGMP inhibits activation of the MAP kinase pathway in response to growth factors or serum. The subsequent rate of cell proliferation is dictated by whether the guanylyl cyclase or mitogen-stimulated pathways are dominant.

Both GCA and GCB appear to exist in a phosphorylated state in the absence of ligand, and it is the phosphorylated form that is most sensitive to the addition of ligand. Based on the work of Potter and Hunter [14], 6 principal sites of phosphorylation exist, all located within or just to the amino terminal side of the consensus protein kinase homology domain. A number of reports have suggested that dephosphorylation at these sites leads to desensitization of either GCA or GCB [14]. However, neither the protein kinase(s) nor the protein phosphatase(s) responsible for the apparent regulation of these receptors have been identified, although the protein phosphatase(s) responsible for dephosphorylation have been shown to be particularly sensitive to inhibition by microcystin [15,16] (Fig. 3). A significant number of reports suggest that activation of protein kinase C is one of the important upstream events that lead to desensitization of the natriuretic peptide receptors [14,17], where even the dephosphorylation of a single serine through activation of the protein kinase C pathway appears to desensitize GCB [18]. However, the recent work of Abbey and Potter [17] suggest that not only protein kinase C but also the other arm of the phospholipase C pathway (IPS/Ca^{2+}) is capable of causing desensitization of GCB. Their work in A10 smooth muscle cells further suggests that it is the elevations of Ca^{2+} that are required for the desensitization. Interestingly, however, the degree of desensitization obtained through elevations of Ca^{2+} alone are not equivalent to those seen through activation of the phospholipase C pathway [17]. Identification of the guanylyl cyclase protein kinase(s) and phosphatase(s) now seems essential for the understanding of the mechanisms by which the various mitogens, acting through either G-protein-coupled or receptor tyrosine kinase receptors mediate a rapid and marked decrease in guanylyl cyclase activity, where Ca^{2+} or protein kinase C may often serve as upstream regulators.

Acknowledgments

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CHAPTER 192

Phosphodiesterase Families

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Introduction

The cyclic nucleotides are ubiquitous second messengers that regulate a large number of processes, including proliferation, chemotaxis, differentiation, contraction, gene transcription, and inflammation. These second messengers are produced and utilized by nearly all eukaryotes from amoebae to man. Regulation of the intracellular levels of cGMP or cAMP, both in the resting state and in response to stimuli, is therefore critical for the proper functioning and survival of many organisms. The levels of a cyclic nucleotide in the cell are determined by the relative rates of synthesis by the cyclases, adenylate cyclase, and guanylate cyclase and degradation by the phosphodiesterases (PDEs). Although the generation of cyclic nucleotide signals in many systems has been the subject of intense study, the understanding of the mechanism by which these signals are terminated by the PDEs is, in most cases, still in its infancy. This is curious as the cyclase and phosphodiesterase activities were discovered within a few years of one another [1,25].

"Cyclic nucleotide phosphodiesterase" was first described as a widely distributed enzyme that could catalyze the hydrolysis of cAMP and cGMP to their respective 5′ monophosphates [2]. The initial studies of PDE activity used either tissue homogenates or partially purified preparations of these enzymes from various tissues. The characteristics of PDE activity from these studies varied greatly depending on the PDE source. It was unclear whether these differences were a consequence of the purification scheme of these enzymes (that is, the presence of different contaminating proteins) or of the existence of multiple forms of PDE. PDEs were therefore referred to in terms of the tissue from which they were purified (for example, rat liver PDE or bovine brain PDE). Later anion exchange chromatography experiments demonstrated the presence of several PDE activities in an individual tissue or cell type. These observations were later confirmed in experiments by use of immunocytochemistry, immunobloting, and *in situ* hybridization. With the purification of multiple enzymes to apparent homogeneity and more stringent characterization of their properties, PDEs were subsequently named according to their regulatory properties and substrate specificities (for example, calcium/calmodulin-stimulated PDE or cGMP-stimulated PDE). With the advent of molecular biology, there has been a virtual explosion of new information, including the cloning of the previously known and many new PDE genes, as well the identification of a number of new splice variants. Nucleotide sequence data for the PDEs have also allowed for the organization of them into gene families according to homology. As more data emerge regarding the distribution, characteristics, and roles of the many PDE isozymes, it is clear that the regulation of cyclic nucleotide signaling by PDEs is far more complex than could have been imagined when they were first studied in the 1960s.

The Gene Families

There currently exist 11 PDE gene families. The current nomenclature for a PDE contains, in order, two letters to indicate species, followed with a number indicating gene family, a letter to represent an individual gene, and finally a letter to identify the splice variant. For example, HSPDE7A1 represents *Homo sapiens* PDE gene family 7, gene A, splice variant 1. For a correlation between older and current PDE nomenclature, see Beavo *et al*. [3]. The kinetic properties, substrate specificities and drug sensitivities of these families (Table I) have been described extensively elsewhere [3,23,26,27] and will not be discussed at length here. In general, the phosphodiesterases share the same organizational structure. Each protein has an N-terminal domain that confers regulatory properties to the protein, followed by a more C-terminal ~270 amino acid catalytic domain and a

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Table I Characteristics of the PDE Families

PDE family	Genes	# Splice variants	Regulatory domains, role	Phosphorylation	Substrate(s)	Commonly used inhibitors
PDE1	1A, 1B, 1C	9	CaM, activation	PKA	cGMP, cAMP	KS-505
PDE2	2A	3	GAF, activation	Unknown	cAMP, cGMP	EHNA
PDE3	3A, 3B	1 each	Transmembrane domains, membrane targeting	PKB	cAMP	Milrinone
PDE4	4A, 4B, 4C, 4D	>20	UCR1, UCR2, unclear	ERK, PKA	cAMP	Rolipram
PDE5	5A	3	GAF, unclear	PKA, PKG	cGMP	Sildenafil, Dipyrimadole, Zaprinast
PDE6	6A, 6B, 6C	1 each	GAF, activation	PKC, PKA	cGMP	Dipyrimadole, Zaprinast
PDE7	7A, 7B	6	Unknown	Unknown	cAMP	None Identified
PDE8	8A, 8B	6	PAS, unknown	Unknown	cAMP	None Identified
PDE9	9A	4	Unknown	Unknown	cGMP	None Identified
PDE10	10A	2	GAF, unknown	Unknown	cAMP,cGMP	None Identified
PDE11	11A	4	GAF, unknown	Unknown	cAMP, cGMP	None Identified

short C-terminal tail. The sequence identity in the catalytic domain between genes is only about 35 percent, yet all PDEs possess the signature sequence H–D–X_2–H–X_4–N [4]. The substrate specificities of the different PDE families run the gamut from dual-specificity PDEs to those that are highly specific for either cAMP or cGMP. Further, the relative substrate specificity can vary even between members of a gene family. For example, within the PDE1 family, PDE1A2 has a K_m for cGMP that is approximately 20-fold higher than that for cAMP, yet PDE1C2 has a K_m that is equal for both. In addition to variation in specificity, the activity of a PDE toward one nucleotide may depend upon the concentration of the other. For instance, PDE2s hydrolyze cAMP and cGMP with relatively similar K_m values. However, the presence of a small amount of cGMP (which binds allosterically) stimulates the enzymes' catalytic activity toward cAMP several-fold [5]. To make things more complex, there are also PDEs for which the cyclic nucleotides are competitive inhibitors for one another. Cyclic AMP is a competitive inhibitor of cGMP hydrolysis by PDE10 [6], and cGMP is a potent competitive inhibitor of cAMP hydrolysis for PDE3 [7]. This variety and flexibility in substrate specificities of the PDEs makes them a family of enzymes with tremendous diversity, suitable for the fine tuning of many cyclic nucleotide-mediated signaling systems.

As mentioned above, most of the PDEs also possess domains within their N-termini that regulate the activity of the catalytic site (Fig. 1). The PDE1 proteins have two Ca^{2+}/calmodulin binding domains, and binding of calmodulin to these PDEs stimulates their activity [8]. The PDE2, PDE5, PDE6, PDE10, and PDE11 proteins all have allosteric, cGMP-binding domains known to be part of the larger GAF domain family [9]. The consequence of binding of cGMP to these domains varies with the PDE. As discussed above, cGMP binding to PDE2 stimulates activity. For PDE5, binding of cGMP to this domain alters the protein's susceptibility to phosphorylation, but a direct effect on activity has not been demonstrated. For further discussion of the GAF domains in PDEs, see Martinez, Tang *et al.* (Chapter 207) in this volume. The PDE3 family proteins have six predicted transmembrane segments in their amino terminal domains, consistent with the observation that PDE3 activity is at least partially membrane-associated. The PDE4 family, a large family of enzymes with four genes and many splice variants, is responsible for the majority of basal cAMP-hydrolyzing activity in most cells. For further discussion of the PDE4 family, see Conti (Chapter 71) in this volume. The N-terminus of PDE8 contains a PAS domain, a domain that generally is found in proteins that are involved in sensing and responding to the cellular environment (for example, redox state, light or energy levels) [10]. The PAS domains in many proteins bind small molecules such as heme, NAD, or chromaphores and can also serve as a site for homodimerization. It will be interesting to see whether some small molecule also binds the PDE8 PAS domain, what effect that may have on activity,

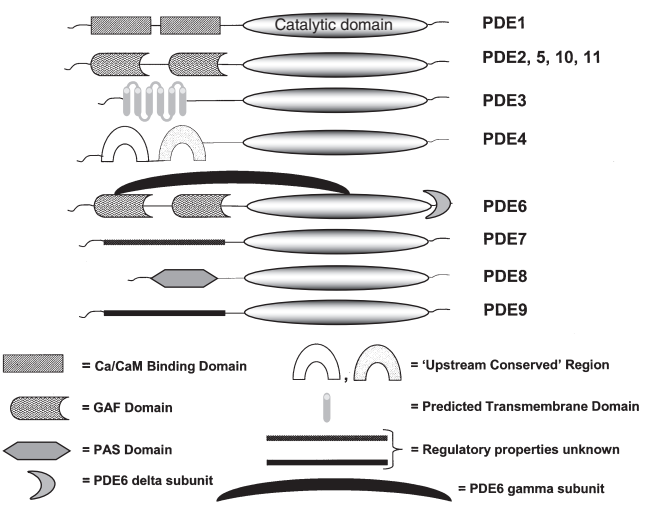


Figure 1 Domain organization of the PDE families.

and also whether the PAS domain of PDE8 serves to dimerize the protein. PDE7 and PDE9 have substantial N-terminal segments that bear no resemblance to known proteins. What role these portions of PDE7 and PDE9 have in the protein remains to be seen.

Thus, with great variation in nucleotide specificity and regulatory properties, the PDE superfamily comprises a complex set of enzymes that can provide cross talk between the cGMP and cAMP pathways, with Ca^{2+}/CaM dependent pathways, and allow the cell exquisite control of cyclic nucleotide dynamics.

Implications of Multiple Gene Families/Splice Variants

Another remarkable feature of the PDE superfamily is the expression and localization patterns of its members. Individual PDEs, within gene families, and even between splice variants, have unique expression patterns. For example, the PDE1 genes PDE1A, PDE1B, and PDE1C are all expressed in brain. However, *in situ* localization studies have shown that PDE1A is expressed primarily in the cortex, PDE1B in the striatum, and PDE1C in the cerebellum [11]. The expression pattern of PDE1C splice variants has been further broken down. PDE1C5 is highly expressed in testis; PDE1C1 is more generally distributed, found in heart, testis, cerebellum, and olfactory epithelium; and PDE1C2 is primarily expressed in the olfactory neuroepithelium.

The PDE3 genes provide another example of the complex localization of family members. PDE3A and PDE3B are generally found in distinct cell types, with PDE3A expressed in platelets and PDE3B in adipose cells and hepatocytes. Both are apparently expressed in vascular smooth muscle cells, although probably in different compartments. For further discussion of the PDE3 family, see Weston *et al.* (Chapter 72) of this volume.

The ability to produce multiple N-terminal variants allows for specific differential targeting of the PDE2 family members. The three PDE2A splice variants differ only at their extreme N-terminus. However, the PDE2A2 N-terminus contains a putative transmembrane domain, and the PDE2A3 variant contains an N-terminal myrisoylation site, both of which probably allow for targeting to membrane compartments of the cell and are responsible for the membrane associated forms of PDE2 activity observed in tissue homogenates [12].

All of the above examples of differential localization of PDE genes/splice variants imply that each of these PDEs probably plays specialized roles in the regulation of cyclic nucleotide signaling in cells. Perhaps the definitive example of precise localization of a PDE to achieve specialized function is in the case of the PDE6 gene family. In the photoreceptor cells of the retina, a visual signal is generated through the activation of a cascade of proteins that ultimately result in the activation of PDE6. PDE6 rapidly hydrolyzes the cGMP in the cell, the resident cGMP-gated cation channels close, and the cells become hyperpolarized. All of the proteins involved in this cascade, including PDE6, are expressed primarily in the retina and specifically targeted to the membrane disks of the photoreceptors. Although this PDE family is the first known and best characterized example of a highly specialized PDE, there are certainly others to follow. A prime candidate example is PDE1C2. As mentioned previously, PDE1C2 is highly expressed in the olfactory neuroepithelium. The other major PDE expressed there is PDE4A. However, expression of PDE1C2 is restricted to the cilia of the epithelium, where it co-localizes with adenylate cyclase III. PDE4A is not expressed in the cilia of the neurons, but rather throughout the remainder of the neuronal layer [13]. Clearly, in the olfactory neuroepithelium, PDE1C2 and PDE4A are playing different roles in regulating cAMP during olfaction.

Another demonstration of compartmentalization of PDE activities was accomplished by Dousa and colleagues while investigating the effects of cAMP-elevating agents on kidney mesanglial cells. In these cells, mitogenesis and superoxide production are both stimulated through the production of cAMP. Rolipram (a PDE4-specific inhibitor) inhibited the production of superoxide in these cells while having no effect on mitogenesis. Likewise, cilostamide (a PDE3-specific inhibitor) inhibited mitogenesis while having no effect on superoxide production. These results indicate that these two PDEs have access to different cAMP pools within the cell [14].

The phenomenon of differential localization of PDEs speaks to the idea of compartmentalization of cyclic nucleotide signals. Compartmentalization was originally proposed to explain how a second messenger as ubiquitous as cAMP can mediate different effects within a cell [15,20]. The idea that there are different cyclic nucleotide "pools" available only to certain kinases, channels, exchange factors, or PDEs is a compelling one. However, the details of how these compartments are established are only beginning to emerge. For the cAMP-dependent protein kinases, differential localization is accomplished through their association with anchoring proteins, AKAPs. It is becoming increasingly clear that signaling "modules" are built through the association of receptors, mediators, and targets with each other, AKAPs, and other scaffolding proteins. To that end, in recent years the AKAPs have been shown to associate with receptors, channels, G proteins, and PDEs [16,18,28,29,30]. PDEs also may have their own scaffolding partners. Recently, it was shown that PDE4D5 interacts with RACK1, a WD-repeat protein that also serves as a scaffold for PKC, Src, and integrin [17]. In addition, PDE4D interacts with myomegalin, a protein that appears to target PDE4 to the Golgi and centrosomes of cells [18]. As better tools become available to investigate PDE targeting, we are likely to see more examples of this type of compartmentalization of PDE function.

PDE Inhibitors as Therapeutic Agents

The wide variety of PDE isozymes implies that PDEs have tremendous therapeutic potential. Indeed, some of the

oldest drugs man has known (for example, caffeine, ginseng) are PDE inhibitors! Some PDE inhibitors such as theophylline, papaverine, and dipyridamole were in fact used before their mechanism of action was known. However, with a new appreciation of the complexity of the PDE signaling systems and the availability of more sophisticated endpoint assays, a new generation of PDE-inhibiting drugs is being developed.

PDE inhibitors have long been known to have anti-inflammatory properties and have been used for the treatment of asthma, stroke, and chronic obstructive pulmonary disease (COPD). Early treatments for these diseases with PDE inhibitors have unfortunately been hampered by the side effect of emesis. Novel PDE4 inhibitors (Ariflo, GlaxoSmithKline; and Roflumilast, Byk Gulden) are now in clinical trial for the treatment of asthma and COPD with milder emetic side effects [19]. Milrinone, a PDE3 inhibitor that has been used to treat patients in congestive heart failure, has recently been shown *in vitro* to increase conductance of the CFTR transporter, indicating promise for the treatment of cystic fibrosis [20,31]. Cilostazol, also a PDE3 inhibitor, is currently being applied to the treatment of patients with intermittent claudication, and clinical trials suggest that cilostazol may also be useful in the prevention of restenosis after angioplasty [21,32]. Dipyridamole inhibits PDEs in platelets and is commonly used in combination with aspirin (ASA) to reduce clotting, despite the initial lack of clinical data demonstrating an added benefit of dipyridamole over ASA [22]. However, the recent European Stroke Prevention Study (ESPS2) clearly demonstrated an additive effect of dipyridamole and ASA in the prevention of second stroke [23]. Thus, dipyridamole will likely continue to be prescribed in combination with ASA. And finally, Viagra, a PDE5-specific inhibitor, has been used successfully for the treatment of male erectile dysfunction with minimal side effects.

The successful treatment of patients with specific PDE inhibitors is encouraging but not surprising given the specific, diverse distribution of many of the PDEs. In addition, the recent identification of the PDE8, PDE9, PDE10, and PDE11 should lead to the development of novel inhibitors with as yet unknown application. As we begin to fully appreciate the full complement of PDEs in a system of interest and how they are compartmentalized and regulated, new therapeutics (as well as novel application of older drugs) will no doubt be applied to a wide variety of disease states.

Where Do We Go from Here?

Although it is certainly possible that more PDEs exist that bear little resemblance in linear sequence to the currently known proteins, it is also certain that most if not all of the proteins responsible for the major PDE activities in tissues have been identified and their genes cloned. With the upcoming completion of the sequencing of multiple genomes, the identification of new PDE gene families by the "traditional" method of BLAST searching with current known sequences is probably drawing to a close. One of the new challenges in the field lies in determining exactly how many PDEs exist. Many of the newly identified splice variants are based on EST searching or RACE from cDNA libraries. It is often difficult to distinguish alternative splicing from library construction artifacts or mis-splicing. It is therefore important to confirm the existence of these variants by using a variety of techniques, including RNAse protection and immunoblotting. There are currently few good antibodies available for PDEs, especially the newly discovered ones. It will take the development of a full complement of good, sensitive antibodies specific for all PDEs and spice variants to accomplish this goal.

Another challenge in the field lies in the potential differences in PDE activities and their regulation *in vitro* versus '*in cellulo.*' Only a few of the PDEs have potent, highly selective, cell-permeable inhibitors. Therefore, current methods for studying PDE activity/regulation require either purification of the protein or overexpression in heterologous systems. Localization of PDEs is determined through subcellular fractionation or immunocytochemistry by using tissues that have been fixed and mounted to a slide. Little is known about how well these studies correlate to the situation in the cell. Removing a PDE from its native environment also removes it from other regulatory factors. It is possible that factors that mildly stimulate a PDE *in vitro* have much greater effects *in situ*. In addition, PDEs are also vulnerable to proteolysis in the *in vitro* assays, which can affect their activities. Two things are in great need. One is selective inhibitors for all the PDE families, preferably for the different splice variants as well. This need is exemplified by the discovery that the newest PDEs are insensitive to IBMX, which has been used for decades as a nonspecific PDE inhibitor. It is not clear whether the development of splice variant-specific PDE inhibitors is possible, but efforts in this area are under way. A second need is the accurate, sensitive, real-time measurement of PDE activity in live, intact cells. New techniques have already been developed for the real-time measurement of cyclic nucleotide levels in cells [24,50,51]. Although further refinement of these technologies is needed, the real-time measurement of cyclic nucleotide levels, combined with specific PDE inhibitors, will prove to be a powerful step in furthering the understanding of PDE functions in cells.

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CHAPTER 193

The cAMP-Specific Phosphodiesterases: A Class of Diverse Enzymes That Defines the Properties and Compartmentalization of the cAMP Signal

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The second messenger cAMP generated by adenylyl cyclases either interacts with and activates intracellular effectors or is degraded and inactivated by phosphodiesterases (PDEs). Of the eleven PDE families thus far identified, three families of isoenzymes are specific for cAMP, including family 4,7, and 8 (see chapter *xx.* for the nomenclature). Given their high affinity for cAMP in the submicromolar to micromolar range, they are the primary enzymes involved in the inactivation of this cyclic nucleotide under resting or stimulated conditions, thus playing a critical role in signaling.

Isoenzymes that belong to the PDE4 family were the first to be identified on the basis of their pharmacological and biochemical properties. Indeed, rolipram, a PDE inhibitor developed in the fifties as an antidepressant that specifically targets PDE4, has been of tremendous value in dissecting the properties and functions of PDE4. Because of their potential therapeutic use in inflammatory disorders, drugs specific for PDE7 and PDES are also under development.

The cloning of the PDE4 genes by virtue of their homology with the *Drosophila* PDE [1] was soon followed by the identification of the other two families using either a strategy of complementation of yeasts deficient in phosphodiesterases for PDE7 [2], or database homologous searches for PDE8 [3]. It is now established that four genes code for the PDE4s, whereas two genes each encode the PDE7s and PDESs. The presence of multiple genes represents only a first layer of complexity in the cAMP-PDE enzymes, as multiple transcripts originate from these genes either by alternate splicing or the use of different promoters, greatly expanding the number of cAMP-PDE proteins expressed in mammalian cells. Although the number likely will be corrected upward, 15–18 different PDE4, 4 different PDE7, and potentially 6 PDE8 proteins thus far have been identified [4].A total of 28 different proteins specifically hydrolyze cAMP in the cell (Fig. 1). Given this extreme heterogeneity, it has been proposed that cAMP-PDEs have a modular structure with

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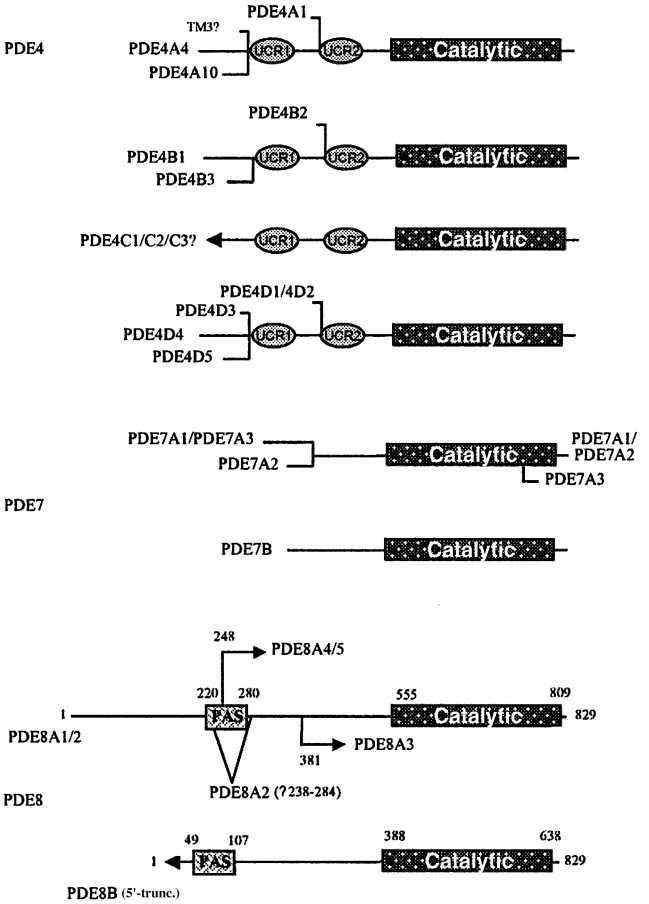


Figure 1 Schematic representation of the different cAMP-PDE expressed in human cells. The gene family is reported on the left. The open reading frame is indicated as a continuous line with the catalytic region indicated as a box. The point of divergence of a splicing variant is indicated by a bracket. The conserved UCR1 and UCR2 regions present in all PDE4 are designated as ovals. The PAS domain in PDE8 is indicated as a cross-hatched box.

different regulatory cassettes specific for each variant, and that cells use distinct cAMP-PDE proteins with subtle differences in their properties to adapt cAMP signaling to their specialized functions.

Structure of the cAMP-PDEs: Catalytic and Regulatory Domains

Cyclic AMP-PDEs are proteins with molecular weights ranging between 50 and 130 kDa and are composed of a catalytic domain surrounded by domains with regulatory functions. Deletion and site-directed mutagenesis, as well as analysis of sequence conservation, have defined the boundaries of the catalytic domain and mapped it to the carboxyl-terminus end of the cAMP-PDE. The structure of this domain in a PDE4 has been recently resolved at the atomic level, demonstrating a compact globular bundle of 17 helices subdivided in 3 subdomains that define a catalytic pocket [5]. Two metal ion binding sites are present in this pocket: one of these metal binding sites is thought to be permanently occupied by Zn^, whereas binding of the second metal ion at the second site, likely Mg^{2+}, can be rapidly exchanged. Both metals are essential for substrate binding and for the catalytic activity of PDE4 and presumably for the other cAMP PDEs. Changes in affinity for Mg^ follow posttranslational modification of PDE4 and may impact the hydrolytic capacity of the enzyme [6].

Domains flanking the catalytic domain of the cAMP-PDEs have been identified by different strategies and can roughly be divided into regulatory and targeting domains. Two regions highly conserved from worm and fly to mammals are present at the amino terminus of the PDE4 long forms and are termed upstream conserved regions 1 and 2 (UCR1/UCR2). These domains, signatures for PDE4, serve regulatory functions because a phosphorylation site for PKA has mapped to the amino-terminus end of UCR1. Yeast two-hybrid or pulldown assays have indicated that UCR1 and UCR2 interact with each other and that phosphorylation modulates this interaction [7].

Targeting domains have been identified in several PDE4s as well as at the aminoterminus of PDE7 (see the following). In addition, motifs corresponding to a PKA pseudosubstrate are present at the amino terminus of PDE7A1 and PDE7A2 [2]. Although their function has not been confirmed, they may play a role in the regulation of PKA. Finally, PDES contains a PAS domain likely involved in protein/protein interaction [3].

Subcellular Targeting of the cAMP-PDEs and cAMP Signal Compartmentalization

The cAMP PDEs are not uniformly distributed throughout the cell. Conversely, they are targeted to discrete compartments via mechanisms that are only partially known. This nonrandom distribution of PDEs is described, for instance, in the olfactory sensory neurons. The cAMP PDE 4A is present in the body of the neuron in a region surrounding the nucleus, whereas a PDE1 is targeted to the cilia, suggesting that the two enzymes control different cAMP pools [8].

Targeting domains have been identified in PDE4s, and in most instances they coincide with domains that mediate protein/protein interaction. The interaction of a PDE4D5 with RACK-1 [9], a scaffold protein initially identified as a PKC binding protein, is the best characterized, even though the significance of this interaction is unclear. The localization of PDE4D to the Golgi/centrosome structures is probably mediated by the PDE4 interaction with myomegalin, a large coiled-coil protein discovered by yeast two-hybrid screening [10]. Interestingly, in skeletal muscle where it is expressed at high levels, myomegalin colocalizes with PDE in a region where P adrenergic receptors, adenylyl cyclases, PKAs, and AKAPs are also localized [11,12]. A PDE4D3 is targeted to the centrosome or the perinuclear region through interaction with AKAP450 or mAKAP, two scaffold proteins that also bind PKA (see the following). PDE4A5 binds to SH3 domain-containing proteins and is localized at the plasma membrane [13]. An additional mechanism of targeting may be

dependent on the direct interaction with the lipid bilayer, as the amino terminus of PDE4A1 is highly hydrophobic and is sufficient for membrane localization of this PDE isoform [14]. One of the splicing variants of PDE7, PDE7A2, contains a unique hydrophobic amino terminus which likely targets it to insoluble structures of the skeletal muscle [15].

The physiological consequence of this distribution of PDEs in different cell districts is still a matter of debate. However, in one case, it is clear that interaction of PDE with scaffold protein serves to create a signaling unit with PKA. The PDE4D3 variant co-immunoprecipitates with the RII regulatory subunit of PKA and with two AKAPs, indicating the presence of a PKA/PDE signaling complex organized on the AKAP scaffold [16,17]. AKAP350/450, also termed Yotiao, is a true signaling scaffold because it complexes PKA, a PDE, PP1, and in some cases, is anchored to receptors [18]. Given the rapid phosphorylation and activation of this PDE isoform by PKA, this colocalization allows the rapid termination of the cAMP signal in a discrete domain of the cell.

Regulation of cAMP-PDEs

The activity of cAMP-PDEs is tightly regulated and integrated in several signaling pathways. Two major mechanisms of regulation have been described. These include regulation by phosphorylation and regulation at the level of transcription/translation of the genes [19]. In addition, interaction with other proteins or with lipids may affect the conformation and activity of these enzymes, even though this area of research is in its infancy.

Regulation of cAMP PDEs by Phosphorylation: Feedback Regulation of cAMP

Using a thyroid cell line as a model of PDE regulation, it was demonstrated that TSH rapidly activates a PDE4D and that this activation is dependent on cAMP accumulation and mediated byPKA [4]. The phosphorylation sites in PDE4D3 have been mapped by site-directed mutagenesis and by phosphopeptide analysis. These data, together with experiments in intact cells, have established that a short-term feedback regulation of PDE4 is operating in the cell to dampen or maintain cAMP levels within a narrow range of concentrations. PKA phosphorylation sites are also present in PDE7, though their significance is unclear.

In addition to a PKA-mediated phosphorylation at the amino terminus, all PDE4s, except PDE4A, are phosphorylated by MAPK at a carboxyl-terminus site [20]. This phosphorylation decreases the activity of the long forms or increases that of the short forms. These effects are overridden by the PKA-mediated phosphorylation. It has been proposed that this regulation is a means to terminate the MAPK kinase activation.

Other kinases may use PDE4s as substrates, as an increase in PDE4A activity follows S6 kinase activation by GH or monocytic cell line activation by lipopolysaccharides (LPS) [13]. PDE4B may be the target of kinases functioning in the T-cell-receptor-activated pathway [13].

Regulation of cAMP PDE Expression During Cell Adaptation and Differentiation

In the seventies, it was observed that changes in cAMP produced an increase in PDE activity and that this increase required protein synthesis. The cloning of PDE4 provided the tools to demonstrate that the regulation of transcription of the PDE4D gene and mRNA accumulation are regulated by cAMP. hi the Sertoli cell of the testis, FSH, which increases cAMP, produces more than a 100-fold increase in PDE4D mRNA and accumulation of PDE4D2 protein. This upregulation and consequent increase in cAMP hydrolysis contributes to the state of desensitization that follows hormonal stimulation [19]. Similar findings have been reported for T cells and several cell lines. PDE4B is subject to a similar cAMP-mediated regulation, eventhough both transcription and mRNA stabilization may contribute to the regulation of the corresponding mRNA [4]. This long-term feedback regulation of cAMP-PDE may play a particularly important role in neuronal cell adaptation and in gonadal cell function. This is inferred by recent observations on the phenotype of mice deficient in a PDE4 where several functions including fertility and behavioral effects follow inactivation of PDE4D [13,21].

An increase in PDE4 expression is also associated with the activation of other signaling pathways, including activation of T-cell receptors by mechanisms that are mostly unknown. In the same vein, PDE7 and PDE8 expression are induced by activation of T lymphocytes by CD3/CD28 antibodies [22]. This induction seems to be critical for the activation of these cells because PDE7 mRNA antisense treatment blocks replication and IL-2 production. PDE4B2 expression is induced in monocytes by LPS activation of the Toll-like receptor pathway [23], a regulation that may be critical for cytokine production, since PDE4 inhibitors block TNF-α production. These regulations in immune cells provide the rationale for the development of cAMP-PDE inhibitors for the treatment of inflammatory disorders.

The above summarized properties and regulations indicate that cAMP-PDEs play an important role in cAMP signaling as well as acting as integrators of multiple signaling pathways. Although much needs to be done to fully understand their functions, these PDEs should be regarded as homeostatic regulators of signaling.

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CHAPTER 194

cAMP/cGMP Dual-Specificity Phosphodiesterases

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Introduction

The mammalian PDE superfamily contains at least 11 functionally distinct, highly regulated, and structurally related gene families. PDE families differ in their primary sequences, substrate affinities and catalytic properties, sensitivity to effectors and inhibitors, responses to regulatory molecules, and cellular functions [1–5]. Intracellular cAMP and cGMP pools are tightly regulated and seem to be temporally, spatially, and functionally compartmentalized [6]. Most cells contain representatives of more than one PDE gene family (and different variants of the same family) but in different amounts, proportions, and subcellular locations. By virtue of their distinct intrinsic characteristics, their intracellular targeting to different subcellular locations, and their interactions with molecular scaffolds, cellular structural elements, and regulatory partners, different PDEs integrate multiple cellular inputs and modulate the intracellular diffusion and functional compartmentalization, as well as the amplitude, duration, termination, and specificity of cyclic nucleotide signals and actions [6–8]. PDEs are critical determinants of the unique cellular phenotypes (or "fingerprints") that characterize cyclic nucleotide signaling pathways.

Some PDE families are rather specific for cAMP hydrolysis (PDEs 4,7,8); others are cGMP-specific (PDEs 5,6,9); some hydrolyze both cGMP and cAMP (PDEs 1,2,3,10,11). This chapter will briefly discuss various aspects of the molecular diversity, structure/function, regulation, and functions of these dual-specificity PDEs.

The catalytic core of mammalian PDEs (~270 amino acids near the carboxy terminus) is more highly conserved among members of the same gene family than between different gene families [1,9]. This core contains common structural elements responsible for hydrolysis of cAMP and cGMP, as well as family-specific sequences responsible for differences in substrate affinities, catalytic properties, and sensitivities to family-selective inhibitors, such as SCH51866 and methoxymethyl-isobutylxanthine (PDE1), EHNA (erythro-9-(2-hydroxy-3-nonyl)-adenine) (PDE2), and milrinone, cilostazol, and cilostamide (PDE3) [1,9]. Selective inhibitors—that is, drugs that target individual PDE families with 10 to 100-fold greater potency than for other PDE families—are not yet available for the more recently identified PDEs 10 and 11, although dipyridamole (IC_{50}, ~0.37 μM) is a potent inhibitor of PDE11.

Widely divergent N-terminal portions of PDEs contain information that determine responses of different PDEs to specific regulatory signals, such as binding sites for Ca^{2+}/calmodulin and autoinhibitory domains (PDE1), membrane-targeting domains (PDEs 2,3), and sites for phosphorylation by cAMP-, cGMP- and Ca^{2+}/calmodulin-dependent protein kinases, protein kinase B (PKB/Akt), and protein kinase C (PDEs 1,2,3,10,11) [1–5].

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PDE2s contain two homologous, noncatalytic, cGMP-binding regions upstream of the catalytic core. These domains, conserved in PDE5, PDE6, PDE10, and PDE11 and a wide variety of proteins, are referred to as GAF domains (because of proteins containing these domains: cGMP-binding PDEs, Anabena adenylyl cyclase, and the *E. coli* transcriptional regulator, fhl A) [5,10]. In PDE2, PDE5, and PDE6, GAF domains bind cGMP with high affinity but with different functional consequences. Binding of cGMP to GAF domains results in allosteric activation of PDE2, enhances PKG-induced phosphorylation/activation of PDE5, and enhances interactions between PDE6 catalytic and inhibitory subunits and transducin. Functional consequences of interactions with GAF domains in PDEs 10 and 11 are unknown. Since the K_d for cGMP binding to the PDE10A GAF domains (>9 μM) is much higher than physiological cGMP concentrations, cGMP binding is probably not the primary function of these GAF domains [5,10]. Other small molecules can bind GAF domains; for example, fhl A binds formate in an N-terminal region containing two GAF domains [11].

PDE1 (Ca^{2+}/Calmodulin-dependent PDE)

Of the three PDE1 subfamilies (1A–C), PDE1A and PDE1B have higher affinity for cGMP (K_m ~5 μM and 2.7 μM, respectively) than for cAMP (K_m ~113 μM and 24 μM, respectively) [15], whereas PDE1C has high affinity for both cAMP and cGMP (K_m ~1 μM). PDEs1B and 1C hydrolyze cAMP and cGMP at similar rates, with V_{max} for cAMP by PDE1A twice that for cGMP [15].There are five PDE1A [16,17], two PDE1B [17,18], and five PDE1C [15,19] splice variants; N-terminal, not C-terminal, diversity apparently accounts for functional and regulatory differences. Alternative splicing generates structural changes in calmodulin-binding domains, with PDE1A1 and PDE1C2 having greater affinity for calmodulin than PDE1A2 [20] or other PDE1C isoforms [15], respectively. *In vitro* phosphorylation of PDE1A1 and PDE1A2 by PKA [21,22], or PDE1B by calmodulin-dependent protein kinase II [23], renders the enzymes less sensitive to Ca^{2+}/calmodulin. PDE1A possesses an N-terminal PEST recognition motif for m-calpain which generates an activated cleavage fragment that is independent of calmodulin [24] and could provide an alternative intracellular mechanism for cyclic nucleotide regulation.

PDE1s may mediate cross-talk between Ca^{2+}, lipid, and cyclic nucleotide signals. Stimulation of CHO cells with PMA or agonists that activate lipid-signaling pathways, or transfection with specific PKC isoforms, rapidly induces PDE1 activity and expression [25,26].In mammalian brain, PDE1 is relatively highly expressed , with distinct isozyme-specific distributions. PDE1B distribution in the striatum correlates with that of dopamine receptors, inferring a role in modulating dopamine-mediated cAMP signaling [27]. PDE1C2 and adenylyl cyclase AC3 colocalize to olfactory sensory neuronal cilia that extend to nasal epithelium, where they may regulate transient cAMP responses to odorants [27]. PDE1A upregulation has been implicated in developing tolerance to vasodilator effects of chronic nitroglycerin treatment [28]. Quiescent smooth muscle cells (SMCs) from intact normal human aorta express PDEs 1A and 1B, whereas proliferating SMCs in human arterial primary cultures and cultures from atherosclerotic lesions contain high levels of PDE1C(12).

In pancreatic islets [29,30] and cultured β-cell lines [31], PDE1, especially PDE1C, may regulate cAMP and glucose-induced insulin secretion, since PDE1 inhibition augments glucose-stimulated insulin release from βTC3 cells and exposure to glucose activates PDE1 [31].

PDE1 may be elevated in certain tumors [32]. PDE1B1 is induced in activated T cells and expressed in human lymphoblastoid cell lines but not in normal, quiescent, peripheral blood lymphocytes [33]. PDE1 inhibitors, 8-methoxymethyl-IBMX and vinpocetine, can attenuate IL-13 production and induce apoptosis of lymphoma cells [34], suggesting the potential for PDE1-targeted therapy of leukemia and inflammatory disorders.

PDE2 (cGMP-stimulated PDE)

Unique N-terminal regions in three variants of the single PDE2A gene, most likely generated via alternative exon splicing [4,35–41], may localize PDE2 to soluble (PDE2A1) and particulate (PDE2A2, PDE2A3) subcellular fractions. In general, PDE2A mRNA expression is similar in human [38], rat [42], and bovine [39] tissues, with relatively high levels in brain and intermediate levels in heart, liver, skeletal muscle, and pancreas.

PDE2 isoforms exhibit similar catalytic properties, hydrolyzing cAMP (K_m, ~30 μM) and cGMP (K_m, ~10 μM) at similar maximal velocities (100–160 μmol/min/mg) with positively cooperative kinetics. At physiological concentrations, cGMP is the preferred substrate and effector for PDE2, and can stimulate PDE2-mediated cAMP hydrolysis up to 50-fold with a K_d ~0.35–0.5 μM. By interaction with non-catalytic cGMP-binding regions in the two GAF domains upstream of the catalytic domain [43], cGMP induces a conformational change and converts PDE2 from a ligand-free state, displaying low affinity for cAMP and positively cooperative or sigmoidal kinetics, to an activated high-affinity conformation, displaying classical Michaelis-Menton kinetics without any change in $V_{max,}$ [36]. Because of this unique allosteric regulation, PDE2 is referred to as the cGMP-stimulated cAMP PDE. At higher concentrations, cGMP inhibits cAMP hydrolysis, due to competition at the catalytic site.

Physiologically, PDE2 is a locus for cross-talk between cAMP- and cGMP-signaling pathways and may play an important role in cells where cAMP and cGMP regulate opposing functions. Regulation of PDE2 by cGMP may be more important when intracellular cAMP is elevated, rather than in the basal state [4,36]. The relatively recent development of a selective PDE2 inhibitor, EHNA [44], has been

critical in elucidation of putative pathways regulated by PDE2s. However, although EHNA potently inhibits PDE2 (IC50, ~0.8–2 μM, at least 50-fold less than for other PDEs), it also inhibits adenosine deaminase.

In heart and platelets of different animal species, effects of NO and cAMP and cGMP seem to be regulated by the interplay of different PDEs, especially PDE2, PDE3, and PDE5. In frog ventricular myocytes and human atrial myocytes, NO-induced activation of guanylyl cyclase increases cGMP, which can activate PDE2, resulting in increased hydrolysis of cAMP and decreased L-type channel Ca^{2+} current I_{ca} [45–48]. PDE2 may be located in the same subcellular compartment as PKA and the L-type channel [49], and might specifically regulate L-type channel phosphorylation. In rabbit platelets, nitrovasodilators and prostacyclins act synergistically to inhibit platelet aggregation via increased cAMP, generated by NO-induced activation of guanylyl cyclase, cGMP accumulation, and subsequent inhibition of cAMP hydrolysis by PDE3 [50]. In human platelets, however, although PDE3 may be important in the absence of nitrovasodilators or at low cAMP concentrations, increases in cAMP seem to be restricted by cGMP-induced activation of PDE2 [51]. [Stimulation by nitrovasodilators, via increases in cGMP, results in inhibition of PDE3 as well as activation of PDE2, resulting in net cAMP hydrolysis. Thus, clinical effectiveness of nitrovasodilators as platelet inhibitors, which could be compromised by activation of PDE2, may be enhanced by PDE2 inhibitors [50–52].

In cultured bovine adrenal glomerulosa cells, ANP inhibits ACTH-stimulated aldosterone secretion, presumably via cGMP-induced activation of PDE2 and hydrolysis of cAMP [53]. PDE2 may also be important in regulation of phytohemaglutinin-induced activation (but not by anti CD3) of the T-cell receptor [54], the hypoxic pressor response in rat lung [55], NO-induced inhibition of prolactin release from the pituitary gland [56], NaCl absorption by the rat thick ascending tubule [57], olfactory neuroepithelial signaling [58,59], and effects of NGF on cAMP-induced PC12 cell differentiation [60,61].

PDE3 (cGMP-inhibited cAMP PDE)

Both PDE3 subfamilies, PDE3A and PDE3B, generated from separate genes located on chromosome 11 and 12, respectively [62,63], hydrolyze cAMP and cGMP with high affinity (K_m values of ~0.1–0.8 μM) in a mutually competitive manner, with V_{max} for cAMP higher (~4–10-fold) than for cGMP [14,64]. Both are specifically inhibited by compounds such as milrinone, enoximone, and cilostazol [64].

PDE3A and B exhibit cell-specific differences in expression. PDE3B is relatively highly expressed in cells important in energy metabolism, such as white and brown adipocytes, pancreatic β cells, and hepatocytes [64–67]; PDE3A is highly expressed primarily in the cardiovascular system, for example platelets, smooth muscle, and cardiac myocytes [64,68]. Full-length PDE3s (Mw~135 kDA) are found in association with membranes; smaller PDE3A forms are found in cytosolic fractions [68].

The structural organization of PDE3A and PDE3B proteins is identical [14] with the catalytic domain found in all PDEs located in the C-terminal portions of the molecules. The catalytic domains of PDE3A and B are highly conserved, except for an insertion of 44 unique amino acids that is not found in the catalytic domains of other PDE families and that also differs in, and thus distinguishes, PDE3A and B isoforms. PDE3A and B N-terminal portions are quite divergent, consisting of large hydrophobic regions containing 5 to 6 transmembrane helical segments and separated from the catalytic domain by a regulatory domain with consensus sites for phosphorylation by PKA and PKB [14,64].

Studies with specific, cell-permeable, PDE3 inhibitors (some of which are used in clinical situations [64,65]) suggest that PDE3s regulate several important physiological functions, including adipose tissue lipolysis, insulin secretion, platelet aggregation, vessel relaxation, cardiac function, oocyte maturation, and cell proliferation [14,64]. In intact cells, insulin/IGF-1 and cAMP-increasing agents activate (and presumably phosphorylate) PDE3s in adipocytes [14], hepatocytes [67,69], platelets [64], oocytes [70], and pancreatic β cells [71].

In adipocytes, insulin-induced phosphorylation and activation of membrane-associated PDE3B, via insulin receptor substrates (IRS)/phosphatidylinositol-3 kinase (PI3K)/PKB signaling pathways [72–77], is a major mechanism whereby insulin acutely antagonizes catecholamine-induced lipolysis and release of fatty acids. Activation of PDE3B leads to increased degradation of cAMP and consequent lowering of PKA activity, net dephosphorylation of hormone-sensitive lipase, and reduced lipolysis [78]. Protein phosphatase 2A catalyzes PDE3B dephosphorylation [79]. cAMP-elevating hormones also phosphorylate and activate PDE3 in adipocytes and several other cells, including platelets, as a feedback mechanism to limit excess production of cAMP.

Several studies suggest that reduced PDE3B gene expression in adipocytes is associated with insulin resistance. PDE3B activity and gene expression are decreased in adipose tissue from JCR: LA-cp rats, a strain that develops obesity, insulin resistance, and vasculopathy [80], and from obese, insulin-resistant, diabetic KKAy mice [81,82]. Administration of pioglitazone to KKAy mice increased adipocyte PDE3B expression and restored its responsiveness to insulin. Long-term incubation (~24 hr) of 3T3-L1 adipocytes with TNFα and ceramides results in increased lipolysis that is associated with downregulation of PDE3B; troglitazone reverses the effect on PDE3B [83]. Whether downregulation of PDE3B is important in development of TNFα-induced insulin resistance is not certain.

In pancreatic islets, insulinoma cells, and clonal β cells [30,31,71,84,85], PDE3, most likely PDE3B, seems to be important in regulation of insulin secretion. Activation of PDE3B by insulin-like growth factor I (IGF-1) [71] or leptin [88] attenuates insulin release stimulated by the cAMP-elevating hormone glucagon-like peptide 1 (GLP-1).

Selective PDE3 inhibitors increased plasma insulin in normal rats [86,87]. In pancreatic islets and insulinoma cells, they enhanced insulin secretion stimulated by glucose [30,84–86], indicating that PDE3-mediated control of cAMP may also be important for nutrient-stimulated release of insulin (although inhibition of the cAMP/PKA pathway has been reported not to adversely affect glucose-induced release [89,90]).

In hepatocytes, activation of PDE3B is thought to be important in the antiglycogenolytic actions of insulin and leptin [69,91]. In frog *(Xenopus laevis)* and murine oocytes and in intact rodents, specific PDE3, not PDE4, inhibitors blocked oocyte maturation [92,93]. In the frog oocyte, IGF-1-induced meiotic maturation is associated with PKB-induced activation of oocyte PDE3A and reduction in cAMP [70]. Taken together, these studies suggest that activation of PDE3B (perhaps via PI3-K/PKB-signaling) is important in counterregulatory actions of insulin, IGF-1, leptin, and other cytokines on certain cAMP-mediated processes.

PDE10

Two variants of the single PDE10 gene, PDE10A1 and PDE10A2, with unique N-termini but otherwise identical sequences, and several rat variants, PDE10A3, 10A4, 10A5, and 10A6, have been cloned and characterized [94–97]. PDE10 activities were detected in extracts of rat striatum and testis [98,99]; human PDE10A1 transcripts, in brain, testis, heart, kidney, lung, liver, and pancreas; PDE10A2 in brain, kidney, and placenta. Recombinant PDE10 hydrolyzes both cAMP (K_m ~0.05–0.26 μM) and cGMP (K_m ~3–8 μM), with V_{max} for cGMP ~ five-fold higher than cAMP. Since cGMP hydrolysis is potently inhibited by cAMP, PDE10 may serve as a cAMP-inhibited cGMP PDE *in vivo*, but characterization of PDE10 function(s) awaits development of specific inhibitors.

Two PDE10 N-terminal GAF domains, although similar to GAF domains in PDE2, PDE5, and PDE6, are probably not important in functional cGMP-binding [96]. The unique N-terminal region of PDE10A2 contains a consensus site for PKA phosphorylation, but effects of phosphorylation on activity in intact cells and *in vitro* are unknown.

PDE11

Several rat [100] and human [100–102] splice variants of the single PDE11 gene, with unique N-termini, have been cloned and characterized. Recombinant PDE11A hydrolyzes both cGMP and cAMP, with K_m values of 0.52 μM and 1.04 μM, respectively [103]. With similar V_{max} values for both cAMP and cGMP, despite the two-fold higher affinity for cGMP, PDE11A may function as a genuine dual substrate PDE at physiologically relevant concentrations. PDE11A splice variants exhibit differences in V_{max} (PDE11A4 >> PDE11A2 > PDE11A3 >> PDE11A1), and in their sensitivities to inhibitors, some of which were two- to three-fold more potent against PDE11A3 than PDE11A4 [102], indicating that the N-terminus of PDE11A may affect the conformation of the protein and thus regulate catalytic activity. The splice variants possess different GAF domains in their N-terminal regions: PDE11A4, with two GAF domains; PDE11A3, one complete and one incomplete GAF domain; PDE11A2, one complete GAF domain; and PDE11A1, an incomplete GAF domain. Although PKA and PKG phosphorylate PDE11A4, but not other variants, it is uncertain whether phosphorylation occurs *in vivo* or serves a regulatory function [102].

PDE11A transcripts are highly expressed in prostate and testis, with moderate expression in salivary gland, pituitary gland, thyroid gland, and liver. PDE11A3 transcripts are specifically expressed in testis, whereas PDE11A4 is particularly abundant in prostate [102], suggesting a potential role for PDE11 in these tissues.

Conclusions

In general, dual-specificity PDEs seem to be regulators of many cyclic nucleotide signaling pathways, including proliferation of vascular smooth muscle (PDE1) [12,13], myocardial contractility and platelet aggregation (PDE2 and PDE3) [4,14], adrenal steroidogenesis (PDE2), and insulin/IGF-1 action (PDE3) [3,4,12–14]. In addition, because of their intrinsic characteristics and regulatory properties, dual-specificity PDEs can serve as a locus for cross-talk among Ca^{2+}, cAMP, and cGMP signaling pathways, since Ca^{2+}, calmodulin, and calmodulin kinase regulate PDE1, and since, depending on physiological cyclic nucleotide concentrations, cGMP can modulate intracellular cAMP concentrations by either stimulating or inhibiting cAMP hydrolysis by activating PDE2 or inhibiting PDE3. Little is known of intracellular functions of recently identified PDE10 and 11, but PDE10 might be expected to function as a cAMP-inhibitable cGMP PDE.

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CHAPTER 195

Phosphodiesterase-5

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Introduction

Cyclic GMP-specific cGMP-binding phosphodiesterase (PDE5) is a class I PDE, and it is abundant in smooth muscle, platelets, Purkinje cells, and renal tissues. It is the pharmacological target for sildenafil citrate (Viagra™), vardenafil (Levitra™), and Fadalafil (Cialis™), all of which are treatments for male erectile dysfunction. It is also an emerging target for treatment of other disorders involving smooth muscle, such as pulmonary hypertension. PDE5 is dimeric; each 100-kDa monomer contains a catalytic domain and a regulatory domain that includes two GAF domains associated with the allosteric cGMP-binding function and a single phosphorylation site for either cGMP-dependent protein kinase (PKG) or cAMP-dependent protein kinase (PKA). The role of the allosteric cGMP-binding in enzyme regulation is poorly understood. There is no apparent homology between the allosteric cGMP-binding site(s) and the catalytic site; they have distinctly different analog specificities, and neither is homologous with the cyclic nucleotide-binding sites of PKA and PKG. There are three splice variants that differ at their amino-termini, and they are derived from a single gene. Expression of PDE5 is subject to long-term and developmental regulation. Phosphorylation of the single phosphorylation site requires binding of cGMP to the regulatory domain and results in activation of catalysis as well as increased cGMP-binding affinity at the allosteric sites. Increased phosphorylation occurs in intact cells when cGMP is elevated in response to atrial natriuretic peptide. PDE5 plays a central role in selectively controlling cGMP levels in numerous tissues through breakdown of cGMP at the catalytic site and binding of cGMP at the allosteric sites.

PDE5 has recently gained prominence with the realization that the activity of this enzyme in combination with that of guanylyl cyclases plays a key role in modulating cGMP levels in smooth muscle cells and therefore in determining contractile tone in this tissue (Fig. 1). PDE5 is highly abundant in corpus cavernosum of the penis and is inhibited by several drugs that are a highly effective are for treatment of male erectile dysfunction. The demonstrated role of PDE5 activity as a major determinant in modulating cGMP signaling in numerous tissues has led to renewed interest in this enzyme and its potential as an important pharmacological target.

Gene Organization and Regulation of Expression

The single human PDE5 gene is located on chromosome 4q26, contains 23 exons, and encodes mRNA for three variants: PDE5A1, A2, and A3. These proteins, 100 kDa, 95 kDa and 95 kDa, respectively, share similar properties and have different amino-termini, a result of alternative mRNA splicing [1–4]. Alternative first exons arranged in the order A1-A3-A2 account for the three forms [5]. PDE5 mRNA is present in many tissues [3,4,6], but PDE5 protein level is relatively low in most tissues. PDE5 protein occurs in relative abundance in smooth muscle, platelets, and Purkinje cells and has been detected in proximal renal tubules, collecting renal ducts, and epithelial cells of pancreatic ducts [2,7]. It is by far the major cGMP-hydrolyzing PDE in penile corpora cavernosae of human, dog, and rabbit. PDE5A3 appears to predominate in smooth muscle, and its expression may only occur in smooth muscle [4,8]. PDE5A1 and PDE5A2 are co-expressed in a variety of tissues, but PDE5A2 is the major form found in most human tissues [4,9].

PDE5 is subject to long-term and developmental regulation [1,9–11]. Both cAMP and cGMP stimulate PDE5A promoter activity, and cAMP increases PDE5A2 expression [9,12]. PDE5A1 promoter contains multiple Sp1- and AP2-binding sequences and involves a portion of the first exon. PDE5A2 utilizes an intronic promoter located downstream from the

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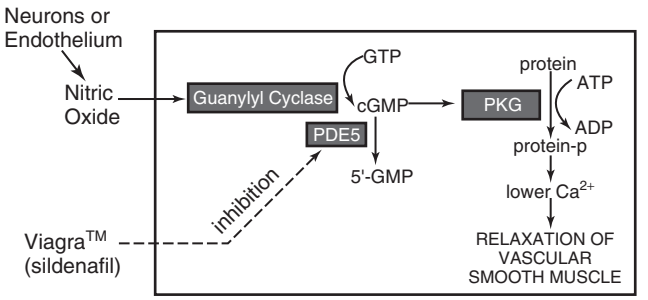


Figure 1 Nitric oxide and cGMP signaling in smooth muscle cells. Cyclic GMP level in smooth muscle cells is determined by the balance between the rate of cGMP synthesis by guanylyl cyclases and the rate of cGMP breakdown by phosphodiesterases (PDE). When cGMP is elevated, it binds to cGMP-dependent protein kinase (PKG) and activates that enzyme to bring about relaxation of smooth muscle. PDE5 is abundant in vascular smooth muscle and contributes to the downregulation of cGMP levels. Inhibitory agents such as sildenafil (Viagra™), Vardenafil (Levitra™), or tadalafil (Cialis™) that block catalytic activity of PDE5 enhance elevation of cGMP in response to stimuli that activate guanylyl cyclases.

first exon of PDE5A1. This promoter also contains sequences that bind Sp1 and AP2 transcription factors [5]. Several of the Sp1 sites are important for promoting PDE5A2 gene transcription and in providing for cGMP stimulation of transcription [13].

General Structure

PDE5 is a dimer of identical subunits each with M_r ~100,000 (Fig. 2). A regulatory domain is located in the amino-terminal portion of each monomer, and a catalytic domain occupies a more carboxyl-terminal region. The regulatory domain of PDE5 contains a phosphorylation site (Ser-92 in bovine PDE5A1 and Ser-102 in human PDE5A1) for PKG or PKA [14]. The regulatory domain binds cGMP at highly specific, allosteric binding sites that show kinetic heterogeneity [15]. The catalytic site is also highly specific for cGMP and, like all known PDEs, contains binding sites for divalent cation(s) that are required to support catalysis. The amino acid sequence of the PDE5 allosteric cGMP-binding region is not homologous with the catalytic site, and neither of these is homologous with cyclic nucleotide-binding sites in PKA, PKG, cyclic nucleotide-gated cation channels, or cyclic nucleotide-regulated guanine nucleotide exchange factors [16].

Phosphorylation of a single serine near the aminoterminus of PDE5 by PKG or PKA requires cGMP binding to the regulatory region and would be predicted to occur only when cellular cGMP is significantly increased and PKG is concomitantly activated (Fig. 3). PDE5 is phosphorylated in intact cells in response to stimuli that elevate cGMP. Elevation of cAMP does not mimic this. PDE5 is at least a ten-fold better substrate for PKG than for PKA. This preference results from a phenylalanine located at P^{+4}, which is a negative determinant for PKA. PKG has ~20-fold higher affinity for PDE5 regulatory domain as substrate (K_m ~2.7 μM) than

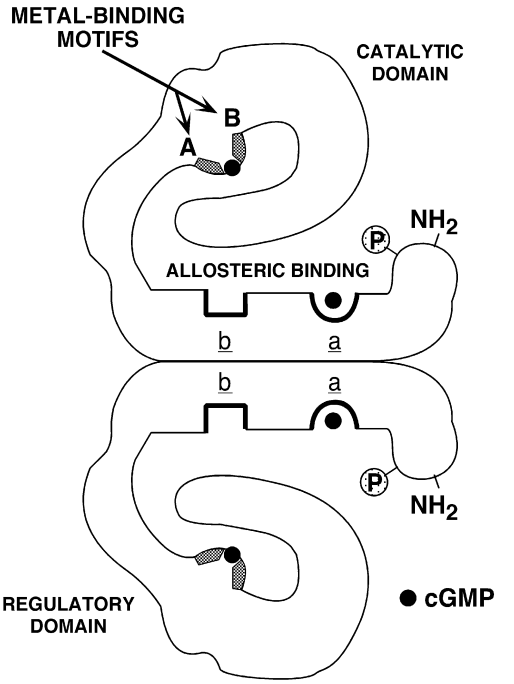


Figure 2 Working model of PDE5. The regulatory domain in the amino-terminal portion of PDE5 contains the phosphorylation site (Ser-102), two GAF domains (*a* and *b*) associated with allosteric cGMP-binding and the dimerization domain. Cyclic GMP binding to the isolated GAF domain *a* has been demonstrated, but whether the GAF domain *b* binds cGMP remains to be determined. The catalytic domain in the carboxyl-terminal portion of PDE5 contains a cGMP-binding substrate site and two Zn^{2+}-binding motifs (A and B), portions of which form a novel metal-binding site.

for the peptide containing the phosphorylation site (K_m ~ 68 μM), indicating that additional features in the PDE5 regulatory domain contribute to high-affinity interaction with PKG [17].

PDE5 Catalytic Domain

Catalytic domains of all mammalian PDEs include ~270 residues that are 25 to 50 percent conserved and contain 18 invariant amino acids [16,18]. PDE5 is highly specific for cGMP and exhibits a k_{cat} of 4.3 sec^{-1} and K_m of 1–6 μM for that nucleotide. Catalytic site affinity for cAMP is at least 100-fold lower than that for cGMP, but V_{max} for cAMP is actually higher than that for cGMP. Cyclic nucleotide analog specificity of the catalytic site differs from that for cGMP-binding to the regulatory domain, and the catalytic site tolerates more modifications to the cGMP molecule. Cyclic GMP analogs with modifications at the 2′-OH or at N1 and C2 are accommodated well at the catalytic site. High catalytic site affinity vardenafil and PDE5 inhibitors such as sildenafil, tadalafil which exhibit slight resemblance to cGMP, further indicate the promiscuous nature of this site. Recombinant PDE5 containing only the catalytic domain is monomeric, and the catalytic properties (K_m, V_{max} for cGMP, and IC_{50} for inhibitors) compare well to those of full-length PDE5, indicating that requirements for catalysis exist within a single catalytic domain [16,19].

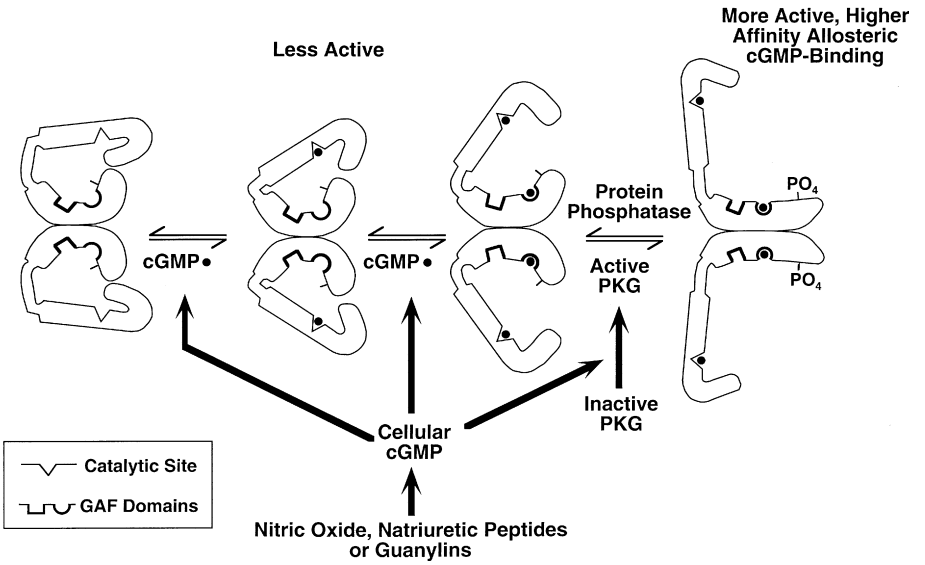


Figure 3 Working model of conformational changes that occur in PDE5. Upon elevation of cellular cGMP, cGMP interacts with its major intracellular receptors, PDE5 and PKG. For PDE5, evidence suggests that cGMP interacts first with the catalytic site where it is hydrolyzed. That initial interaction induces a conformational change in PDE5 to increase cGMP binding to the regulatory domain. The possibility that cGMP binding to the regulatory domain increases the affinity of the catalytic site for cGMP, and maximum catalytic activity is also increased. Cyclic GMP-binding to the regulatory domain produces yet another change in the amino-terminal portion of PDE5 to expose the serine that can be phosphorylated by PKG. Under these circumstances, PKG would be activated due to specific binding of cGMP to its allosteric sites. Evidence suggests that the phosphorylated PDE5 is the most active form and exhibits highest affinity for cGMP binding in the regulatory domain. Existence of each conformation is based on extensive biochemical studies.

Cloning of PDE5 cDNA led to identification of two conserved Zn^{2+}-binding motifs (A and B) in catalytic domains of all mammalian PDEs. The importance of Zn^{2+} in catalytic function of PDEs was first demonstrated experimentally with PDE5. Zinc binds to PDE5 with high stoichiometry (~3 mol/mol PDE5 monomer), is not competed by 10,000-fold excess of either Mn^{2+} or Mg^{2+}, and is effective at far lower concentration (submicromolar) than either Mn^{2+} or Mg^{2+} in supporting catalytic activity. Site-directed mutagenesis of PDE5 confirms the catalytic importance of residues in Zn^{2+}-binding motifs A and B and reveals involvement of residues from each motif to form a novel Zn^{2+} binding site [20,21]. Two conserved downstream aspartates are also implicated as critical for efficient catalysis. Mutation of two other residues, Tyr-602 and Glu-775, causes marked changes in K_m for cGMP.

A variety of PDE inhibitors block catalysis by competing with cGMP at the catalytic site. The order of potency, in descending order, of some common PDE inhibitors for PDE5 is vardenafil, tadalafil ≥ sildenafil, zaprinast, dipyridamole, 3-isobutyl-1-methylxanthine (IBMX), cilostamide, theophylline, caffeine, rolipram [19]. PDE5 is the specific target of sildenafil citrate, a potent competitive inhibitor (IC_{50} of 1–4 nM) marketed as Viagra™. Affinity of sildenafil for PDE5 is 10- and 100-fold greater than that for PDE6 and PDE1, respectively, and much greater than that for other PDE families. Other commercialized compounds [Levitra™ (vardenafil), Cialis™ (tadalafil)] have now been produced that inhibit PDE5 with equal or better potencies than sildenafil and that show even stronger selectivity than sildenafil for inhibition of PDE5 compared to other PDEs [22,23].

Occupation of PDE5 catalytic site by cGMP, cGMP analogs, or inhibitors such as sildenafil increases cGMP binding to allosteric cGMP-binding sites in the regulatory domain (Fig. 3). This increaes catalytic activity and also leads to exposure of the phosphorylation site, thereby allowing for phosphorylation, further activation of PDE5, and enhanced cGMP-binding affinity of the allosteric sites. Inhibitors such as sildenafil bind exclusively to the PDE5 catalytic site and do not compete with cGMP for binding in the regulatory domain sites [16,19].

PDE5 Regulatory Domain

Allosteric sites in unphosphorylated PDE5 bind cGMP with a K_d ~0.2–1.5 μM; affinity varies depending on conditions. Cyclic GMP binding is associated with one or two GAF domains comprised of ~110 residues each [29]. The GAF acronym derives from a group of apparently unrelated proteins that contain these ancient domains, bind diverse ligands, and share low amino acid sequence similarity: cGMP-binding PDEs, cyanobacterial *Anabaena* adenylyl cyclase, and *Escherichia coli* transcriptional factor fhlA. Whether both GAF domains in a single PDE5 monomer bind cGMP is not known. Two kinetically distinct cGMP-binding sites with ~four-fold difference in affinity exist in the enzyme. Over the years, much evidence has been presented and interpreted

as supporting the likelihood that cGMP binds to both GAF domains in PDE5. However, stoichiometry of cGMP binding to the regulatory region is best estimated at 1 mol cGMP per monomer. A recombinant protein containing only the more amino-terminal GAF domain of PDE5a binds cGMP with an affinity similar to that of the high-affinity component in native and wild-type PDE5 [17]. It is clear that at least the cGMP-binding associated with the more amino-terminal GAF domain is a very stable and self-contained entity (Figs. 2 and 3).

Functional roles of allosteric cGMP-binding in PDE5 has been partly clarified (see below), but the biological advantage of having two GAF domains in most cGMP-binding PDEs (PDE2, PDE5, PDE6, PDE10, PDE 11) is a mystery [16]. Site-directed mutagenesis of residues in the more amino-terminal GAF or in both GAFs diminishes or compromises cGMP binding and causes a three- to four-fold increase in K_m of PDE5 for cGMP. Integrity of both GAFs and retention of cGMP binding by the regulatory domain influences exposure of the phosphorylatable serine for PKG or PKA action (Fig. 3). The phosphorylation in turn increases the affinity with which the regulatory domain binds cGMP, and the K_m for cGMP as substrate is lowered. Whether this latter effect results from phosphorylation alone or from a concomitant increase in the cGMP-binding affinity of the regulatory domain is not known.

Dimerization occurs through stable interactions located near or within the allosteric cGMP-binding sites. The nature of the contacts and the advantages of a dimeric enzyme are not known. Dimerization could contribute to stability or function of the cGMP-binding domains. It is clearly not required for catalytic function.

Concluding Remarks

Studies of PDE5 have provided major insights into molecular characteristics of that enzyme and have advanced our understanding of general properties shared by all members of the PDE superfamily. PDE5 has been successfully targeted for a widely marketed treatment for male erectile dysfunction. The success of sildenafil (Viagra™) in this regard has renewed interest in PDEs as potential pharmacological targets. The relatively restricted expression of PDE5 along with its well-defined biochemical properties enhances its attraction as a target for future pharmacological interventions.

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CHAPTER 196

Structure, Function, and Regulation of Photoreceptor Phosphodiesterase (PDE6)

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Introduction

The remarkable single-photon sensitivity of the visual pathway in rod photoreceptors requires that a photon of light be converted into a cascade of biochemical reactions. This process starts with activation of the visual pigment molecule (rhodopsin, R^*). During the lifetime of one R^*, ~100 heterotrimeric G-proteins (transducin) can be catalytically activated per second. Each activated transducin (specifically, the GTP-loaded α subunit, α_t^*-GTP) then binds to and activates the effector enzyme, cGMP phosphodiesterase (PDE). The hydrolytic action of PDE rapidly reduces the cytoplasmic cGMP concentration, causing dissociation of cGMP from cyclic GMP-gated ion channels. A single R^* can suppress over 1 pA of current that normally flows through these plasma membrane channels in the dark. Termination of the rod photoresponse, along with modulation of photoresponse sensitivity during dark and light adaptation, requires biochemical reactions distinct from the excitation pathway described above. Cone photoreceptors have phototransduction pathways similar to rods, but the biochemical machinery of cone phototransduction must differ significantly to account for physiological differences in the light sensitivity and adaptational properties of cones. For reviews of rod and cone phototransduction, see [1–4].

Photoreceptor cells express a single type of PDE (classified as PDE6). In rods, the PDE6 holoenzyme consists of a catalytic heterodimer ($\alpha\beta$) to which two inhibitory γ subunits bind ($\alpha\beta\gamma_2$). Different classes of cone photoreceptors exist in mammalian retina, and each expresses cone-specific PDE6 catalytic (α') and inhibitory (γ') subunits. There is some evidence that PDE6 subunits—particularly the γ subunit—are expressed and function in nonretinal tissues [5,6], but the level of expression is low compared to photoreceptor cells (20 μM). The PDE6 family is one of five families of PDEs that contain noncatalytic, cGMP-binding GAF domains (see Chapter 192 by Glick and Beavo, this volume). The catalytic and regulatory features of the PDE6 family make it uniquely suited as the central effector of the visual transduction pathway.

Structure and Subcellular Localization of Rod PDE6

The primary sequence of the catalytic subunits of PDE6 largely consists of highly conserved regulatory and catalytic domains along with a C-terminal sequence that is the site of posttranslational modifications conferring membrane attachment (Fig. 1). The N-terminal half of the sequence contains homologous GAFa and GAFb domains. The GAF domain

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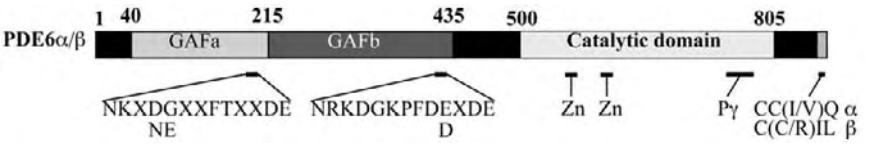


Figure 1 Domain organization of the catalytic subunits of rod PDE6. The bovine rod α (859 a.a.) or β (853 a.a.) subunit each contain two tandem GAF domains (blue and red), a catalytic domain (yellow), and unique C-terminal isoprenylation motifs (green). The consensus sequences for GAFa and GAFb represent amino acid identity [except for positions occupied by two (alternate shown below) or more (denoted by X) different residues]; sequence alignment was performed with human, bovine, mouse, dog, and frog α and β subunits. For the isoprenylation motifs, the frog sequences were omitted.

is a structural module responsible for binding allosteric regulatory molecules [ref. [7,8]; see review by Hurley, this volume]. Based on the crystal structure of the related PDE2 GAF domains [9] and amino acid sequence analysis of the known cGMP-binding phosphodiesterases (PDE2, PDE5, and PDE6), it is likely that the GAFa domains of PDE6 contain high-affinity cGMP binding sites (Fig. 1). An interaction site of the γ subunit with GAFa has been identified by cross-linking experiments [10]. The fact that the PDE6 holoenzyme has 4 GAF domains but only 2 high-affinity cGMP binding sites [11,12,13] raises intriguing questions about the function of the GAFb domains. Whereas 26% of the residues in GAFa are identical in known rod and cone PDE6 sequences, 60% of the GAFb amino acids are identical, including a unique 24 amino acid insert present in PDE6 GAFb but not in other GAF-containing phosphodiesterases.

Progress in elucidating the structure-function relationship of the catalytic domain of PDE6 has relied on its structural similarity to the PDE5 catalytic domain [14], the ability to express recombinant PDE5/cone PDE6 chimeras [15–17], and homology modeling of the catalytic domain based on the PDE4 crystal structure [18]. Two metal ion binding sites critical for catalysis are present in all PDE families [19,20], and substitution of PDE6-specific residues from this region into the PDE5 sequence partially restores PDE6 catalytic efficiency [17]. Using a similar approach, PDE6-specific residues near the catalytic pocket of the enzyme that interact with the Pγ subunit have been delineated [16,17,21].

The arrangement of the GAF and catalytic domains within the quaternary structure of PDE6 has been obtained at 2.8 nm resolution by using electron microscopy and image analysis of individual PDE6 molecules [22]. Three distinct domains, probably representing two GAF domains and the larger catalytic domain, were observed. The molecular organization of PDE5 was highly homologous. The isolated GAFa domain [23] or the catalytic domain [24] of PDE5 have been expressed and retain their ability to bind or hydrolyze cGMP, respectively. These results strongly suggest that individual domains fold into functional units that can interact to generate the final quaternary structure of the catalytic dimer.

Regulation of Rod PDE6 Catalysis by γ

The γ subunit has multiple sites of interaction with the PDE6 αβ dimer and with the α_t^*-GTP subunit of transducin;

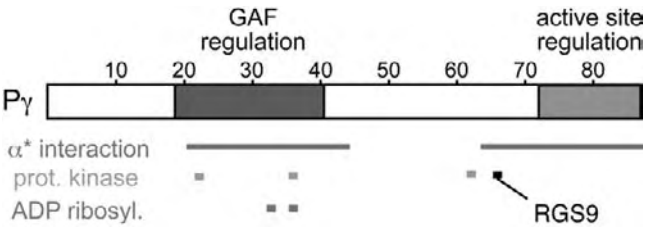


Figure 2 Sites of interaction and regulation of the inhibitory γ subunit of PDE6. The rod γ subunit (87 a.a.) binds to PDE6 at its central GAF interaction domain (red) and its C-terminal inhibitory domain (green). Two major sites of interaction with activated transducin α_t^* subunits are shown in green, and interaction with RGS9 at Val^{66} is in black [50]. Phosphorylation sites are shown in orange. ADP ribosylation at either Arg^{33} or Arg^{36} is in purple [51].

in addition, it serves as a potential substrate for posttranslational modifications (Fig. 2). The C-terminus of γ binds at the entrance to the active site and physically blocks entry of substrate [16,17,25,26]. The central, polycationic region of γ also interacts with the catalytic dimer, and with 50-fold higher affinity than the C-terminal γ region [27]. Binding of this region of γ to the catalytic dimer enhances cGMP binding to PDE6. The combined interactions of the central and C-terminal domains of γ with PDE6 result in very high binding affinity of full-length γ [28]. Recent work has revealed two nonidentical γ binding sites on αβ, one of which is regulated by cGMP binding to the GAF domain [27].

Post-translational modifications of γ have been postulated as feedback mechanisms for PDE6 regulation (Fig. 2). Protein kinases present in rod outer segments can phosphorylate γ at Thr^{22} [29–31] or Thr^{35} [31–33]. The primary effect of phosphorylation at either site is to markedly reduce γ interaction with activated transducin [29,31,33]. However, feedback regulation of PDE6 or transducin by phosphorylated γ is unlikely, based on the low stoichiometry of γ phosphorylation observed in the above-cited studies. Phosphorylation of γ at Thr^{62} in nonretinal tissue culture cells has been linked to regulation of the MAP kinase signaling cascade [6], suggesting that γ might be involved in regulating other signaling pathways in photoreceptor cells.

Catalytic Properties of Nonactivated and Activated PDE6

In rod outer segments, there is equimolar γ subunit relative to the PDE6 catalytic subunit concentration [34].

The very high (K_D~1pM) binding affinity of γ for the catalytic dimer ensures that only 1 out of 2200 αβ dimers lacks bound γ and are activated in the dark-adapted condition [35], in good agreement with electrophysiological estimates of spontaneous PDE6 activation [36].

Removing both γ subunits from the PDE6 catalytic dimer activates the enzyme more than 300-fold, with a catalytic constant (k_{cat}) of 5500 s^{-1} for bovine rod PDE6 [27,37] and close to 8000 s^{-1} for frog PDE6 [34,35]. The catalytic efficiency ($k_{cat}/K_M = 4 \times 10^8$ $M^{-1}s^{-1}$) for cGMP approaches the diffusion-controlled limit. cAMP is a relatively poor substrate ($k_{cat}/K_M = \sim 3 \times 10^6$ $M^{-1}s^{-1}$), primarily because of its reduced affinity for the active site [27,35]. Under conditions tested to date, the GAF domains of PDE6 do not allosterically activate or inhibit the active site [27,38].

During visual excitation, PDE6 activation results from binding of α_t*-GTP to the PDE6 holoenzyme, thereby releasing the inhibitory constraint of γ. However, the maximum extent of activation of cGMP hydrolysis ($k_{cat} = 4400$ s^{-1} for frog PDE6. [34,35,39,40]) is about half of the fully activated value. In combination with observations that full activation of the PDE6 holoenzyme requires a single α_t*-GTP [41], it is likely that only one of the two catalytic sites on the PDE6 dimer is activated by transducin during phototransduction.

Roles of the GAF Domains in PDE6 Regulation

The GAF-containing PDE2, PDE5, and PDE6 families each utilize the GAF domain in a different manner. cGMP binding to PDE2 allosterically stimulates catalysis directly [42,43], whereas cGMP binding to PDE5 induces a conformational change enhancing phosphorylation of the enzyme that subsequently increases catalysis [44].

For PDE6, cGMP occupancy at the GAF domains enhances the affinity of γ for nonactivated PDE6 [31,41]; conversely, binding of γ to the PDE6 catalytic dimer enhances cGMP binding to the two nonidentical GAF domains [35,45]; conversely, binding of γ to the PDE6 catalytic dimer enhances cGMP binding to the two non-identical GAF domains [34,46]. This positive cooperativity of cGMP and γ binding serves to lower the basal catalytic activity of PDE6, and reduces cGMP metabolic flux in the dark-adapted state. Binding of cGMP to the GAF domains also serves to sequester a large majority of the total cellular cGMP, because the concentration of PDE6 is so large (see Chapter 195 by Francis and Corbin, this volume, for detailed discussion).

Upon transducin activation of frog PDE6, displacement of one γ subunit on PDE6 not only fully stimulates catalysis but also lowers cGMP-binding affinity at one GAF domain. Accelerated dissociation of cGMP at this site is accompanied by release of γ from the PDE6 holoenzyme. Rebinding of cGMP to PDE6 causes re-association of γ to the enzyme (see Fig. 8 of [34]).

It has been proposed that the primary purpose of the GAF domains is to release sequestered cGMP to assist guanylyl cyclase in restoring cytoplasmic cGMP levels during the recovery phase of the photoresponse [13,47]. However, the kinetics of cGMP dissociation from the GAF domain are too slow, and the rates of cGMP hydrolysis by activated PDE6 too fast to allow bound cGMP to significantly affect cytoplasmic cGMP levels during recovery to the dark-adapted state.

A more likely role for the GAF domains is to regulate the state of association of γ to catalytic dimer [46]. Free γ has been shown to act in concert with RGS9 to accelerate inactivation of α_t*-GTP [48,49]. Only when the GAF domains are unoccupied can γ dissociate from PDE6 and serve in this capacity [38,50]. Dissociation of cGMP (and hence γ) from PDE6 is likely only during prolonged light exposures when PDE6 is persistently activated and cytoplasmic cGMP levels remain low. This negative feedback mechanism involving cGMP, the GAF domains of PDE6, and the γ subunit acting as a GTPase accelerating factor for transducin could underlie the accelerated kinetics and reduced amplitude of the photoresponse characteristic of light-adapted rod photoreceptors.

Conclusion

The lifetime of activated rod PDE6 during rod phototransduction is a highly regulated process. Much work remains to fully describe the molecular events within the PDE holoenzyme and its interactions with transducin that precisely regulate the extent and duration of PDE6 activation. The interaction of PDE6 with other proteins such as the "δ subunit" [51] or GARP2 [52] is poorly understood, but may represent novel mechanisms for regulating the subcellular localization or catalytic activity of PDE6. Finally, it is possible that crosstalk between the visual transduction pathway and as yet uncharacterized signaling pathways may be fundamental to a complete understanding of rod PDE6 regulation during daytime illumination conditions when rod photoreceptors are near or at response saturation.

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CHAPTER 197

Spatial and Temporal Relationships of Cyclic Nucleotides in Intact Cells

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The Complexity of Cyclic Nucleotides Signaling

Protein phosphorylation is the most common posttranslational mechanism for regulating cellular functions. Regulation by this covalent modification of proteins is not a simple on-off mechanism occurring homogeneously within cells. On the contrary, activation and inhibition of protein kinases and phosphatases are tightly controlled both in time and in space. The exact timing of signal kinetics, the quantitative determination of the speed of signaling molecule diffusion, and their precise location within living cells are necessary prerequisites to a better understanding of cell physiology and pathology [1]. Protein kinases and phosphatases are often discretely localized within the cell in close proximity to receptors and targets, and this location appears critical for speed and specificity of response. The finding that signaling components are highly organized at the plasma membrane, in the cytoplasm, and in the nucleus has led to the proposal of "signaling domains" [2], that is, specific compartments within the three-dimensional matrix of the cell where the signal is generated and/or specifically targeted. cAMP-mediated signaling, for example, complies with tight local control, and the structural basis for such compartmentation is being uncovered. In hippocampal neurons the presence of a macromolecular signaling complex including the β2-adrenergic receptor, a G protein, adenylyl cyclase (AC), protein kinase A (PKA), the phosphatase PP2A, and the ultimate effector of the receptor, the L-type Ca^{2+}, channel has been documented [3], and a highly localized signal transduction from the receptor to the channel has been demonstrated. A crucial role in the nucleation of such signaling domains is played by A kinase anchoring proteins (AKAPs) [4], a family of functionally related proteins that anchor the regulatory subunit of PKA and possess unique targeting sequences that direct the PKA-AKAP complex to specific subcellular compartments. Within the "signaling domain" not only receptors, effectors and their ultimate targets are found in close proximity, but evidence is also accumulating that compartmentation involves the small diffusible molecules that act as intracellular second messengers and that second messenger limited diffusion can contribute to the efficiency and specificity of signal transmission [5].

The cyclic nucleotides cAMP and cGMP are freely diffusible intracellular second messengers that mediate specific responses to a staggering number of extracellular stimuli by activating downstream effectors, mainly the cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively). PKA and PKG in turn can potentially phosphorylate hundreds of different cellular targets. The enormous load of specific information that enters the cell and is funnelled through these two second messengers is relayed to the final effector systems without any loss of informational detail. The issue raised is how a single molecule can generate the appropriate response to so many different stimuli. One possible way that cyclic nucleotides could expand their signaling capability enough to accomplish this task is by adopting multiple signaling codes. According to this view, transmission of information is not exclusively dependent on the amplitude of the response but can be frequency-dependent [6], similar to what has been shown for Ca^{2+} oscillations, or space-dependent [7]; that is, signal transmission can take place in restricted subcellular compartments, while other

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signals are excluded. Several features of the cyclic nucleotides signaling cascades provide a potential molecular basis for such diversity in signaling modes [8]. First, intracellular cyclic nucleotide concentrations appear to be highly flexible and transient in nature because of a tightly controlled equilibrium of synthesis and breakdown. Second, both cyclases (the enzymes that make cyclic nucleotides) and phosphodiesterases (the enzymes that degrade cyclic nucleotides) exist in several isoforms with different tissue distribution, different intracellular targeting, specific regulation, and cross-talk with other signaling pathways. Third, Ca^{2+}, for which a frequency-encoded and a space-encoded mode of signaling has been clearly established, modulates both the synthesis and the breakdown of cyclic nucleotides by regulating, in an isoform-specific manner, both cyclases and phosphodiesterases [8].

A thorough and accurate analysis of the spatio-temporal aspects of cyclic nucleotides signaling appears to be crucial for our understanding of cell physiology [9,10]. So far, such analysis has been very difficult due to technical limitations. Lately, however, there have been methodological advances that allow monitoring of cyclic nucleotide in real time in live cells, thus opening the way to a detailed description in space and time of cAMP and cGMP biochemistry *in vivo*.

Methodological Advances

Traditionally, cyclic nucleotide concentration has been measured by immunoassay on cell lysates. Such a destructive method has several limitations: it is rather insensitive and often phosphodiesterase inhibition is necessary to achieve adequate sensitivity [11,12]; only the average concentration of the cyclic nucleotide in the cell population is recorded; the total, rather then the free cyclic nucleotide content, is detected; the temporal resolution is rather limited; any topographical information on the location of the response is cancelled out. A few attempts have been made to localize cyclic nucleotides in fixed tissue by immunocytochemistry [13] but accurate quantification is a problem, and time-courses are difficult to record.

Two nondestructive approaches have been pursued to monitor dynamically and in real time the levels of cyclic nucleotides in single live cells: (1) recombinant cAMP-cGMP sensitive channels, and (2) fluorescent probes sensitive to cAMP-cGMP and/or cAMP-cGMP dependent phosphorylation. The first method utilizes cyclic nucleotide-gated (CNG) ion channels genetically engineered to be especially sensitive to either cGMP [14] or cAMP [15]. One version of such methodology artificially introduces CNG channels into various cell types via the "patch-cramming" technique: an excised, inside-out membrane patch containing the channels is "crammed" into a recipient cell to measure cGMP concentration in the cytosol near the tip of the pipette [16]. The advantages of this approach are the possibility of easily calibrating the system and the minimal alteration or buffering of the intracellualar level of cGMP. One limitation is that detection of the cyclic nucleotide level is confined to the restricted area where the patch pipette is inserted. Also, given the relatively large size of the patch pipette, this approach is applicable only to relatively large cells (above about 40 µm in diameter). Alternatively, CNG channels have been used to study the membrane-localized, cAMP-signaling pathway by expressing the channel at the plasma membrane, where the adenylyl cyclase is known to reside, and by measuring either cAMP-induced currents [17] or cAMP-induced Ca^{2+} influx via Ca^{2+}-sensitive fluorescent dyes [18].

The second approach for real-time monitoring of cyclic nucleotide levels in live cells relies on imaging of cyclic nucleotide-activated protein kinases (or of their target peptides) that have been fluorescently labeled and are competent for fluorescence resonance energy transfer (FRET) [19]. FRET is a physico-chemical phenomenon whereby the excited state energy of a donor fluorophore is non-radiatively transferred to a nearby acceptor fluorophore. For FRET to occur, the following requirements must be satisfied: (1) substantial overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor, (2) alignment relative to each other in space of the fluorophore transition dipoles, and (3) distance between donor and acceptor fluorophores within 1 to 10 nm. The efficiency of FRET decreases with the sixth power of the distance between the fluorophores [20], making FRET a phenomenon that is exquisitely suitable for detecting conformational changes.

Most FRET-based probes for cyclic nucleotides exploit either PKA or PKG as the natural sensors for cAMP and cGMP, respectively. They take advantage of the conformational change that results from binding of the cyclic nucleotide to the kinase and that leads to a change in the distance between the FRET-donor and acceptor fluorescent labels.

Inactive PKA is a heterotetramer made of a dimer of regulatory subunits (R_2) and two catalytic subunits (C). When cAMP levels rise, two molecules of the second messenger bind to each of the R subunits, thus inducing a conformational change that leads to dissociation of two active C subunits. The prototype FRET-based sensor for cAMP consists of a fluorescein-labeled C subunit and a rhodamine-labeled R subunit [21]. This probe requires *in vitro* labeling of purified C and R subunits and subsequent microinjection in the cell of interest, which limits its use only to a restricted number of applications. Moreover, this very laborious approach suffers from several limitations [22,23], including probe instability, nonspecific compartmentation, and some toxic effect on the injected cell. A variant of this sensor has been generated that is entirely genetically encoded and in which the donor and acceptor fluorophores are the blue (BFP) and green (GFP) variants of the green fluorescent protein [24]. When cAMP is low PKA is mainly in the holotetrameric conformation R-BFP_2C-GFP_2 and the two fluorophores are close enough to generate FRET. Upon excitation of BFP at its proper excitation wavelength (380 nm), part of its excited state energy is emitted as blue light (460 nm) and part is transferred to the nearby acceptor GFP, which in turn is excited and emits green light (510 nm). When cAMP levels increase, C-GFP subunits are released, the two fluorophores

diffuse apart, and FRET is abolished. In this condition excitation of BFP at 380 nm only generates emission of blue light and no green light is emitted. FRET can be conveniently measured as the ratio of donor to acceptor emission with the advantage of cancelling out fluorescence intensity variations due to probe concentration, optical path length, and excitation intensity [25]. More recently this probe was modified by changing the BFP-GFP couple with the more convenient mutants CFP-YFP [3].

Based on the same general principle, sensors for cGMP have been generated [26,27]. In the probe denominated "cygnet," the cyan (CFP) and yellow (YFP) variants of the green fluorescent protein have been genetically fused to the amino- and carboxy-terminus of PKG, respectively. CFP and YFP act as donor and acceptor for FRET. The relatively large conformational change generated by cGMP binding to the PKG regulatory domain moves the donor CFP away from the acceptor YFP, thus reducing FRET.

Two other genetically encoded probes have been recently generated to monitor the cAMP dynamics in living cells. These two probes are also based on FRET between two mutants of GFP. Rather than directly probing cAMP levels, these sensors report the PKA-mediated phosphorylation of a substrate peptide. In the first case the two variants of GFP are joined by the kinase-inducible domain (KID) of the transcription factor CREB (cAMP-responsive element binding protein) [28]. The phosphorylation of KID by PKA decreases FRET among the flanking GFPs. The cAMP probe described by Zhang and coworkers is based on a similar principle. In this latter case the fusions of CFP, a phosphoamino-acid-binding domain (14-3-3τ), a consensus substrate for PKA, and YFP results in a change of FRET dependent on PKA-mediated phosphorylation [29].

Functional Compartments of cAMP in Heart Cells

Mayer and coworkers [30] were the first to propose, more than 20 years ago, the existence of spatially restricted domains of cAMP. The hypothesis was formulated to explain a series of data obtained in cardiac myocytes. In particular, the authors found that the positive inotropic and lusitropic effects induced by β-adrenergic stimulation correlated with activation of a particulate, membrane-bound fraction of PKA; similar elevations of cAMP due to prostaglandin 1 resulted in the activation of a cytosolic form of PKA and in minimal functional consequences [31]. Similarly, stimulation of cardiac myocytes with glucagon-like peptide-1 (GLP-1) generates a rise in $[cAMP]_i$ comparable to that elicited by isoproterenol, a β-AR agonist, but causes modest negative inotropy and no lusitropic effect, in sharp contrast with isoproterenol [32]. Moreover, in contrast to β_1-AR, β_2-AR stimulation fails to induce a cAMP-dependent phosphorylation of non-sarcolemmal proteins (such as phospholamban, or the myofilament proteins troponin I and C protein), but it does activate sarcolemmal L-type Ca^{2+} channels [33], suggesting a differential spatial organization of the PKA activated by the two β-ARs.

More convincing, yet indirect evidence, for localized domains of cAMP was provided by Jurevicius and Fishmeister [34]. In a series of elegant experiments carried out by whole-cell patch-clamp recordings of Ca^{2+} currents in frog heart cells they showed that a local stimulus of the β-adrenergic receptor causes a local activation of I_{Ca}, whereas local application of the adenylyl cyclase activator forskolin induces activation of I_{Ca} throughout the cell. The authors suggested that β-adrenergic stimulation generates a localized accumulation of cAMP sufficient to efficiently activate only nearby located Ca2+ channels, whereas nonspecific activation of AC induces a generalized rise of cAMP concentration [34]. Due to the experimental setup, the spatial resolution of these experiments was in the order of 20 μm, making it difficult to anticipate the physiological relevance of such cAMP compartments in a 10 to 15 μm wide mammalian cell. New insight into this issue came from experiments in which cAMP dynamics were monitored in real time in rat neonatal cardiac myocytes by using a FRET-based, genetically encoded sensor for cAMP [24]. In these cells, β-adrenergic stimulation generates multiple microdomains with increased concentration of cAMP in correspondence of the T tubular system (Fig. 1). T tubules in heart cells run along sarcomeric Z lines whose distance from one another is about 2 μm. The local gradients of the second messenger spread for less than half such a distance, implying a range of action for cAMP as small as about 1 μm [35].

Local control and limited diffusion of cAMP is not a prerogative of heart cells. In fact, in human embryonic kidney cells it has been demonstrated, by using recombinant cyclic nucleotide-gated channels as a cAMP sensor, that a uniform extracellular stimulus with PGE_1 initiates a transient increase in cAMP concentration near the plasma membrane and a sustained rise of the second messenger in the bulk cytosol [18], suggesting that spatially and temporally distinct cAMP signals can coexist within simple cells.

An important role in cAMP diffusional restriction seems to be played by phosphodiesterases, the cAMP degrading enzymes. In fact, PDE inhibition allows uniform spreading of the second messenger from the restricted microdomains along the T tubular network into the bulk cytosol [35] (Fig. 1). It is interesting that certain PDE inhibitors seem to cause "spill-over" of PGE_1-induced cAMP from the soluble into the particulate compartment of ventricular myocytes [7]. A reduction in the localized cAMP effects upon inhibition of PDE was observed also in the frog heart cells locally stimulated with isoproterenol [34], and a role for PDE in shaping the local cAMP response underneath the plasma membrane has been uncovered in embryonic kidney cells [18]. In support of the notion that PDE can control cAMP spreading is the finding that PDEs have also been found to be targeted to specific subcellular compartments [36] and in cardiac myocytes PDEs have been found to be anchored to AKAPs [31,37]. Alternative mechanisms, however, cannot be excluded. In the case of the β_2-AR, in fact, local signaling has been suggested to result from the coupling of β_2-AR to Gi proteins that may act locally, possibly through a protein phosphatase-dependent mechanism [38].

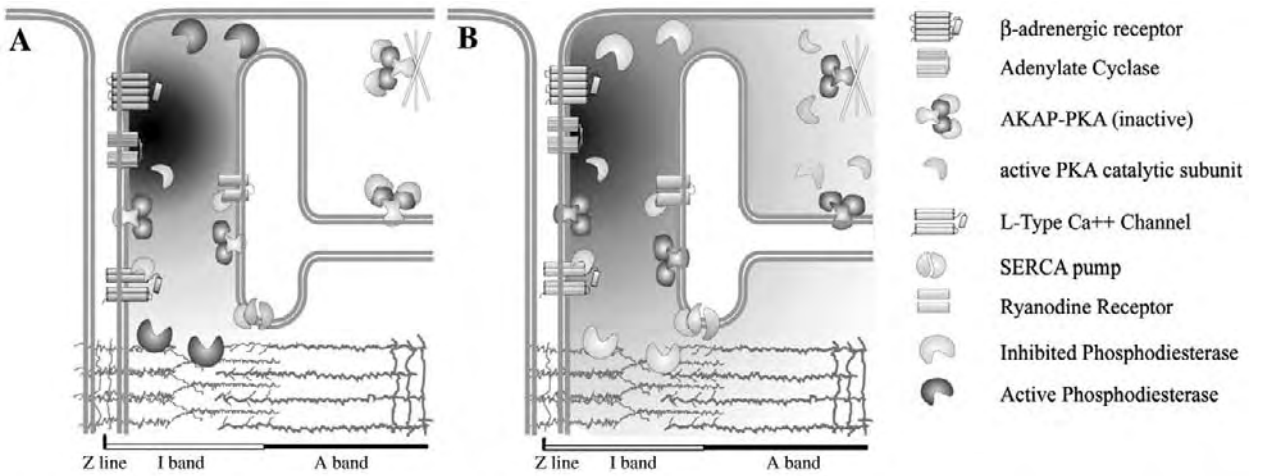


Figure 1 PKA, the main effector of cAMP, is a key regulator of excitation-contraction coupling in muscle cells. In the heart, sympathetic control of the frequency and strength of contraction is exerted by β-AR stimulation, activation of G proteins, and in turn activation of adenylyl cyclase and synthesis of cAMP. The second messenger activates PKA, which by phosphorylating L-type Ca^{2+} channels and the ryanodine receptor increases the amount of Ca^{2+} ions available for contraction of the sarcomere, and by phosphorylating phospholamban (PLB) it favors Ca^{2+} reuptake by the calcium pump SERCA-2. In heart cells, G proteins [44], AC [44], PKA [45], A kinase anchoring proteins (AKAPs) [37,45], the L-type Ca^{2+} channels [44], and the ryanodyne receptor [46] have all been found within the T tubular network/junctional SR membrane, thus forming a "signaling domain" that provides a potential anatomical basis for the confined activation of the cAMP signaling pathway.

When the β-adrenergic agonist interacts with the β-AR, a series of G-protein-mediated changes leads to activation of adenylyl cyclase and synthesis of cAMP. The latter acts by means of PKA to phosphorylate the L-type Ca^{2+} channel, which increases the inward movement of Ca^{2+} ions through the membrane of the T tubule. These Ca^{2+} ions release more Ca^{2+} from the sarcoplasmin reticulum (SR) through the ryanodine receptor, and more Ca^{2+} is thus available for binding to sarcomeric proteins for contraction. PKA also activates phospholamban (PLB), thus increasing Ca^{2+} reuptake through the Ca^{2+} pump SERCA-2, located on the SR membrane. All the molecular elements involved in the β-adrenergic signaling pathway are clustered in the region of the T tubular network/junctional SR membrane. PKA itself is anchored in this area through binding to AKAPs [45]. Activation of the β-AR generates in these cells a localized increase of cAMP, which in turn activates a subpopulation of PKA that is confined within the same signaling domain [35] (panel A). Compartmentation of cAMP is due to the activity of phosphodiesterases. Indeed, inhibition of PDE allows homogeneous diffusion of cAMP in the cytosol [35] (panel B). The bars at the bottom of panels A and B indicate sarcomeric elements.

Spatio-temporal Aspects of Cyclic Nucleotides Signaling in Neurons

Although both cAMP and cGMP have been shown to be involved in the molecular processes that govern certain types of learning and memory and, in particular, in activity-induced synaptic plasticity [39,40], most of the spatiotemporal intricacies of cyclic nucleotide signaling in neural circuits still remain to be elucidated [41,42]. Some of the available data, however, point to an important role of the spatial organization of the second messenger in controlling the output. cAMP is involved in both short- and long-term synaptic changes, and functional compartmentation of the second messenger has been proposed as one of the possible mechanisms that control which effect, either short- or long-term, is put in place [42]. In the monosynaptic connection between sensory and motor neurons of the gill-withdrawal reflex in the marine snail *Aplysia* the generalized application of serotonin very rapidly produces (20 sec) a spatial gradient of free cAMP, with the higher concentration being recorded at the distal neuronal processes and the lower concentration at the cell body. On the contrary, only a very prolonged stimulation (2 hrs) is accompanied by translocation of the PKA catalytic subunit to the nucleus [43]. The interpretation of these results is that the observed gradients confine cAMP where it is most needed for short-term plasticity, that is at the tip of the processes where most of the presynaptic terminals reside. The attenuation of the cAMP signal at the cell body could represent a safety mechanism to ensure that only repeated stimulation leads to active PKA C subunits entering the nucleus, activating transcriptional programs, and therefore generating long-term effects [43].

Conclusions

In the last decades it has become clear that signal transduction does not rely on stochastic events but rather on the sophisticated organization of molecular interactions that are precisely regulated both in time and space. Cyclic nucleotides are key players in the relay of intracellular signals, and are bound to be subject to the same strict control. The fine description of cAMP and cGMP dynamics has been hampered in the past by the lack of adequate technology for real-time monitoring of the levels of these second messengers in live cells. The recent development of imaging-based methodologies that allow single cell analysis and very high spatial and temporal resolution will certainly be crucial in our understanding of cAMP and cGMP biochemistry *in vivo*. At present, the large majority of the data available on the spatio-temporal

aspects of cyclic nucleotide signaling refers to cAMP, and although some evidence for subcellular heterogeneity in cGMP has been presented in the past, no direct single cell data on this topic have yet been published (at the moment this manuscript was submitted). Certainly, in the coming years we can expect to see the uncovering of a finely detailed map of the molecular mechanisms that orchestrate both cAMP and cGMP signal transduction.

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CHAPTER 198

Regulation of Cyclic Nucleotide Levels by Sequestration

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Introduction

Cyclic nucleotides bind to specific proteins in cells and are thereby sequestered into different functional compartments. Some of the bound cyclic nucleotide serves to activate target proteins such as protein kinases or ion channels while another portion is bound to sites in other proteins and therefore inactive for stimulating protein kinases or ion channels. Sequestration into or release from such inactive sites could be modulated in response to certain stimuli. In order for sequestration to be physiologically relevant, cyclic nucleotide-binding proteins should have ample affinity and be present in sufficient amounts to bind a significant portion of the total cellular cyclic nucleotide. Moreover, the sequestration should be a regulated process. These conditions appear to be met for cGMP binding to the allosteric (noncatalytic) sites of phosphodiesterase-5 in penile corpus cavernosum or of phosphodiesterase-6 in retina.

It is usually emphasized that extracellular signals modulate levels of cyclic nucleotides in cells by regulation of the activities of adenylyl and guanylyl cyclases. Changes in concentrations of signals presented to cells are thus critical determinants for rate of formation of cAMP or cGMP. Rate of loss, or inactivation, of cAMP or cGMP also contributes importantly to determining the cellular concentration of these nucleotides, but this aspect has been studied much less. There are at least three possible routes of loss of active cellular cyclic nucleotides. These are illustrated for cGMP in Fig. 1.

First, loss of cellular cAMP into the extracellular space can occur by efflux such as through leakage or specific transport processes [1,2]. This energy-dependent transport process for cAMP and cGMP may involve an organic anion transport process that utilizes multidrug resistance proteins in some tissues [3–5]. The rate of cyclic nucleotide efflux appears to be dependent on intracellular cyclic nucleotide levels and independent of direct signal regulation. Furthermore, loss of cyclic nucleotides by hydrolysis greatly exceeds that achieved by efflux [1,6–9].

Second, loss of cyclic nucleotides through degradation by cAMP and cGMP phosphodiesterases (PDEs) has been widely investigated [10–16]. PDEs have been shown to be regulated by a variety of signaling pathways [10–16]. See several chapters in this volume for additional information on specific PDE families.

Third, sequestration of cyclic nucleotide, which is the focus of this chapter, is also a possible route of loss of the cytoplasmic, active pool of cyclic nucleotide. In contrast to loss of cyclic nucleotide by either efflux or degradation, this process would be largely reversible.

Physiological sequestration of second messengers and other signaling agents is not a new concept. For example, Ca^{2+} sequestered in cardiomyocyte sarcoplasmic reticulum is unavailable to stimulate contraction of heart muscle until its release due to depolarization of the cardiomyocyte [17]. Compartmentalization and regulated trafficking of protein kinases and myriad other proteins among plasma membrane, cytoplasm, nucleus, and other organelles is commonplace.

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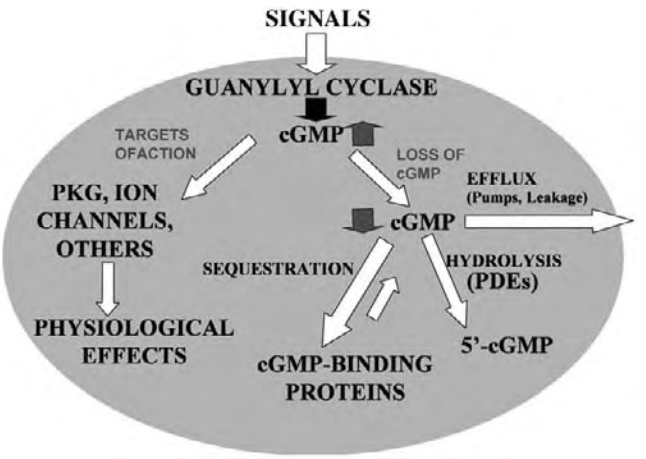


Figure 1 Routes of formation and loss of cGMP in cells.

Likewise, there is increasing evidence for compartmentalization of cAMP into subcellular microdomains that impose diffusional restriction on the nucleotide [18,19]. A portion of the cellular cyclic nucleotide pool can be bound as inert forms to certain cellular proteins. As such, this pool is diffusionally restricted and less available for degradation or efflux, but upon dissociation, this cyclic nucleotide can join the pool of free cyclic nucleotides or traffic among cyclic nucleotide-binding proteins. Thus, some proteins could act as sinks for cyclic nucleotides, not only providing for inhibition of action by sequestration but also as a source of active cyclic nucleotide after release by certain stimuli.

Localization of cyclic nucleotide-binding proteins to particular compartments within cells would concentrate both the proteins and bound cyclic nucleotide within these locales [18]. Compartmentalization of cyclic nucleotides with their target proteins such as PKA and PKG, or with PDEs, almost certainly occurs and is likely to be physiologically advantageous. Co-localization of cyclic nucleotides with PDE activity could also be brought about evolutionarily by fusing noncatalytic cyclic nucleotide-binding domains with catalytic domains responsible for cyclic nucleotide hydrolysis. This would effectively concentrate the cyclic nucleotide in the microenvironment of the catalytic site. Upon dissociation from the binding site, the nucleotide would most likely be hydrolyzed rather than reentering the active pool.

Conclusive evidence for sequestration of cAMP into inactive pools by cellular proteins has not been demonstrated. Type I regulatory subunit (R subunit) of PKA occurs in stoichiometric excess of catalytic subunit (C subunit) in some tissues [20]. This R subunit contains bound cAMP. However, the apparent excess of R subunit in some cases could be an artifact, since the instability of free C subunit makes quantification difficult. If excess R subunit is conclusively demonstrated, it might act as a buffer to dampen cAMP responses or act as a reservoir for cAMP. R subunit is in slight excess of cAMP in unstimulated rat heart [18].

Cellular sequestration of cGMP in certain instances seems entirely plausible. Of particular emphasis is the likelihood that cGMP could be sequestered by a group of cGMP-binding phosphodiesterases (PDEs) (PDE2, PDE5, PDE6, PDE10, PDE11) that contain noncatalytic GAF domains. Several of these families of PDEs (PDE2, PDE5, PDE6) degrade cGMP or cAMP at catalytic sites and have been shown to bind cGMP at noncatalytic (allosteric) sites associated with the GAF domains. These binding sites provide the potential for sequestration of cGMP away from its targets such as cGMP-dependent protein kinase (PKG) and cyclic nucleotide-gated channels. Some signals could cause release of this latent form of cGMP for stimulation of certain pathways, or alternatively, they could release the nucleotide near the catalytic site for its efficient breakdown and contribute to negative feedback regulation of the cGMP pathway. If sequestration of cGMP by a cGMP-binding PDE is to be physiologically meaningful, the affinity of cGMP for this PDE should be high. Furthermore, the stoichiometric amount of this PDE should be significant relative to the level of cGMP itself. There should also be mechanisms for modulating the affinity state of the cGMP-binding sites of the PDE. Whether the levels of PDE2, PDE10, or PDE11 are sufficient or capable to bind a significant amount of total cGMP in any cell has not been investigated [21–24]. It should be emphasized that cellular sequestration by any of the cGMP-binding PDEs would be enhanced by appropriate co-compartmentalization with cGMP production. This also applies to cGMP-gated channels, although it is unlikely that number and affinity of binding sites of these proteins are sufficient to bind a significant portion of total cGMP in most cells.

Sequestration of cGMP in Rod Photoreceptor Cells by PDE6

Cyclic GMP is the primary intracellular second messenger for visual transduction and is present in high concentrations in photoreceptor cells. Light stimulation activates PDE6 more than 100-fold, causing free cytoplasmic cGMP to drop to sub-micromolar levels. Recovery of the dark-adapted level of cGMP is aided by PDE inactivation in concert with guanylyl cyclase activation. cGMP-gated cation channels in the plasma membrane close in response to the light-induced decrease in cGMP, resulting in hyperpolarization of the cell (for reviews, see [25, 26]).

Sequestration of cGMP is central to this pathway, since the dark-adapted level of cytoplasmic cGMP (2–4 μM) inferred from electrophysiological studies [27,28] is less than 10 percent of total cGMP concentration (60 μM) in the signal-transducing outer segment portion of the photoreceptor [29]. Both PDE6 holoenzyme ($\alpha\beta\gamma_2$) and the cGMP-gated channel bind cGMP and could sequester it [30,31]. However, the relative number of cGMP-binding sites and the affinity with which each protein binds cGMP (Table I) indicate that the cGMP-gated ion channel plays a minor role in sequestration of cGMP in the outer segment.

PDE6 is present in high concentrations (20 μM holoenzyme concentration) in rod outer segments [30,32]. Two high-affinity

Table I Calculated Intracellular Concentrations in Frog Rod Outer Segments

Component	concentration[1] (μM)	[cGMP site] (μM)	Binding affinity (μM)	[cGMP] bound[3] (μM)
PDE6 (high affinity)	20	40	0.06	39.0
Low affinity		75	7.0	18.4
cGMP-gated channel	1	4	30[2]	0.3
cGMP	Total: 60 free: 2.3			

1. Concentrations in the rod outer segment are referenced to the cytoplasmic volume (see text).
2. The cited value is the half-maximal activation constant for this binding reaction.
3. Bound and free values were estimated using EQCAL (Elsevier Biosoft) and assuming non-interacting, non-cooperative binding.

cGMP-binding sites per holoenzyme are occupied in the inactive PDE6 [34], binding two-thirds of total cellular cGMP in amphibian rod outer segments [35]. A second class of cGMP-binding sites with 100-fold lower affinity has been detected [35]. Assignment of this binding to the second GAF domain within each catalytic subunit of PDE6 is largely based on the fact that no other high-abundance cGMP-binding protein has been reported in photoreceptors. Together, the high- and low-affinity sites sequester 95 percent of total cellular cGMP, bringing the free cGMP into the low micromolar range (Table I).

Light activation of PDE6 causes a sub-second decline in free cytoplasmic cGMP to sub-micromolar levels, causing closure of cGMP-gated ion channels in the plasma membrane. Notably, total extractable cGMP is decreased only approximately 50 percent (to approximately 30 μM). Activation of PDE6 converts one of its high-affinity cGMP-binding sites to a lower affinity that allows for rapid dissociation of this cGMP. The other high-affinity site on PDE6 undergoes a more modest loss in affinity, and cGMP probably remains bound to this site—and therefore protected from hydrolysis–during excitation and recovery [33,36–38]. Termination of the photoresponse requires inactivation of PDE6 catalysis by its γ subunit, restoration of the second high-affinity binding site on PDE6, acceleration of cGMP synthesis by guanylyl cyclase, and rebinding of cGMP to the cGMP-gated channel and to both sites on PDE6.

The functional significance of light-induced changes in the amount of sequestered cGMP is unknown. One hypothesis is that cGMP released from light-activated PDE6 could elevate cytoplasmic cGMP and assist guanylyl cyclase during the recovery phase of the response [35,36]. However, sequestered cGMP that is released upon illumination will be quickly hydrolyzed and is unlikely to contribute to restoring free cGMP levels during recovery [37]. A more plausible hypothesis is that cGMP release from activated PDE6 serves as a negative feedback mechanism to inactivate PDE6 during bright light adaptation of photoreceptors [33]. Cyclic GMP dissociation from PDE6 exerts an allosteric effect on inhibitory γ subunit [33,38,39], causing γ to accelerate transducin GTPase (in concert with RGS9; [37,40,41]). In this view, cGMP-binding sites on activated PDE6 would respond to a persistent reduction in free cGMP levels (that is, bright, continuous illumination) by releasing bound cGMP, causing transducin to more rapidly inactivate itself and allow the γ subunit to inhibit PDE6 once more. This is consistent with the speeded recovery of the photoreceptor light response under conditions of bright light adaptation.

In summary, cGMP sequestration by PDE6 meets the criteria for physiological relevance: (1) concentrations of PDE6 cGMP-binding sites and cGMP in photoreceptor cells are comparable, (2) GMP-binding to PDE6 is regulated, and (3) affinity of the cGMP-binding sites is sufficient for quantitatively significant cGMP binding in these cells. Cyclic GMP binding by PDE6 is likely to serve multiple functions in regulating the signal transduction in photoreceptors. In the dark-adapted state, PDE6 binding of cGMP maintains free cytoplasmic cGMP at the low levels needed for opening a small fraction of cGMP-gated ion channels in the plasma membrane. Following light activation, release of cGMP from PDE6 may shorten the lifetime of excitation and provide a mechanism for adapting photoreceptor cells to sustained illumination.

Sequestration of cGMP by PDE5

PDE5 was the first recognized mammalian cyclic nucleotide receptor other than PKA and PKG [42,43]. It is highly specific for hydrolysis of cGMP at a single catalytic site on each subunit, and it contains two potential cGMP-binding sites (GAF domains) in the regulatory domain of each subunit [44,45] with an average cGMP-binding affinity of about 200 nM. Phosphorylation of PDE5 by PKG at Ser-92 (bovine enzyme) increases cGMP-binding affinity ten-fold [46,47] and also stimulates the catalytic activity of the enzyme [47]. Furthermore, occupation of the catalytic site of bovine PDE5 by cGMP or cGMP analogs stimulates binding of cGMP to the allosteric sites, which in turn stimulates phosphorylation at Ser-92 [43,48]. It follows from these reciprocal, allosteric effects that elevation of cGMP in cells would initiate PDE5-mediated negative feedback by stimulation of both cGMP breakdown at its catalytic site and sequestration of cGMP at its allosteric sites. These two mechanisms for lowering free

Table II Calculated Intracellular Concentrations in Rabbit Corpus Cavernosum

Cell conditions		Free [cGMP] nM[2]	Bound [cGMP] nM[2]	
[cGMP] nM	PDE5		To PDE5	To PKG
Basal	Unphospho-	7	6.7	4.0
Elevated	Unphospho-	23	20.0	11.0
Elevated	Phospho-	8	41.1	4.5
Basal	Phospho-	2	14.2	1.4

1. Basal total cGMP concentration 18 ± 4 nM. Nitric oxide-stimulated cGMP concentration, ~54 nM. Total PDE5 concentration (referenced to cGMP binding sites), 188 ± 6 nM. Total PKG concentration (referenced to cGMP binding sites), 58 ± 6 nM. Calculations based on specific enzyme activity of pure proteins, and intracellular water of 0.5 g per g tissue.

2. Calculated assuming the following K_D values: PKG = 100 nM (form Km of PKGIα at 20°C), unphospho-PDE5 = 200 nM at 0°, phospho-PDE5 = 30 nM, using EQCAL and assuming noninteracting, noncooperative cGMP binding.

cGMP levels should diminish cGMP binding to PKG or other cGMP receptor proteins.

In order for sequestration of cGMP to play a significant role in modulating the free cGMP levels in PDE5-regulated signaling pathways, the number and affinity of cGMP-binding sites must be sufficient to buffer cGMP, and the rate of binding/sequestration must be faster than the rate of loss via hydrolysis. At least in some tissues, these conditions appear to be met. In rabbit corpus cavernosum smooth muscle cells (Table II), PDE5 allosteric binding site concentration is more than five times higher than basal cGMP [49]. The binding affinity of these sites is great enough (even for the unphosphorylated PDE5) to sequester a significant portion of cGMP, even after elevation of this nucleotide by various agents. Loss of active cellular cGMP by this sequestration could be quantitatively meaningful since loss of total cellular cGMP by PDE5 catalytic breakdown of this nucleotide is calculated to require about 16 sec [49].

Under basal intracellular conditions, most cGMP molecules would be bound to PDE5 or PKG given the high affinities of these two proteins for cGMP. Using the K_D value of 200 nM that we determined for unphospho-PDE5 [48,50], PKG K_D for cGMP of 100 nM [51], and the intracellular values of 188 nM PDE5, 58 nM PKG and 18 nM for cGMP (Table II), we used EQCAL (Elsevier Biosoft) to calculate free cGMP and bound cGMP under various conditions.

Figure 2 summarizes our model for regulation of cGMP signaling pathways by sequestration of this nucleotide by allosteric sites of PDE5. In the basal condition (low cGMP, unphosphorylated PDE5), both PDE5 and PKG contribute to binding about half of the total cellular cGMP (free concentration = 7 nM). A three-fold increase in total cGMP levels by guanylyl cyclase activation initially would cause corresponding increases in free cGMP and the amount bound to both PKG and PDE5. Increased PKG activation resulting from cGMP binding might phosphorylate PDE5, enhancing ten-fold its binding affinity at the noncatalytic binding sites. This would immediately reduce the free cGMP and reverse

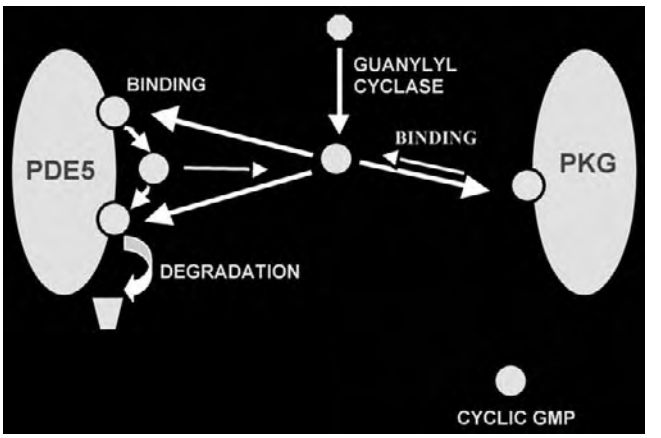


Figure 2 Proposal for regulation of some signaling pathways by sequestration of cGMP by allosteric sites of PDE5.

PKG activity to its basal state. If PDE5 dephosphorylation were relatively slow, free cGMP levels as well as PKG bound to cGMP might both drop several-fold lower than their basal values (Table II). In summary, cGMP elevation would cause increased sequestration, resulting in dampening of the cGMP signal and facilitating termination of this signal.

The process described above could be part of a concert of negative feedback processes for cGMP that have evolved for fine regulation of cGMP signaling in a number of tissues: (1) increased PDE5 catalytic activity due to mass action of elevated cGMP, (2) increased cGMP binding to PDE5 allosteric sites due to mass action of elevated cGMP, (3) increased PDE5 catalytic activity due to phosphorylation and activation of PDE5 by activated PKG [50], and (4) increased cGMP binding to PDE5 allosteric sites due to this phosphorylation. A fifth possible process is direct stimulation of the PDE5 catalytic site by allosteric cGMP binding to the enzyme, which would be predicted by the principle of reciprocity [52]. A final possibility is that concentrating cGMP near the catalytic site by its release from proximal

cGMP-binding sites could increase efficiency of cGMP hydrolysis. The presence of such an array of mechanisms for negative feedback of the cGMP pathway suggests that cells cannot readily tolerate excessive activation of PKG or other target proteins. Thus, for both PDE5- and PDE6-containing cells, sequestration may act to (1) buffer cGMP levels in the basal state and (2) act as negative feedback regulator to prevent overstimulation and accelerate response termination of the signaling pathway.

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CHAPTER 199

cAMP-dependent Protein Kinase

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Introduction

cAMP-dependent protein kinase (PKA) is one of the best characterized members of the large protein kinase superfamily. The catalytic subunit serves as a structural prototype for the entire family. The inactive holoenzyme comprises a regulatory (R) subunit dimer and two catalytic subunits. Binding of cAMP to the R subunits unleashes the active C subunits. The structure of the C subunit is described and correlated with its function. The structure of the dimerization/docking domain of RIIα and the cAMP binding domains of RIα and RIIβ are also described and correlated with the dynamic properties of the R subunits.

cAMP-dependent protein kinase (PKA) was one of the first protein kinases to be discovered [1], the first to be sequenced [2], the first to be cloned [3], and the first protein kinase for which a crystal structure was solved [4]. It thus serves in many ways as a prototype for the entire protein kinase superfamily, which represents approximately 2 percent of the human genome. cAMP is an ancient stress response signal; for example, it is a universal indicator of glucose deprivation. Whereas in bacteria, the cAMP second messenger is linked to the catabolite gene activator protein, in mammals it is linked primarily to the activation of PKA. PKA is ubiquitous in mammalian cells and regulates many diverse pathways.

The inactive holoenzyme complex consists of a regulatory (R) subunit dimer and two catalytic (C) subunits. Binding of cAMP to the R subunits unleashes the active C subunits, thus allowing them to phosphorylate a variety of protein substrates, both cytosolic and nuclear [5,6]. In addition to serving as inhibitors of PKA activity and receptors for cAMP, the R subunits also serve as adapters that tether the C subunit to specific cellular locations by binding to A kinase anchoring proteins (AKAPs) [7]. PKI, another inhibitor of the C subunit that is independent of cAMP [8], also contributes to trafficking of the free C subunits between the cytoplasm and the nucleus [9]. The inhibitors of PKA activity are both modular and multifunctional proteins. A review of PKA structure thus must include the diverse set of proteins that contribute overall to PKA regulation. The structures of the C subunit and its inhibitors, both the R subunits and PKI, are described here.

Catalytic Subunit

In mammals three isoforms of the C subunit have been identified: α, β, and γ [3,10,11]. The Cα subunit is expressed constitutively in all cells, whereas expression of Cβ is tissue specific and especially prominent in brain. Cγ is found primarily in testes. Several splice variants of both Cα [12] and Cβ [13] also exist; all differ in the first exon. In the primary form of Cα, β, and γ, exon I codes for 14 amino acids that include an N-terminal myristylation site [14]. The other C subunit splice variants are typically not myristylated. In addition to co-translational myristylation, the C subunit is phosphorylated posttranslationally at two essential sites [15]. Phosphorylation at Thr197 in the activation loop is essential for efficient catalysis [16]. Phosphorylation at Ser 338 is essential for stability and is very likely to be an important part of the maturation of the initial transcript into an active enzyme [17]. Phosphorylation at Ser10 [18] and deamidation of Asn2 [19] are other posttranslational modifications that have been identified.

Catalytic Properties

The C subunit is a highly concerted enzyme; all its energy is focused on transferring the γ–phosphate of ATP to an appropriate substrate protein [20]. There are two general recognition motifs for PKA substrates [21]: Arg-Arg-X-Ser/Thr-Hyd and Arg-X-X-Arg-X-X-Ser/Thr-Hyd, where X is any residue and Hyd is a hydrophobic residue. The mechanism

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for catalysis has been carefully defined by Adams [22]. Pre-steady state kinetics established that the actual rate of phosphoryl transfer is very fast (>500/s) whereas the kcat is only 20/sec. For PKA, the kcat correlates, in general, with the release of ADP and the conformational changes that allow for its release [23]. The K_m for the heptapeptide, kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly), is 10–20 μM; however, this K_m does not reflect a true binding affinity. The K_d (200–300 μM) more accurately reflects affinity [24].

Structure

Multiple forms of the C subunit have been crystallized, and these structures provide a molecular understanding of nucleotide binding, peptide binding, and conformational flexibility [4,25–27]. The C subunit comprises a highly conserved core containing a smaller ATP binding domain (residues 40–126) that is dominated by β structure and a larger, mostly helical lobe (residues 127–300) that provides a docking site for peptides/proteins as well as several essential residues that contribute to catalysis (Fig. 1). The adenine ring of ATP is buried at the base of the cleft between the two lobes, and the peptide docks to the surface of the large lobe at the edge of the cleft. This core is conserved in all protein kinases that phosphorylate Ser, Thr, or Tyr [28].

In PKA, as seen in Fig. 2, the core is flanked by 40 additional residues at the N-terminus that begin with a myristyl moiety attached to the N-terminal Gly. This is followed by an amphipathic helix that is anchored by hydrophobic interactions to both the small and large lobe of the core [29]. The core is followed by a 50 residue "tail" that is anchored to the large lobe (residues 301–318), has a flexible anionic "gate" that draws basic peptides to the active site cleft, and terminates with a hydrophobic motif at the C terminus (Phe-Ser-Glu-Phe) [30,31]. This hydrophobic motif is anchored to a hydrophobic pocket on the small lobe and probably helps orient the C-helix into its active conformation.

Conserved Core

As recognized initially by Hanks and Hunter [32], the conserved kinase core consists of a set of sequence motifs that span the entire core (Fig. 1). Although affinity labeling provided clues about the roles of some of these motifs [5,33,34], the first crystal structure revealed the unique architecture that brings most of these conserved motifs to the active site cleft where they contribute primarily to the binding of ATP and phosphoryl transfer [35]. The detailed characterization of the C subunit is reviewed in Johnson *et al.* [36].

Small Lobe

In general, the small lobe is more "loosely" structured than the large lobe. One of the most essential features of this enzyme is the glycine-rich loop that links β strands 1 and 2. In most of the crystal structures, this loop is disordered or ordered poorly [31]. Only in the ternary complex where ATP, or an ATP analog, and an inhibitor peptide, PKI (5–24), are bound [26,37] and in a recently solved aluminum fluoride complex that mimics a transition state intermediate, is the tip of the loop firmly anchored [38].

The hydrogen bond between the backbone amide of Ser53 and the γ-phosphate of ATP is probably the driving force for catalysis [39]. The two other essential residues in the small lobe are Lys72 in β strand 3, which anchors the α- and β-phosphates of ATP, and Glu91 in the C helix, which interacts with Lys72. All crystal structures of the C subunit so far have been of the active, fully phosphorylated protein.

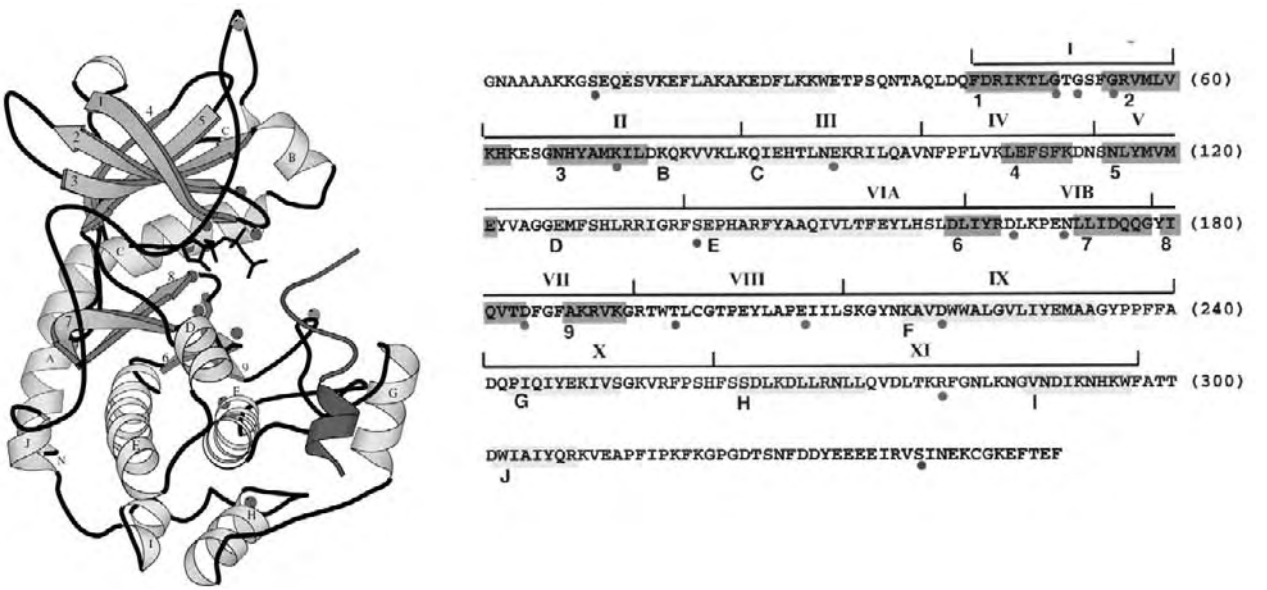


Figure 1 Structure and sequence of the catalytic subunit of PKA. A ribbon diagram of the mouse C subunit bound to ATP and an inhibitor peptide PKI(5-24) is on the left [26]. β strands are in green; α helices are yellow. PKI(5-24) is red. Conserved residues are indicated as red balls, phosphorylation sites as purple balls. On the right is the sequence with the same color coding.

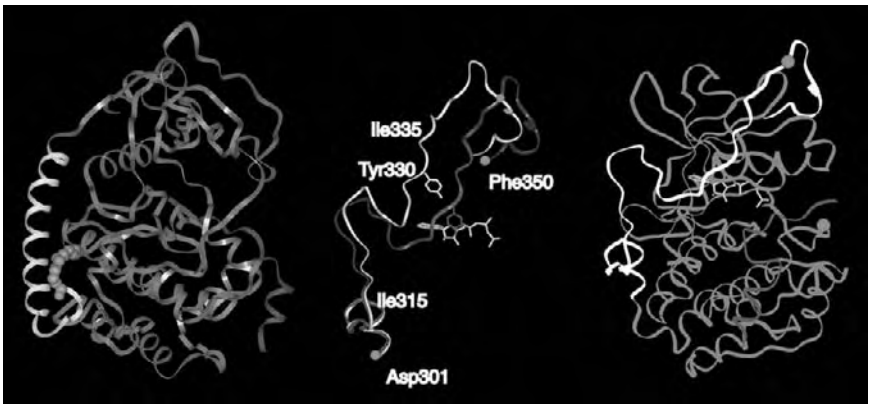


Figure 2 The N and C terminal tails of the catalytic subunit. The structure of the myristylated N terminus (residues 1–40) was observed in the mammalian C subunit (left); this structure represents an open conformation. On the right is a structure of a ternary complex of the recombinant C subunit with the C terminal tail highlighted in white. In the center is shown the conformation of the C terminal tail in an "open" and "closed" conformation. Tyr330 in the closed conformation forms a nucleation site by interacting with the ribose of ATP, the linker through Glu127, and the P-3 Arg through a water molecule. Replacement of Tyr330 with Ala leads to significant loss of activity. In the absence of ATP, the tail tends to be disordered.

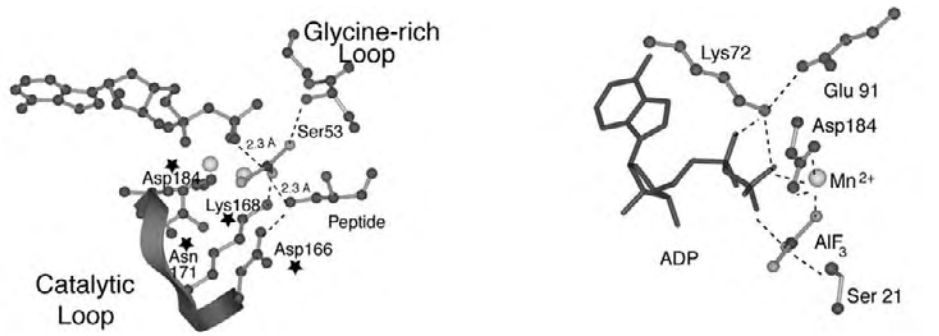


Figure 3 Aluminum fluoride complex mimics a transition state intermediate. On the left is the active site cleft in the presence of MgATP and PKA (5-24) [26]. On the right is the convergence of active site residues in a complex of C subunit with ADP, AlF_3, and a substrate peptide. This structure reveals how many of the conserved residues cluster around the active site cleft and contribute to the transfer of the γ phosphate of ATP to the peptide substrate. Conserved residues are highlighted with a yellow dot.

Phosphorylation decreases the K_M (ATP) 50-fold and increases the rate of phosphoryl transfer from $500s^{-1}$ to $20s^{-1}$ [16]. For many other protein kinases the proper orientation of the C helix is dependent upon phosphorylation of the activation loop in the large lobe [40]. Most likely, this is also true for the C subunit when the protein is unphosphorylated.

LARGE LOBE

Most of the conserved residues in the large lobe are localized on a β sheet that is anchored firmly through hydrophobic interactions to the large lobe (Fig. 3). The catalytic loop linking β strands 6 and 7 contains 3 conserved residues; Asp166 and Asn171 are universally conserved whereas Lys168 is conserved in all Ser/ Thr specific kinases. Although Asp166 is positioned to be a catalytic base, it contributes only minimally to phosphoryl transfer and is thought to be used primarily for orienting the peptide hydroxyl moiety rather than contributing significantly to the nucleophillic properties of the attacking group [41]. Asn171 binds to the second metal ion that interacts with the α and γ phosphates of ATP. It also hydrogen bonds to the backbone carbonyl of Asp166, thereby stabilizing the backbone of the catalytic loop. The magnesium-positioning loop, residues 184–187, links β strands 8 and 9. Asp184 binds the activating Mg ion that bridges the β and γ phosphates of ATP. β strand 9 is followed by the activation loop, which is positioned for optimal phosphorylation by the phosphorylation of Thr197. When expressed in *E. coli*, Thr 197 can be autophosphorylated. However, Thr197 is also an excellent substrate for PDK1 [42], and in mammalian cells it is more likely that the C subunit is phosphorylated by a heterologous protein kinase, not by autophosphorylation [43]. Thr197 is followed by the P+1 loop, named because three residues (Leu198, Pro202, and Leu205) fold inward

and form a docking site for hydrophobic P+1 residue. In fact, however, this loop can be more appropriately referred to as the peptide-organizing loop, since almost every residue contributes to some aspect of peptide recognition. Gly200 and Thr201 are essential and conserved in all Ser/Thr protein kinases. Gly200 abuts the backbone of the P-site residue and forms a hydrogen bond to the P+1 backbone amide. In contrast, the side chain of Thr201 interacts directly with catalytic loop residues, where it is wedged between and hydrogen bonds to the side chains of Lys168 that positions the γ-phosphate of ATP and Asp166 that positions the hydroxyl acceptor in the peptide substrate. The bridging role of Thr201 is seen most clearly in the structure of ADP, AlF_3, and a substrate peptide (Fig. 3) [38]. Glu203 in PKA provides a docking site for the P-6 Arg and Tyr204 hydrogen bonds to Glu230, a primary recognition site for the P-2 Arg. The aromatic ring of Tyr204 also contributes to peptide binding.

Protein Kinase Inhibitor

PKI contains a 20 residue inhibitor domain that binds to the free subunit ($K_d = 2$ nM). In solution, PKI, which contains 75 amino acids, is mostly disordered with the exception of two helical regions [44]. The first helical region provides high-affinity binding for PKI to the C subunit [8]. This amphipathic helix precedes the consensus site, which for PKI contains an Ala at the P site. The high-affinity binding of PKI requires ATP. While the consensus site segment of PKI binds to the active site cleft region, the high-affinity binding of PKI requires the amphipathic helix that docks to a hydrophobic pocket composed of Tyr^{235}-Pro-Pro-Phe-Phe [25]. The second helix in the C subunit lies in the region that harbors the nuclear export signal [9].

Regulatory Subunits

As seen in Fig. 4, the R subunits are modular proteins that are multifunctional and highly flexible [45]. There are two major isoforms, types I and II, with α and β subtypes in each class. RIα and RIIα are expressed in most mammalian tissues whereas the expression of the β isoforms is more tissue specific. There are also unique isoform distribution patterns: RIα is expressed predominantly in growing and transformed cells and RIIα predominates in differentiated cells [46]. The isoforms are clearly not functionally redundant. The only R subunit that is essential is RIα. Deletion of RIα is embryonically lethal and leads to cardiac defects [47]. Knockouts of other isoforms give unique phenotypes but are not lethal, and RIα tends to compensate when other R subunits are deleted [48]. For example, deletion of RIIβ gives a lean phenotype with a resistance to alcohol toxicity [47,49]. Myxomas and Carney disease are caused by premature stop codons in RIα [50].

Clearly, there is still much to be learned about the physiological importance of the PKA isoforms. RIα requires ATP and 2 Mg^{2+} ions to form a tight complex with the C subunit [51]; the high-affinity binding of ATP (60 nM) and C (0.2 nM) are synergistic. Type I holoenzyme is activated at lower levels of cAMP than type II holoenzyme [52,53]. RII binding to the C subunit is independent of MgATP; instead RII subunits are autophosphorylated at the consensus inhibitor site by the C subunit.

Molecular Architecture

All mammalian R subunits share the same organization. At the N-terminus is a dimerization/docking domain that locks the enzyme into a stable dimer. In the RI subunits the two protomers are actually linked by a disulfide bond [54].

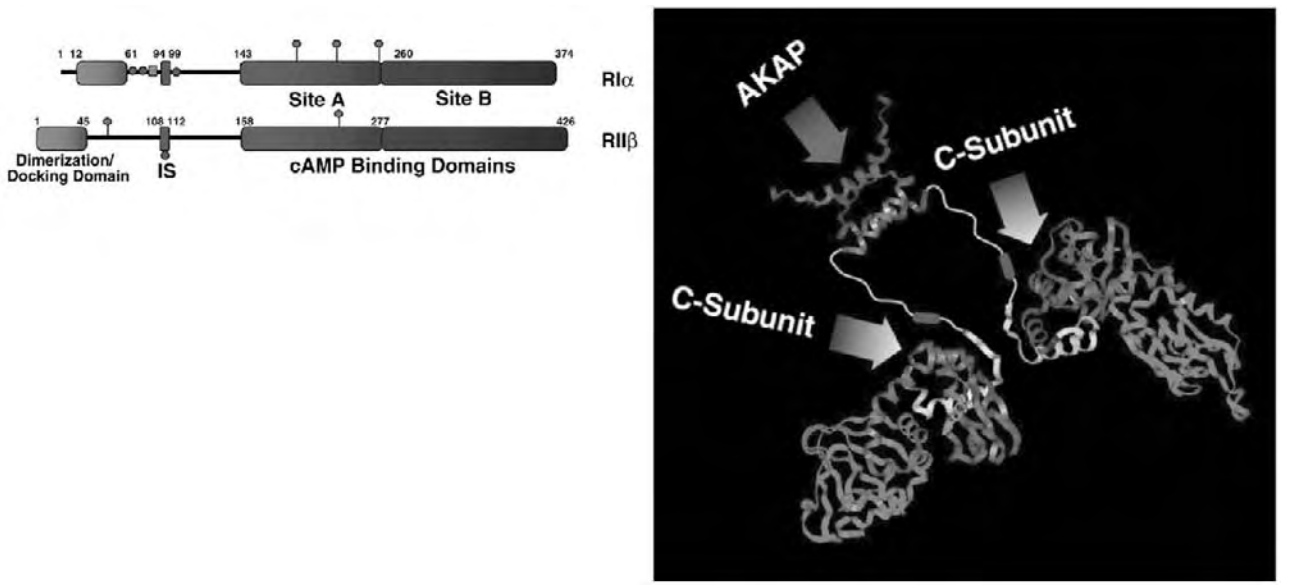


Figure 4 Domain organization of the regulatory subunits. The modular organization of RI and RII is shown on the left and a model of the subunit showing a flexible linker is on the right. Figure on the left is done by Ashton D. Taylor.

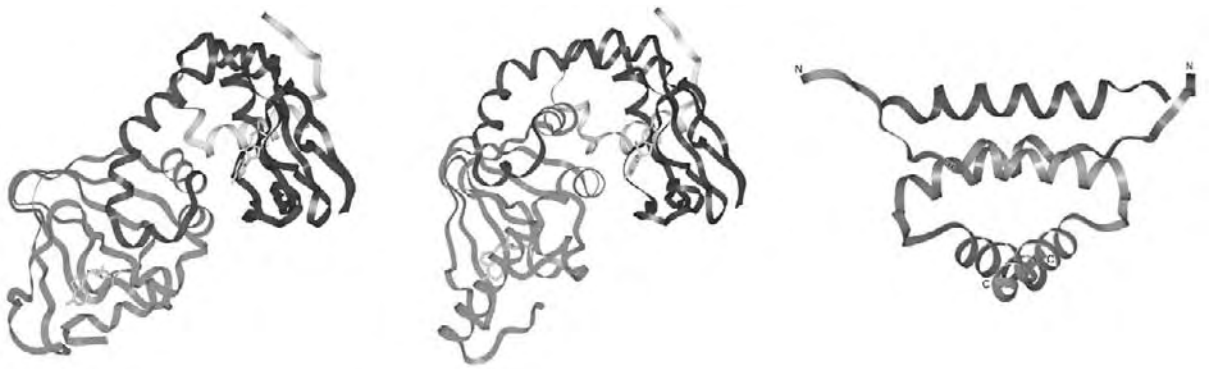


Figure 5 Structures of the regulatory subunits. On the left is the structure of the cAMP-binding domains of RIα [61] and RIIβ [62]. On the right is the dimerization/docking domain of RIα.

This is followed by a flexible and variable linker region that also contains a psuedo-substrate inhibitor site. In the absence of cAMP, this inhibitor site binds to the active site cleft of the C subunit, thus blocking access of other substrates. At the C-terminus lie two stable, tandem cAMP-binding domains. In RIα the first cAMP-binding domain also contributes to the docking of the C subunit [55]. Domain A thus shuttles between two conformations: a C bound form associated with the holoenzyme and a cAMP-bound conformation. The second cAMP-binding domain serves as a gatekeeper and regulates access of cAMP to site A [56].

cAMP is an ancient signaling molecule that has been conserved from bacteria to man. The cAMP-binding domain that serves to shield the cyclic phosphate from solvent and from phosphodiesterases is also ancient [57]. In bacteria the cAMP-binding domain is linked to a DNA-binding domain in the catabolite gene activator protein, whereas in mammals it is linked to protein kinase activation and is also found in cyclic nucleotide gated channels [58] and in a cGMP exchange factor, EPAC [59]. The highly conserved phosphate-binding cassette that surrounds the cyclic phosphate is the recognition motif for this [57]. One side of this motif interacts with cAMP while the other side interacts with the rest of the domain and is the heart of an extended network of interactions that reach to both the C-subunit docking site and the B domain [60]. Although the motif and the overall domain are highly conserved in RI and RII, the network of interactions (Fig. 5) that lead to the cooperative binding of cAMP and the release of the catalytic subunit are remarkably different in RIα and RIIβ [61,62].

The D/D domains are composed of a very stable four-helix bundle (Fig. 4), but once again there are striking differences between RI and RII [63]. The AKAP-binding surface is formed at the dimer interface, and dimerization is essential for AKAP binding. An amphipathic helix from the AKAP docks to this dimer interface [64].

Dynamics

In the absence of C subunit, the region that links the D/D domain to cAMP-binding domain A is quite mobile, as demonstrated by time-resolved fluorescence anisotropy [65]. Even in the holoenzyme the linker remains quite mobile. Small angle X-ray scattering reveals a highly asymmetric structure [66]. Hydrogen/deuterium exchange in the presence and absence of C-subunit and in the presence and absence of cAMP has provided a glimpse of the dynamic network that links cAMP binding to the release of the C-subunit [60].

Acknowledgments

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CHAPTER 200

Cyclic GMP-Dependent Protein Kinase

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Introduction

The emergence of nitric oxide (NO) signaling between cells in biological systems over the past two decades has opened up new fields of exploration in such areas as vascular biology and neuroscience. Because NO signaling involves one of the more established intracellular signaling molecules, namely cyclic GMP, there has been a renewed interest among investigators to learn more about this signaling pathway. In this review, I will highlight one major player in the cyclic GMP signaling pathway, the cyclic GMP-dependent protein kinase (PKG). Although this particular protein kinase was among the first half-dozen protein kinases discovered more than 30 years ago, its role in biological systems is still not as well-defined as some of the more recently discovered protein kinases. I will review a few of the earlier seminal findings regarding the function of this enzyme and examine more thoroughly information that has come to light recently.

Biochemical and Molecular Biology of PKG Isoforms

Up to two genes in the genomes that have been studied to date encode cyclic GMP-dependent protein kinase [1,2]. In vertebrates, the products of the genes are termed the type I and the type II cyclic GMP-dependent protein kinase (referred to hereafter as PKG-I and PKG-II, respectively). In the mammalian tissues studied, PKG-I seems to be the more widely expressed form and is present at easily measured levels in smooth muscle cells, platelets, cerebellar Purkinje cells, and cardiomyocytes [3–6]. Other cells contain measurable but lower levels of the enzyme, and these include endothelial cells, leukocytes, and many endocrine-secretory cells. Still other cells do not appear to express measurable levels of PKG-I, and these include erythrocytes and skeletal muscle myocytes. PKG-II was first discovered in intestinal epithelial cells, where it is highly expressed and localized to the particulate fractions of these cells [7,8]. The presence of PKG-II has been confirmed in brain tissue [9], juxtaglomerular cells [10], and chondrocytes through molecular and genetic studies [11].

PKG is a member of the serine/threonine kinase branch of protein kinases and is a member of the group of cyclic nucleotide-dependent protein kinases. The protein kinases in this group have as their distinguishing feature an N-terminal regulatory component (either a domain of the enzyme or a separate subunit) that binds with high affinity to either cyclic AMP or cyclic GMP. The PKG members bind cyclic GMP on their regulatory domain with an affinity approximately 20–50 times that of cyclic AMP, whereas the cyclic AMP-dependent protein kinases (PKA) bind cyclic AMP to their regulatory subunits with the greater affinity [12,13]. The catalytic domain of the PKG members reside in the C-terminal half of the protein. The PKG isoforms can be divided into four regions: (1) an extreme N-terminal domain consisting of a leucine/isoleucine zipper dimerization motif and an autoinhibitory sequence (the autoinhibitory sequence contains serine and threonine residues that undergo phosphorylation that modulates activity of the enzyme once the enzyme has been activated by the binding of cyclic GMP [12–15]);

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(2) tandem, high-affinity cyclic GMP binding sites probably created through the duplication of the DNA sequences encoding specific amino acid residues in this region [16]; (3) a catalytic domain having homology with all members of the entire protein kinase family; and (4) an extreme C-terminal region with unknown function.

PKG-I is expressed in cells as two isoforms that are derived by the alternate mRNA splicing for the first two exons encoding the N-terminal region of the enzyme [3–6]. The smaller of the two forms (PKG-Iα) has the first exon expressed as the N-terminal region and is the form most abundant in platelets, most vascular smooth muscle cells, and cerebellum. The larger of the two forms (PKG-Iβ) expresses the second exon in the protein and is abundant in vascular and nonvascular smooth muscle cells. However, there appears to be much variability in the expression of these two isoforms even in the same cell type (e.g., aortic smooth muscle cells). Furthermore, mRNA levels usually appear more abundant for the Iβ isoform in cells where the level of Iα protein may be more than twice that of the Iβ protein [17–19]. These results imply that the Iα mRNA may be more unstable than the Iβ, but there are few studies to date published on regulation of the expression of the mRNAs encoding these enzymes.

The concentration of cGMP necessary for half-maximal activation of the purified PKG-Iα isoform has been measured at approximately 0.1 μM, while the concentration of cGMP necessary for half-maximal activation of the purified Iβ isoform is 1 to 2 μM. This is despite both isoforms' having identical sequences that encode the two tandem cGMP binding sites. The reason given for this functional difference is that the N-terminal domain encoding the Iβ isoform contains an autoinhibitory domain that is either "more efficient" at inhibiting the holoenzyme or has a higher affinity for inhibiting the catalytic domain of the holoenzyme [20]. Predictably, therefore, PKG-Iα is activated at lower cGMP levels in the cell than PKG-Iβ. Corbin and colleagues have in fact shown that PKG-I exists as a partially-activated PKG especially after autophosphorylation [13,15]. The two cGMP-binding sites have different affinities for cGMP, and it has been shown that occupation of the high-affinity cGMP-binding site confers partial activation to PKG-I [13–16,20]. Upon elevation of cGMP in the cell, occupation of the second, lower-affinity binding site leads to further activation of the enzyme and autophosphorylation. The role of autophosphorylation of PKG-I has been investigated. Early studies suggested that autophosphorylation may prevent reassociation of regulatory and catalytic domains of PKG, similar to the role of autophosphorylation of the PKA regulatory subunit II. However, it is now clear that autophosphorylation may serve a more complex role for PKG and may somehow stabilize the activated enzyme in the active state even after the dissociation of cGMP. This mechanism has been shown to be operational for the calmodulin-activated protein kinase, CAM Kinase II, where the kinase remains active even after dissociation of the active calcium-calmodulin complex [21]. The molecular regulation of CAM kinase II in this fashion has been suggested to be the basis for synaptic facilitation and of memory and learning in the nervous system. For PKG, it is possible that autophosphorylation is a mechanism to maintain PKG activity and relaxation of the smooth muscle cell even after elevated NO and cGMP have dissipated and returned to baseline levels.

There may be different functional roles for PKG-Iα and Iβ, at least in smooth muscle cells. There has been speculation that PKG-Iβ, with its lower "affinity" for cGMP, may serve to buffer cGMP concentrations in the cell. This seems unlikely given the importance of PKG-Iβ in intracellular calcium regulation (see below). An attractive hypothesis for the functionally different roles of PKG-I isoforms is based on the fact that the sequence difference between the two enzymes is only at the N-terminus. In PKA, the N-terminus of the regulatory subunit determines what targeting proteins PKA binds to in the cell, the AKAPs (A kinase anchoring proteins) [22]. Hence, it has been suggested that the different N-terminal domains of PKG-I allow the kinases to bind to different targeting proteins in the cell. Experimental evidence for this hypothesis has recently been provided and demonstrates that PKG-Iα appears to localize to the perinuclear regions of smooth muscle cells [23,24] whereas PKG-Iβ is more widely distributed in smooth muscle cells [25]. Functionally, the different subcellular distribution could affect which isoform of PKG regulates intracellular calcium mobilization, for example. The PKG-Iβ isoform, but not PKG-Iα, has been shown to bind to a protein termed IRAG [for inositol (1,4,5) P_3-receptor-associated cGKI substrate] [26]. The function of the IRAG protein is to compartmentalize PKG-Iβ with the inositol 1,4,5-trisphosphate (IP_3) receptor in the endoplasmic reticulum (ER) compartment of the smooth muscle cell. PKG has been shown to catalyze the phosphorylation of the IP_3 receptor, and its localization with this substrate is necessary for phosphorylation [27,28]. The role of IP_3 receptor phosphorylation has not been unequivocally defined although there is speculation that PKG-dependent phosphorylation regulates calcium release from the ER [26,28].

Binding proteins specific for PKG-Iα have also been described, and these include a male germ cell 42 kDa protein [29] and the myosin light chain phosphatase binding subunit (MBS) [30]. Of these two potential targeting proteins, the binding to MBS has been most extensively characterized. Surks *et al.* [30] have shown that PKG-Iα, but not PKG-Iβ or Type II PKG, binds via the leucine zipper domain to MBS. As a consequence of PKG binding, the MBS is phosphorylated, resulting in the activation of the phosphatase. In contrast to PKG, both Rho-kinase A and protein kinase C (PKC)-catalyzed phosphorylation of MBS lead to the inhibition of the phosphatase activity [31,32]. Therefore, multisite phosphorylation mechanisms regulate the phosphorylation of myosin light chain and hence contractility in smooth muscle cells. The concepts of PKG-dependent regulation of smooth muscle contractility will be addressed again below. The purpose of this discussion here is to illustrate the importance of the N-terminal domains of PKG-I in targeting the kinase to subcellular compartments.

Other studies have suggested that various PKG isoforms bind to cytoskeletal compartments. PKG-binding proteins

such as myosin [33], vimentin [34], and actin-binding proteins [24,25] have been reported, but except for VASP the physiologic significance is unknown. Recently, fluorescent indicators have been used to determine the localization of PKG in cells. In HEK-293 and A549 epithelial cells, PKG was found to be localized with the best-characterized PKG substrate to date, VASP [24]. VASP is an actin-binding protein that is widely expressed and preferentially localized to focal adhesions of cells [35,36]. The precise anchoring protein for PKG in focal adhesions is not known, however.

PKG-II, as stated earlier, has a different distribution compared with PKG-I. PKG-II was first identified in intestinal epithelial cells as a phosphorylated protein. Since then, PKG-II has been cloned from brain and epithelial tissues. PKG-II clearly is the key kinase that regulates intestinal chloride transport due to phosphorylation and activation of the CFTR [37–39]. PKG-II has also been shown to inhibit renin release from the juxtaglomerular cells in the kidney [10,40]. PKG-II exists as a homodimer, is N-terminally myristolated and anchored in the cell membrane, and has tandem cGMP-binding sites like the PKG-I isoforms, although differences in activation mechanisms exist [41,42]. The sequence homology between PKG-I and PKG-II is approximately 50 percent overall, but the PKG-II isoform possesses unique cGMP-binding properties and activation by cGMP compared with PKG-I. PKG-II also has a very low affinity for cAMP and therefore does not appear to be cross-activated by cAMP in the cell. These findings suggest that drugs may be devised that could selectively target PKG-II and PKG-I in the cell, thus providing better insight into the roles of these two isoforms of PKG in cell function.

Physiologic Roles of PKG

Both PKG-I and PKG-II have been genetically deleted in mice and the phenotypes of the knockout mice studied. PKG-I deficient mice demonstrate a loss of NO-dependent relaxation of smooth muscle and acquire hypertension at approximately four weeks of age [43]. The animals also demonstrate abnormalities in platelet function [44]. These phenotypic properties are predictable based on the known roles for cGMP in relaxing vascular and nonvascular smooth muscle, inhibiting platelet adhesion and activation, and relaxing cavernosal smooth muscle in the penis. The animals succumb at an early age apparently due to digestive and colorectal dysfunction. Recently, Wegener *et al.* [45], using PKG-I conditional knockout animals, demonstrated a clear role for PKG in mediating the negative inotropic actions of NO and cGMP in mouse heart . The role of NO and cGMP in regulating cardiac contractility has been the subject of much controversy over the past two decades, in part because the antiadrenergic effects of muscarinic agonists have been difficult to distinguish from those related directly to cardiomyocyte cGMP, such as the inhibition of voltage-gated Ca^{2+} channels. This study provides a rather conclusive role for PKG in directly mediating the actions of cardiomyocyte cGMP. PKG-II deficient mice show predictable changes in salt and water absorption from intestinal epithelium but also produced an unexpected phenotype of dwarfism. Lack of PKG-II causes the epiphyses of the long bones to close and harden prematurely, suggesting that PKG-II is required for chondrocyte proliferation and matrix production.

The classic roles of PKG in cell function were established in smooth muscle and platelets. Cyclic GMP was identified as the intracellular mediator of nitrovasodilator-drug dependent smooth muscle relaxation, and then shown to mediate epidermal growth factor receptor (EDRF) (that is, NO)-dependent relaxation. Many studies in the 1980s and 1990s established that PKG mediated relaxation by diverse mechanisms. Many of the details of the mechanisms responsible for smooth muscle relaxation have been reviewed elsewhere and will not be repeated here [5,6,46]. However, what should be emphasized is that smooth muscle cell relaxation can be accomplished by a number of very different mechanisms. For example, active contraction of the VSMC is achieved and regulated by two pathways: increases in cytosolic free calcium with the activation of myosin light chain kinase (MLCK), and activation of the rho-rho kinase pathway preventing myosin light chain dephosphorylation by inhibition of myosin light chain phosphatase [47,48]. The latter pathway constitutes the mechanism of calcium-sensitization of smooth muscle [49]. PKG has been shown to catalyze the phosphorylation of proteins that regulate both pathways. For example, PKG-dependent phosphorylation of protein components of calcium-activated potassium channels (K_{Ca} channels) leads to the activation of this channel and hyperpolarization of the cell [50–54]. Hyperpolarization inhibits calcium entry into the cell and hence allows less MLCK activation. PKG also catalyzes the phosphorylation of components of the calcium-sequestering mechanism in smooth muscle cells such as phospholamban [23]. Phospholamban phosphorylation leads to its dissociation from and the activation of the endoplasmic reticulum (ER) Ca-ATPase pump. Not only does this mechanism decrease cytosolic free calcium levels, it loads up the ER (or sarcoplasmic reticulum, the SR) with calcium. More calcium is then available for release near the plasma membrane compartment, where it activates K_{Ca} channels [55]. The increases in the number of "calcium sparks" by PKG is a novel mechanism for regulation of cytosolic calcium levels. Furthermore, the PKG-dependent phosphorylation of the SR calcium release channel, the IP_3 receptor, could contribute to calcium sparks and relaxation by either increasing calcium release toward the plasma membrane or by inhibiting calcium release, thus allowing greater filling of the SR with calcium for release through the ryanodine receptor [27,56,58]. What seems to be the case is that in different types of smooth muscle cells, the regulation of intracellular calcium levels relies on any one of these diverse mechanisms more than the others. Hence, cGMP-dependent regulation of K_{Ca} channels, for instance, might be more important in the relaxation of one type of smooth muscle compared to another. Nonetheless, each calcium-lowering pathway has apparently evolved the

capacity to be regulated by cGMP/PKG-dependent protein phosphorylation. The occurrence of redundant mechanisms controlling cytosolic calcium in smooth muscle apparently provides evolutionary or selective advantage to the cell and organism to prevent excessive calcium accumulation and contractile activity in the cell.

The role of PKG in regulating smooth muscle myosin light chain phosphatase activity followed earlier findings demonstrating that cGMP produced calcium desensitization in smooth muscle cells [49,59,63]. The detailed mechanism by which PKG activates the phosphatase is still uncertain, but it is clear that phosphorylation of the MBS due to PKG localization and phosphorylation of the protein is necessary [30]. The reports of PKG-dependent activation of myosin light chain phosphatase, which is a Type I serine/threonine protein phosphatase, follow other reports in which cGMP and PKG have been implicated in protein phosphatase regulation in the nervous system. In Purkinje cells, a protein substrate for PKG, originally discovered in Paul Greengard's laboratory and termed the G-substrate, is an inhibitor of protein phosphatase 1 [64] and protein phosphatase 2A [65] when phosphorylated. DARPP-32, an inhibitor for protein phosphatase 1 when phosphorylated by PKA, is also phosphorylated by the NO/cGMP/PKG pathway [66], thereby suggesting that both cAMP and cGMP signaling pathways regulate DARPP-32 activity.

In addition to serine/threonine protein phosphatase regulation, protein tyrosine phosphatases have also been reported to be regulated by PKG. In smooth muscle, PTP-1, a soluble form of protein tyrosine phosphatase, is both phosphorylated and activated by NO/cGMP/PKG [67]. The role of the NO/cGMP signaling pathway in smooth muscle cell proliferation has been extensively studied, but specific mechanisms by which PKG regulates proliferation are unknown. Perhaps the regulation of protein tyrosine phosphorylation by phosphatases contributes to the antiproliferative actions of NO and cGMP.

A final mechanism for cyclic nucleotide-dependent smooth muscle relaxation is the regulation of thin filaments and particularly the phosphorylation of small heat shock protein HSP-20 and HSP-27 [68–70]. HSP-20 is a actin-binding protein in smooth muscle whose phosphorylation is catalyzed in the intact tissue by PKA and PKG. Tissues deficient in HSP-20 are less sensitive to cyclic nucleotide-dependent relaxation, thus suggesting an important role for HSP-20 in active relaxation [71,72]. It is interesting that PKG not only catalyzes the phosphorylation of HSP-20 but also induces its expression in VSMC [73].

PKG has been implicated in the regulation of MAP kinase pathways, gene expression and transcriptional activity, and in the regulation of VSMC phenotypic modulation. Although these findings have been reviewed recently and will not be discussed here [5,46], one newer role for cGMP/PKG signaling is in tumor cell apoptosis. It has been known that the nonsteroidal, antiinflammatory sulindac derivatives have potent anticancer activity [74,75]. One derivative, exisulind, induces apoptosis in cells derived from a number of different tumors, and in clinical studies exisulind prevents colorectal polyp formation in patients with familial adenomatous polyposis. Exisulind exerts its actions on tumor cells by inhibiting the type V cGMP phosphodiesterase (PDE-V) [76,77]. PDE-V, which is also the target for sildenafil (trade name, Viagra), is a specific cGMP phosphodiesterase that when phosphorylated by PKG hydrolyzes cGMP at a high rate. Exisulind produces sustained inhibition of PDE-V in tumor cells, leading to increases in cGMP and activation of PKG. PKG promotes the phosphorylation of β-catenin either through direct catalysis of phosphorylation of the protein or through activation of other kinase pathways that lead to β-catenin phosphorylation. When phosphorylated in response to PKG activation, β-catenin is targeted for ubiquination in the cytoplasm of the tumor cell. β-catenin combines with T-cell factor (TCF) to form a transcriptional complex that activates gene expression in tumor cells, resulting in resistance to apoptosis. By virtue of its phosphorylation by PKG activation, anti-apoptotic pathways are apparently turned off while pro-apoptotic pathways are turned on (e.g., caspase genes), leading to tumor cell death [78,79]. The pro-apoptotic effects of PKG may be dependent on activation of the c-Jun NH_2-terminal kinase (JNK) pathway [78–80]. Clearly, the roles for PKG in regulating gene expression, cell growth and differentiation, and apoptosis are only beginning to be uncovered.

Concluding Remarks

The family of protein kinases is huge, with some estimates of the number of gene products expressed in cells that belong to this family at around 5 percent. And protein phosphorylation, being the important and widespread regulator of cell function that it is, is a major area of interest in every biological process. As already mentioned, PKG was perhaps the fourth protein kinase discovered, following phosphorylase kinase, casein kinase, and PKA. Yet until about 15 years ago, understanding and solving the roles of PKG in biological function lagged the understanding of most other kinases' roles because upstream pathways leading to PKG activation were not well-defined. This changed rather dramatically with the uncovering of the biological role of NO. Now it seems that PKG has many important roles in various biological processes—even more than could be imagined by those investigators that began studying PKG from the time of its discovery. As discussed here, PKG plays a central role in smooth muscle cell contraction (e.g., blood pressure regulation, erectile function, gastrointestinal motility), smooth muscle cell gene expression in diseases such as atherosclerosis, salt and water absorbtion, skeletal growth, cardiac contractility, memory and learning, and tumor apoptosis. In the world of fruit flies and honey bees, whether the insects are a "stay-at-home couch potatoes" or "active food foraging providers" seems to depend on whether or not PKG is expressed (the food foragers express PKG and the couch potatoes don't) [81,82]. While I suspect that there is not any message for humans in these findings, one predication is that there will continue to

be active pursuit of therapeutic agents that interact with the PKG pathway. With PKG involvement in so many different biological processes, and with losses in its expression or activity correlated with human diseases, new agents directed to enhance PKG expression or activity may find their way into future therapies.

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CHAPTER 201

Inhibitors of Cyclic Nucleotide-Dependent Protein Kinases

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Introduction

Inhibitors of cyclic nucleotide-dependent protein kinases have served as valuable tools in identifying the pivotal roles of cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) in cellular biology by allowing the elucidation of basic molecular mechanisms of kinase structure and function and the dissection of the specific roles for each kinase in intracellular signaling. The remarkably similar multidomain structures of PKA and PKG simultaneously provide an opportunity and pose a challenge for the design of potent and selective inhibitors. A set of inhibitors now available target one of three distinct regions found in all subforms of cyclic nucleotide-dependent protein kinases: the cyclic nucleotide binding sites, the ATP-binding domains, and the peptide/substrate binding regions. This review will present the apparent experimental advantages and pitfalls of each inhibitor class and will provide a guide for identifying the inhibitor best suited for a given experiment, whether a reconstituted enzyme assay or intact cell or tissue preparation is involved.

The cyclic nucleotide-dependent protein kinases PKA and PKG, primary targets for the second messengers cAMP and cGMP, respectively, have served as rosetta stones in our understanding of a vast number of intracellular signaling mechanisms ranging from smooth muscle cell relaxation to neuronal synaptic plasticity (for reviews see [1–7]). Therefore, the search for potent inhibitors of these kinases has been extensively investigated ever since their discoveries. However, the structural similarities of PKA and PKG have posed a formidable obstacle in the design of selective inhibitors that specifically target cyclic nucleotide-dependent protein kinases and show little inhibitory potency to other more distant Ser/Thr-kinase relatives.

The domain structures of PKA and PKG dictate key target sites for putative inhibitors. Figure 1 compares the domain structures of PKA and PKG and defines three distinct classes of inhibitors and their various sites of actions. The regulatory components of cyclic nucleotide-dependent protein kinases each harbors two tandem cyclic nucleotide binding sites that allow allosteric and cooperative control of kinase activity (for reviews see [1,6,7]). A particular class of cAMP/cGMP derivatives, (R_P)-phosphorothioates, are the only known inhibitors that bind to the cyclic nucleotide binding sites [8–10]. Although their mode of action is still not completely understood, studies have indicated that the binding of these derivatives fails to induce the conformational changes essential for releasing catalytic activity [11,12]. The catalytic components of cyclic nucleotide-dependent protein kinases contain two target sites for inhibitors: the ATP-binding site and the substrate-binding site. Compounds mimicking ATP represent a diverse class of inhibitors, as has been known for

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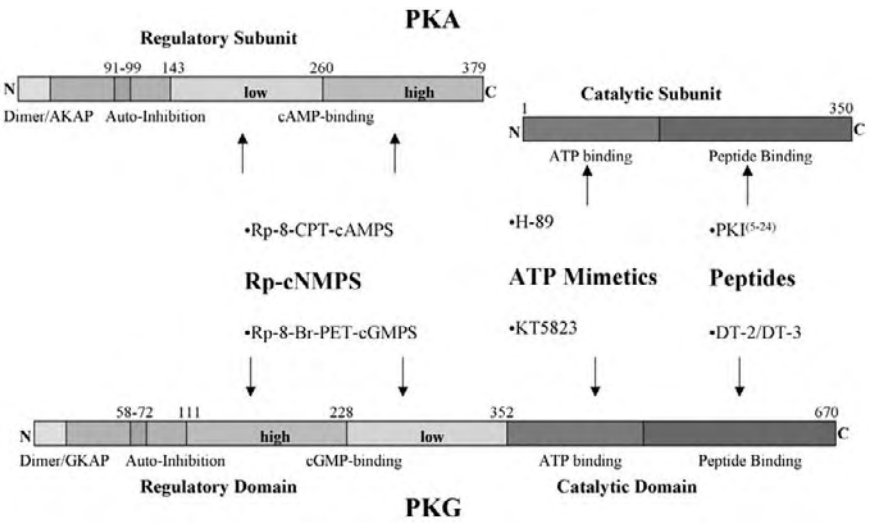


Figure 1 Domain structures of PKA and PKG with target sites for inhibitors and prominent inhibitor examples. Rp-cNMPS denotes (R_P)-diastereomers of cAMP/cGMP phosphorothioate derivatives.

all other major families of protein kinases [13,14]. In contrast, peptide inhibitors designed to block the substrate-binding site of PKA have been relatively straightforward [15–17]. PKA contains a well-defined substrate consensus sequence [18] that serves as an ideal template for peptide inhibitor design [19]. However, selective PKG peptide inhibitors have long remained elusive, partly because the sequence requirements for PKG inhibition do not follow a classic consensus sequence and the kinase appears to nonspecifically favor positively charged amino acids [18,20–26]. Only recently has a new class of potent and cell membrane permeable PKG peptide inhibitors based on combinatorial peptide libraries emerged [27–29].

Cyclic Nucleotide Binding Site-Targeted Inhibitors

The cyclic-nucleotide binding sites of PKA and PKG show remarkable sequence similarity with respect to the recognition motif of the nucleotide phosphodiester (FGE…RAA and FGE…RTA for all PKA and PKG isoforms, respectively). The crystal structures of PKA RIIβ [30] and RIα [31] revealed the architecture of the cyclic nucleotide binding pocket with an invariant arginine situated at its base. It is thought that chelation of the phosphate moiety by the invariant arginine is the first step in cAMP binding. The discovery that sulfur substitution of the axial exocyclic phosphate oxygen (Rp-) of cyclic nucleotides resulted in inhibitors of PKA and PKG, while cyclic nucleotide analogs carrying the equivalent apical sulfur substitution (Sp-) act as kinase agonists [8,32–35], prompted the synthesis and subsequent analysis of an entirely new class of cyclic nucleotide analogs [9,10,36–40] in an effort to identify selective and isozyme-specific inhibitors (for a comprehensive overview and a complete list of references visit *www.biolog.de*). Tables I and II give examples of PKA- and PKG-specific Rp-cyclic nucleotide phosphorothioates (Rp-cNMPS). These studies revealed that the competitive binding of Rp-cAMPS to both cAMP-binding sites in the PKA holoenzyme is thought to prevent dissociation of the regulatory subunits [11,12]. Likewise, it is believed that binding of Rp-cGMPS and analogs to PKG are unable to induce the conformational change needed to expose the enzyme's catalytic cleft.

Recent studies have demonstrated that Rp-cAMPS analogs can function as partial agonists dependent on the presence of MgATP and enzyme concentration [11,41]. These findings raise questions concerning the usefulness of inhibition constants when dealing with Rp-cAMPS analogs. However, this phenomenon is not observed for PKG partly because the holoenzyme complex does not dissociate into regulatory and catalytic subunits. Another important finding is that the sulfur substitution in Rp-cNMPS increases the relative lipophilicity and hence cell-membrane permeability compared to their cNMP counterparts [42], (see also: *www.biolog.de/logkw.html*). In addition, Rp-cNMPS exhibits complete resistance to phosphodiesterases [43]. These properties have markedly contributed to the diverse applications that cyclic nucleotide binding site-targeted inhibitors have enjoyed in intracellular signaling research [10]. It should be noted that Rp-cGMPS derivatives [37,38], in particular Rp-8-Br-PET-cGMPS, as a PKG inhibitor with high selectivity, low toxicity, and superior membrane permeability, has gained supremacy in its class [39].

ATP Binding Site-Targeted Inhibitors

Synthetic protein kinase inhibitors that are competitive with ATP and specific for PKA and/or PKG represent a structurally diverse group of small ligand compounds [44,45]. Polycyclic aromatics, such as Isoquinolinesulfonyl and Naphtalenesulfonyl compounds ("H-series"), and naturally occurring molecules, such as staurosporine analoga ("K-series"), have served primarily as valuable inhibitors of

Table I Potencies of Selected Cyclic Nucleotide-Dependent Protein Kinase Inhibitors

PKA inhibitors	K_i/IC_{50}(μM)	Isoform selectivity	Comments	Refs.	PKG inhibitors	K_i/IC_{50}(μM)	Isoform selectivity	Comments	Refs.
Kempitide		PKA Iα	pep, PKA	60	$H2B^{(29–35)}$	86, PKG Iα	PKG Iα, Iβ	pep, non	24
Rp-cAMPS	12.5	PKA I	cyc, PKA, PDE	33	Rp-cGMPS	20, PKG Iα	PKG Iα, Iβ	cyc, PKG, PDE	38
H-8	1.2		ATP, lip	47	WW21	7.5, PKG Iα	PKG Iα	pep, PKG	28
Rp-8-Br-cAMPS		PKA I	cyc, lip, PDE	9	HA1004	1.4, PKG Iα	–	ATP, lip, AGC, CaMK CMGC	48
Rp-8-CPT-cAMPS		PKA II	cyc, lip, PDE	9	Rp-8-Br-cGMPS	4.0, PKG Iα	PKG Iα, Iβ	cyc, lip, PKG, PDE	81
Rp-8-PIP-cAMPS		PKA II, site B	cyc, lip, PDE	40	Rp-8-CPT-cGMPS	0.5, PKG Iα	PKG II	cyc, lip, PKG, PDE	37
4-Cyano-3-methyl-isoquinoline	0.03, PKA	–	ATP, AGC	50	H-8	0.5, PKG Iα	–	ATP, lip, PKG	47
Balanol	0.004, PKA	PKA I	ATP, PKA	54, 55	KT5823	0.234, PKG Iα	–	ATP, lip, PKG	44, 46
Staurosporine	0.008, PKA	–	ATP, non	83–85	K-252b	0.1	–	ATP, lip, AGC, CaMK	46
H-89	0.048, PKA 0.48, PKG	–	ATP, lip	44, 46	Rp-8-Br-PET-cGMPS	0.035, PKG Iα 0.03, PKG Iβ	PKG Iα	cyc, lip, PKG, PDE	39
KT5720	0.06, PKA	–	ATP, lip		DT-3	0.025, PKG Iβ	PKG Iα	pep, MTS, PKG	29
K-252a	0.018	–	ATP, lip	46	K-252a	0.02	–	ATP, lip, AGC, CaMK	46
$PKI^{(5–24)}$	0.002, PKA 150, PKG	PKA Iα	pep, PKA	62	DT-2	0.012, PKG Iα	PKG Iα	pep, MTS, PKG	29

The following abbreviations are used: AGC specificity for AGC, subfamily protein kinases; ATP, ATP-binding site inhibitor; CaMK, specificity for CaMK subfamily protein kinases; CMGC, specificity for CMGC subfamily protein kinases; cyc, cyclic nucleotide binding site inhibitor; lip, lipophilic, cell permeable; MTS, membrane translocation signal; non, nonspecific; PDE, resistant against PDE hydrolysis; pep, peptide binding site inhibitor; PKA, high selectivity for PKA; PKC, high selectivity for PKC; PKG, high selectivity for PKG.

Table II Representative Commercially Available Inhibitors with Increasing PKA/PKG Selectivity

	K_i(μM)	PKA/PKG selectivity	References
Cyclic Nucleotide Inhibitors			
Rp-cAMPS	7.9 PKA I/II	1.5×10^{-1}	33, 35, 38, 82
	52, PKG Iα		
Rp-cGMPS	20, PKA II	1×10^{0}	37, 38
	20, PKG Iα		
Rp-8-CPT-cGMPS	8.3, PKA II	1.7×10^{1}	37
	0.5, PKG Iα		
Rp-8-Cl-cGMPS	100, PKA II	6.7×10^{1}	38
	1.5, PKG		
Rp-8-Br-PET-cGMPS	11, PKA II	3.1×10^{2}	39
	0.035, PKG Iα		
	0.030, PKG Iβ	3.7×10^{2}	
ATP Analogs			
KT5720	0.06, PKA I	3.0×10^{-2}	46
	>2, PKG Iα		
H89	0.048, PKA I	1.0×10^{-1}	45, 49
	0.48, PKG Iα		
H8	1.2, PKA I	2.5×10^{1}	45, 47
	0.48, PKG Iα		
KT5823	>10, PKA I	$>4.2\times10^{1}$	46, 44
	0.234, PKG Iα		
Peptide Inhibitors			
$PKI^{(5–24)}$	0.002, PKA	1.8×10^{-5}	16, 20
TTYADFIASGRTGRRNAIHD	111, PKG		
$PKI^{(14–22)}$	0.073, PKA	1.5×10^{-3}	17, 28
GRTGRRNAI	47, PKG		
Ala-Kemptide	376, PKA	4.7×10^{-1}	24
LRRAALG	800, PKG		
$[A^{32}]$-$H2B^{(29–35)}$	550, PKA	6.4×10^{0}	20
RKRARKE	86, PKG		
WW21	750, PKA	1.0×10^{2}	28
TQAKRKKALAMA	7.5, PKG		
DT-2	16.5, PKA	1.32×10^{3}	29, 80
YGRKKRRQRRRPPLRKKKKKH	0.012, PKG		
DT-3	493, PKA	1.97×10^{4}	29, 80
RQIKIWFQNRRMKWKKLRKKKKKH	0.025, PKG		

AGC-type protein kinases, notably PKC [44–50]. In fact, the inhibitory potency against PKC is a defining property of most ATP-site inhibitors of the above series. However, a subset of compounds, including the H-series H89 and KT5720 and the K-series H8 and KT5823, are moderately specific inhibitors for PKA and PKG, respectively (see Tables I and II). The relatively simple chemical modifications of isoquinoline-derivatives in particular produced a wealth of selective protein kinase inhibitors with potential for clinical applications [45,50]. In addition, the cell-membrane permeability of these compounds further amplified their versatility in dissecting signaling pathways involving protein kinase signaling. However, concerns regarding toxicity and reports of problems using *in vivo* conditions, specifically with KT5823 as specific PKG inhibitors, have questioned their usefulness in intact cell preparations [51–53] (for a detailed discussion see: *www.biolog.de/ti1003.html*).

Recently it was observed that the natural product balanol inhibits protein kinases of the AGC-subfamily with high potency [54,55]. The crystal structure of balanol in complex with the catalytic subunit of PKA [56] confirmed a structural peculiarity of most ATP-site targeted inhibitors: the molecules

satisfy essential interaction within the ATP-binding site but utilize unique interactions with the enzyme, thus gaining selectivity and specificity. It has been shown that analogs of balanol display variability in protein kinase inhibition, and the structural determinants of their protein kinase selectivity can now be elucidated with computational methods [57–59].

Peptide Binding Site-targeted Inhibitors

The observation that relatively short peptides corresponding to the regulatory subunit's autophosphorylation site were effective substrates for PKA [60] and the discovery of protein kinase inhibitor (PKI) [15,61] prompted a comprehensive search of PKA inhibitory peptides [15–17,19,62–65] and presented a prime example for the concept of "consensus sequences" [18,66,67]. However, full appreciation of the intricate structural web existing between kinase and inhibitor occurred only after the crystal structure of the catalytic subunit of the PKA:PKI adduct was solved [68]. Peptides derived from PKI isoforms α and β [69–71], namely PKI^{5-24} and PKI^{10-22}, are still the most potent and, more important, the most selective PKA inhibitors known today (Tables I and II). However, their use in intact cell studies is limited, for example in patch-clamp techniques, due to their inability to cross the plasma membrane. Unfortunately, fusion peptides of PKI^{15-22} with membrane translocation signal (MTS) peptides derived from Antennapedia-homeo domain or *HIV-1* tat [28,72] showed a profound loss in PKA selectivity (W. Dostmann, unpublished results).

Attempts to identify PKG-selective inhibitor peptides based on the auto-inhibitory domain of the enzyme or *in vivo* substrates have been tedious at best, due to the lack of a well-defined consensus sequence [20–24,73,74]. Only a relative preference for basic residues surrounding the phosphate acceptor site has been established [18,21,22,24–26]. Various synthetic peptides have been used with limited success to analyze and optimize the sequence requirements for PKG substrates and inhibitors [75–79]. Recently, the identification of selective inhibitors of PKG by a novel peptide library screen specifically designed to select for tight binding peptides was reported [27–29]. Cellular internalization of the peptides was accomplished by N-terminal fusion to the membrane translocation sequences from either the HIV-1 Tat protein [47–59], DT-2 or the *Drosophila* Antennapedia homeo-domain [43–58], DT-3 [29,80]. A surprising finding is that these fusion peptides result in an extraordinary synergism with respect to PKG inhibition (Tables I and II). It was shown that DT-2 and DT-3 effectively inhibit NO-induced vasodilation, further emphasizing the central role for PKG in the modulation of vascular contractility [80]. These results suggest that the cell membrane permeability of DT-2 and DT-3, combined with enormous PKG selectivity, will significantly advance our experimental ability to dissect PKG-mediated intracellular pathways from PKA and other kinases.

Conclusions

R_P-phosphorotioate derivatives of cAMP and cGMP competitively inhibit cyclic nucleotide-dependent protein kinases by 'freezing' the enzymes in their inactive holoenzyme states. A large pool of derivatives, moderate selectivity and cell membrane permeability are regarded as their advantages as tools in intact cell studies. However, partial antagonism and limited potencies restrict their versatility. ATP-analogs are a highly resourceful group of protein kinase inhibitors. Cell membrane permeability and limited selectivity highlight their advantages and disadvantages, respectively. Peptide-derived inhibitors present the most potent and selective group of PKA and PKG blockers. Low cell membrane permeability remains their main obstacle in cellular research. Recently, a subset of PKG-selective peptide inhibitors employing MTS sequences as a means of membrane translocation has overcome this problem.

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CHAPTER 202

Peptide Substrates of Cyclic Nucleotide-Dependent Protein Kinases

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Introduction

The cyclic nucleotide-dependent protein kinases cAMP and cGMP-dependent protein kinases (PKA and PKG) are closely related enzymes, with approximately 50 percent sequence identity. These two kinases have similar substrate specificities; in particular, both show a strong preference for Arg residues at positions (−3) and (−2) of peptide substrates. The crystal structure of PKA revealed multiple contacts between these substrate Arg side chains and the enzyme. There are differences in specificity between the two enzymes particularly at the (+1) site, with PKA having a higher preference for hydrophobic residues. However, hydrophobic residues at the (+1) site are only found in approximately 40 percent of PKA substrates, possibly because the phosphorylation sites must be hydrophilic enough to be located on the surface of the protein. Although PKG is more stringent in its requirement for residues at the (−1), (+2) and (+3) positions than PKA, there is substantial overlap in specificity, and the two kinases are expected to share some substrates, as is the case for the cystic fibrosis transmembrane conductance regulator.

The cAMP and cGMP-dependent protein kinases (PKA and PKG, respectively) belong to subgroup 1 of the AGC group of Ser/Thr protein kinases, the group that also contains protein kinase C (PKC) [1]. Early studies on PKA specificity over 25 years ago established that residues in the local phosphorylation site sequence were the primary specificity determinants (reviewed in [2]). Synthetic peptides corresponding to these sites were effective substrates. They have been used extensively to study the substrate specificity of protein kinases and have proved effective to measure PKA and PKG [3] activities in many systems. More recently synthetic peptides have been exploited as substrates in high-throughput screening for inhibitors and activators of protein kinases, including PKG [4]. This chapter focuses primarily on new developments in our understanding of the similarities and differences in the recognition of peptide substrates by PKA and PKG and the identification of important residues in enzyme substrate recognition.

Peptide Substrate Recognition

The catalytic domains [1] of PKA and PKG1 share 48 percent sequence identity; the strongest similarity is found in the central part of the catalytic domain, containing the majority of the substrate-binding residues as well as those involved in catalysis. We can therefore expect that the PKG catalytic domain will have a structure similar to the crystal structure of PKA [5–7]. The PKA crystal structures show the binding of a peptide inhibitor or substrate to PKA [5–9]. By examining these and other structures, we identified a constellation of 20 PKA residues (called acceptor loci)

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Table I Acceptor Loci (Residues) in the Substrate-Binding Cleft of PKA, PKG1α, and PKG1β

Acceptor loci	PKA (Swiss-Prot # P17612)	PKG1α (Swiss-Prot # Q13976)	PKG1β (Swiss-Prot # P14619)	Substrate side chain subsite(s)
1	Gly 52	Gly 368	Gly 384	(−1)
2	**Ser 53**[a]	**Gly 369**	**Gly 385**	(−1)/(+2)
3	Phe 54	Phe 370	Phe 386	(+2)
4	**Leu 82**	**Thr 399**	**Thr 415**	(+2)
5	Gln 84	Gln 401	Gln 417	(+2)
6	Glu 127	Glu 444	Glu 460	(−3)
7	**Phe 129**	**Trp 446**	**Trp 462**	(−3)
8	**Ser 130**	**Thr 447**	**Thr 463**	(−3)
9	Asp 166, catalytic Asp	Asp 483, catalytic Asp	Asp 499, catalytic Asp	(0)
10	Glu 170	Glu 487	Glu 503	(−3)/(−2)
11	Phe 187	Phe 504	Phe 520	(+1)/(+2)
12	pThr 197	pThr 516	pThr 532	(+3)
13	**Leu 198**	**Phe 517**	**Phe 533**	(+1)/(+3)
14	Thr 201	Thr 520	Thr 536	(−2)
15	Pro 202	Pro 521	Pro 537	(+1)
16	Glu 203	Glu 522	Glu 537	(−2)
17	Tyr 204	Tyr 523	Tyr 538	(−2)
18	**Leu 205**	**Val 524**	**Val 539**	(+1)
19	Glu 230	Glu 549	Glu 565	(−2)
20	**Tyr 330**	**Phe 651**	**Phe 667**	(−3)

[a]Where the acceptor loci differ between PKA and PKG, the residues are shown in **bold**. Phosphothreonine, pThr.

predicted to interact with the side chains of peptide substrates between subsites (−3) and (+3) (the specificity determining residues in the phosphorylation site sequence are notated −n to +n, depending on their position on the N or C-terminal side of the phosphorylation site, 0; Table I). Other important peptide substrate enzyme interactions can occur outside the subsites (−3) and (+3) [6] but are not considered here for reasons of brevity. Although acceptor loci determine specificity, historically, residues in substrate phosphorylation site sequences that facilitate peptide substrate binding have been called specificity determinants, and for this reason acceptor loci are used here to notate contact residues on the kinase surface. A substrate Arg at (−3) (see Table I) binds to acceptor loci 6,7,8,10, and 20, plus the 3′-OH and 4′-OH of the ATP ribose moiety. An Arg at (−2), however, binds to acceptor loci 10,16,17, and 19 (acceptor locus 16 corresponding to Glu203 is diagnostic of the AGC group of protein kinase subfamily). The substrate (−3) and (−2) Arg residues make more contacts with the PKA catalytic core than any other substrate side chains, and this accounts for the strong specificity-determining role of Arg at these two positions. The only other subsite approaching this number of interactions is the (+1) hydrophobic pocket comprising acceptor loci 11,13,15, and 18. Acceptor locus 18 (Leu205) is situated at the end of this pocket, facilitating optimal binding to Leu or Ile residues (Val is suboptimal at this subsite). Acceptor loci 1,4, and 13 are located in the (−1), (+2), and (+3) side chain pockets, respectively, and suggest a modest preference for hydrophobic residues at these subsite pockets.

Comparison of Kinase Substrate Acceptor Loci

Due to the similarities in the acceptor loci, PKA and PKG share strong substrate specificity for Arg residues in peptide substrates at (−3) and (−2) positions [2]. By contrast, members of the PKC subgroup have an additional strong specificity for Arg or Lys at the (+2) position [10]. Of the 20 PKA acceptor loci, 13 are shared with PKG1 (Table I), whereas seven are different (2,4,7,8,13,18, and 20). The key difference appears to be acceptor locus 18 at the bottom of the (+1) pocket; the acceptor locus 18 of PKG is a Val, whose side chain is too short to contact a Leu residue bound at (+1). Thus, unlike PKA, a (+1) Val or Leu would make the same number of contacts with PKG. These differences in acceptor loci residues dictate the overall differences in the substrate specificities of PKA and PKG.

Optimum Recognition Sequences

Extensive studies of PKA over the past 30 years have identified a large number of substrates, in fact the largest

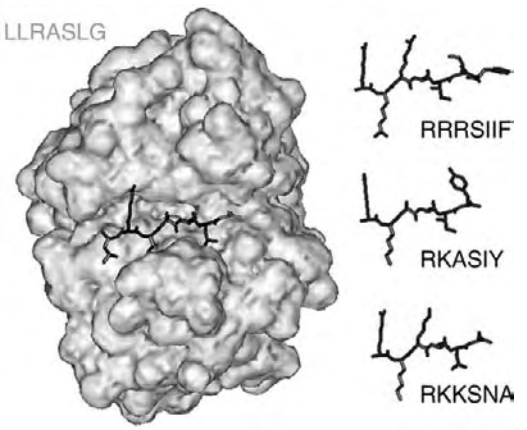


Figure 1 Surface structure of the catalytic domain of PKA with the "Kemptide" substrate (LRRASLG) bound. The structures of peptides representing the optimized recognition sequences for PKA (RRRSIIF [12], RKASIY [13]) and PKG (RKKSNA [13]) are illustrated.

number for any protein kinase thus far; the number of phosphorylation sites currently listed in PhosphoBase [11] is 165, including redundancies such as entries for the same protein from different species. The results of oriented degenerate peptide library experiments have enabled estimates to be made of the optimal substrate sequence for protein kinases. The optimum recognition sequence for PKA [12], RRRSIIF (phosphorylation site underlined), is consistent with conclusions derived from both inspection of the acceptor loci and the early synthetic peptide studies using the Kemptide (LRRASLG). Optimal substrate sequences for PKA, RKASIY- and PKG, RKKSNA-have also been studied by using peptide libraries on cellulose paper [13]. The binding of the "Kemptide" substrate to PKA together with the structures of the optimized peptide substrates for PKA and PKG are illustrated in Fig. 1. Examination of substrate sequences for PKA (Table II) shows that specificity (for Arg) is strong at (−3) and (−2), but the specificity is broad at other positions, particularly at (+2) and (+3). Only a minority of substrate sequences conform reasonably well to the peptide library motif (e.g., RRNSILT, RKVSLAP, RRGSVPI, RRDSLFV, and RRQSVLV). The consensus sequence inferred from known PKA substrates would be RR(R/S)SLSS. Our structural analysis and the peptide library results indicate that the (+1) site should strongly favor hydrophobic residues, however, less than 40 percent of sites in known substrates contain hydrophobic residues at this position. There are a number of smaller residues [Gly, Ala, Ser, and Pro (GASP)] found at (−1), (+1), (+2), and (+3) in substrate sequences, which would be expected to make few, if any, contacts with the enzyme. The presence of these small residues accounts for the wide range of PKA substrate phosphorylation site sequences. This raises the question of whether within the cell the AKAPs (A kinase anchoring proteins) [14] may increase the probability of PKA binding by these suboptimal sequence-containing substrates, and therefore compensating for this suboptimal recognition, as well as localizing the substrate to a signaling complex.

Table II Abundance of Particular Residues in PKA and PKG Substrate Phosphorylation Sites (PhosphoBase), LIMVF, Leu/Ile/Met/Val/Phe; GASP, Gly/Ala/Ser/Pro

Subsite	Residue	PKA	PKG
(−3)	Arg	65.4	69.7
	Lys	16.7	6.1
(−2)	Arg	56.4	45.4
	Lys	16.4	42.1
(−1)	Arg	12.7	24.2
	Leu	9.7	16.2
	LIMVF	23.0	42.4
	GASP	35.2	12.1
(+1)	Leu	19.4	6.1
	LIMVF	36.9	24.2
	Ser	13.9	6.1
	GASP	29.4	27.3
	Arg	7.9	15.1
(+2)	LIMVF	12.4	18.2
	Ser	14.5	12.1
	Ala	19.1	21.2
	GASP	38.6	37.4
(+3)	LIMVF	15.8	9.1
	Pro	6.1	18.2
	GASP	32.9	24.2
	Glu	7.0	27.3

One reason the optimal recognition sequence defined by peptide library experiments is not more highly represented in substrates is that it is rather hydrophobic and therefore difficult to accommodate in an exposed loop of a protein where the phosphorylation site would usually be located. The protein substrate's function also needs to be taken into account. Phosphorylation is required to induce structural and functional changes in the substrate, and these might not necessarily be compatible with a single highly conserved phosphorylation site sequence motif. Substitution of one or more hydrophobic residues in the motif by smaller residues such as Ser, Ala, Gly, or Pro would render the peptide less hydrophobic and increase the probability that the phosphorylation site is in a loop region. In addition, the smaller residues may facilitate structural changes in the substrate triggered by the phosphorylation event that are essential for altering protein function. The major implication of these considerations is that the binding of Arg at (−3) and (−2) provides the bulk of the interactions needed to bind the substrate to PKA, and this permits considerable flexibility in accommodating surrounding residues. A further reason for sequence diversity in local phosphorylation site sequences for PKA is that they may have a kinetic function in signaling, determining the order in which protein substrates are phosphorylated, that otherwise could not be achieved if there was a common

conserved optimum motif [15]. By contrast, the peptide library motif for PKCα (RRRSLRK) [10,16] is more hydrophilic and therefore a better guide for PKC substrates such as MARCKS [17].

Comparison of PKA and PKG Specificity

Despite its close similarity to PKA, PKG is more restricted in its known substrates, with 33 phosphorylation sites listed in PhosphoBase. Some of these phosphorylation sites are common to both PKA and PKG. The consensus motif based on substrate sequences of PKG is R(R/K)(R/L)S(R/A)AE (Table II). Although largely similar to the PKA substrates, the PKG substrates show distinct differences. The (−2) site has a similar preference for Arg and Lys, whereas in PKA, Arg is heavily favored over Lys. The (−3) specificity is basically equal in PKA and PKG, despite the substitution of the acceptor locus 20 of PKA (Tyr to Phe) resulting in a loss of the acceptor group for the Arg side chain. PKG is more specific at the (−1), (+2), and (+3) sites than PKA, and Gly, Ala, Ser, and Pro occur more rarely at (−1) and (+3) positions. The moderate preference for hydrophobic residues at (+1) of PKA is not observed with PKG. Indeed the most dramatic differences in specificity between PKA and PKG were observed in peptide analogs (LRRRRF-aminoalcohol) containing α-substituted alcohols. PKG is able to phosphorylate the hydroxyl group in these peptide analogs in either the equivalent of a D or L-amino acid configuration, whereas PKA has a strict requirement for the L-configuration [18]. The PKG Val acceptor site 18 for the (+1) subsite is likely to explain its capacity to phosphorylate different isomers. The highest preference at (+1) is for Arg or Ala. PKG does display some preference for Arg/Leu, Ala/Ser, and Glu at (−1), (+2), and (+3), respectively. In summary, the specificity of PKG is narrower when compared to PKA, and a correspondingly smaller number of substrates have been identified. The additional specificity constraints for PKG may mean that anchoring proteins play less of a role in PKG signaling.

Overlapping substrate motifs for PKA and PKG suggest that there will be phosphorylation sites that the two protein kinases have in common, as is the case with $RRL^{737}SLVP$ from the cystic fibrosis transmembrane conductance regulator [19,20]. By contrast, $RTL^{7}SVSS$ from glycogen synthase is a substrate for at least eight different protein kinases, including PKA, but not PKG [11,21] possibly because the Ser residues at both (+2) and (+3) do not provide favorable contacts for PKG. The results show how two protein kinases such as PKA and PKG, with considerable sequence similarity, nevertheless exhibit significant diversity in substrate recognition properties.

Conclusion

The use of synthetic peptide analogs and peptide libraries has played a critical role in revealing the major specificity determinants in PKA and PKG substrate phosphorylation site sequences. Crystal structures of PKA with peptide substrates and inhibitors bound to the enzyme have provided a road map to understanding the important acceptor loci residues on the kinase surface responsible for peptide substrate binding. Molecular modeling of these structures has allowed us to identify important similarities and differences in PKA and PKG substrate specificity.

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CHAPTER 203

Physiological Substrates of PKA and PKG

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Introduction

The cAMP- and cGMP-kinases (PKA and PKG, respectively) belong to the ACG-subclass of Ser/Thr-specific protein kinases and generally prefer the phosphate acceptor residue preceded by a row of basic residues. S is the favored phosphate acceptor also when taking into account the 12-fold higher frequency of S over T in eukaryotic proteins. Based on an extensive body of work with peptide substrates *in vitro* (see Chapter 80, this volume) and mapping of phosphorylation sites in physiological substrates *in vivo*, PKA is well known to phosphorylate substrates with the general motif R(R/K)X(S/T) [1–6], whereas the consensus for PKG substrates is (R/K_{2-3}) (X/K)(S/T) and includes more basic residues than the PKA consensus [7]. However, considerable overlap of sites phosphorylated by both kinases is observed *in vivo*. By analysis of physiological substrates (Table II), the preference for PKA in the P^{-3} and P^{-2} positions is RR≥RK>>KR≥KK, and there are weaker preferences for small residues (S, G, P) at P^{-1}, for a basic residue (R) at P^{-4} to P^{-7}, and for a hydrophobic residue (F, I, L, V) at P^{+1} [8]. Substrate specificity of PKG is similar to that of PKA, but in physiological substrates (Table III) a stronger preference for R>>K at P^{-3} (at position P^{-2} K = R), a slight preference for basic or neutral residues (K, R, S) in P^{-4}, and an increased frequency of neutral and hydrophobic residues (S, V, A) at P^{-1} and (S, L, A) at P^{+1} is observed (this chapter).

Here we present data on the total availability of PKA and PKG consensus sites in the human proteome, estimate frequencies of phosphorylation of different motifs, and attempt to give an overview of physiological substrates of both kinases that meet a set of eligibility criteria. However, mechanisms whereby the phosphorylation event alters function of each individual substrate and thereby regulates its physiological role are not included in this short overview.

Abundance of PKA and PKG Phosphorylation Sites in the Human Proteome

We have searched available protein databases (January 2002), including all translated and indexed sequences from the full draft of the human genome, with all the permutations of the canonical PKA and PKG motif, (R/K)(R/K)X(S/T). A little more than 35,000 motifs were found in human proteins, which is approximately 15 percent of all motifs identified (Table Ia). Based on the total abundance of canonical motifs (phosphorylated and nonphosphorylated) in a limited set of approximately 100 substrates for PKA in which the phosphorylation sites have been mapped, Shabb estimated the probability of phosphorylation of permutations of the canonical sequence, which was RRXS (0.8) > RRXT, RKX(S/T) (0.5–0.3) > KKX(S/T), KRX(S/T) (<0.2) [8]. A similar analysis of frequency of PKG phosphorylation (this chapter, from substrates listed in Table III) shows that probability of phosphorylation *in vivo* by PKG is estimated as R(R/K)XS (0.7) > RKXT (0.5) > KRXS, RRXT (0.3) > KKXS (0.2).

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Table I Abundance of PKA and PKG motifs[a]

a. Frequency and probability of phosphorylation of canonical subtstrate sequences

Consensus	Homo sapiens	Estimated phosphorylated motifs of PKA[b]	Estimated phosphorylated motifs of PKG[c]
RRXS	7078	5662	5096
RKXS	5233	2460	3506
KRXS	4670	841	1541
KKXS	5194	831	883
RRXT	3682	1399	921
RKXT	3106	932	1553
KRXT	2783	390	0
KKXT	3801	266	0

b. Frequency of high affinity motifs for PKA [d,e] and PKG [e] identified in physiological substrates[d] or by peptide library screens[e]

PKA Consensus	Homo sapiens	PKG Consensus	Homo sapiens
RRSS(L, V, I, F) [a]	163	RKKS	407
RRGS(L, V, I, F) [a]	106	KRKKS	68
RRPS(L, V, I, F) [a]	93	KARKXS	19
RR(S, G, P)SF [a]	42	KARKKS	3
RRAS [c]	450	AKRKKS	4
AERRAS [c]	3	KRKKSL	5
RAERRASI [c]	0	KXRKKSL	3
LRRASLG [c] (kemptide)	0	KARKKSL	1
		TQAKRKKSLA	0

[a]All available human protein sequences including predicted proteins translated from the full draft of the human genome were analyzed (January 2002: 54687 sequences in the International Protein Index (IPI) version 2.4 at EMBL-European Bioinformatics Institute, Hinxton, Cambridge, UK, which provides a minimally redundant yet maximally complete set of human proteins assembled from SWISS-PROT, TrEMBL, RefSeq and Ensembl, see (*http://www.ebi.ac.uk/IPI/IPIhelp.html*).

[b,c]Probability for the motif being phosphorylated by PKA[b] ([8]) or PKG[c] (this study) *in vivo*.

[d] Preferred in vivo motifs according to [8].

[e]High-affinity peptides [9,10,17]. The authors acknowledge the kind help of Dr. Paul Kersey at EBI with searching IPI.

Using these probabilities, we estimate that PKA, PKG, or both can phosphorylate approximately 15,000 sites in the human proteome *in vivo*. When both frequency of different sites and probability of phosphorylation are taken into account, RRXS followed by RKXS stands out as the most abundant *in vivo* sites for both kinases (Table Ia). Notably, however, this analysis also predicts phosphorylation of significant numbers of substrates with less prevalent motifs such as K(R/K)X(S/T) with lower affinity but which may be physiologically relevant especially in contexts where kinase and substrate are colocalized [8]. In contrast, preferred substrates defined by more detailed analysis of physiological substrates (Tables II and III) or by *in vitro* phosphorylation of generations of peptide libraries with PKA and PKG are clearly less abundant due to their more restricted motifs (Table Ib). High-affinity substrates such as LRRASLG (Kemptide, [9]) and RAERRASI [7] for PKA and TQAKRKKSLA for PKG [10] used *in vitro* enzyme assays were not found in the human proteome.

Physiological Substrates

General criteria for identification of physiological substrates of protein kinases were originally outlined by Krebs and Beavo [11], recently reviewed by Shabb [8], and can be summarized thus:

1. The target protein should be phosphorylated stoichometrically and dephosphorylated by phosphatase *in vitro* at significant kinetic rates.
2. Functional properties of the substrate should change in correlation with the degree of phosphorylation.
3. Phosphorylation of the substrate should be demonstrated *in vivo* or in intact cells with accompanying functional changes.
4. The cellular levels of protein kinase should correspond to the extent of phosphorylation of the substrate.
5. The *in situ* phosphorylation sequence should be identified (new, adds stringency).

Table II Physiological Substrates of PKA

Substrate	*In vivo*/ *in situ* site	Sequence	Accession no./species	Ref
Autophosphorylation				
cAMP-dependent protein kinase regulatory subunit type IIα	Ser-95[a]	pgrfdrrvsvcaet	P00515 (bovine)	[8]
Receptor mediated signaling				
β_2-adrenergic receptor	Ser-262[c]	tghglrrsskfclk	P07550 (human)	[8]
	Ser-345[c]	qellclrrsslkay		
	Ser-346[c]	ellclrrsslkayg		
Prostacyclin receptor (IP)	Ser-357 [c]	qaplsrpasgrrdp	A54416 (mouse)	[13]
Regulator of protein signaling RGS9-1	Ser-427 [c]	epqgttrkssslpf	O46469 (bovine)	[14]
	Ser-428 [c]	pqgttrkssslpfm		
Regulator of G protein signaling (RGS10)	Ser-168 [c]	aqtaakrasriynt	Q9CQE5 (mouse)	[15]
cAMP signaling				
cAMP-specific phosphodiesterase PDE4D3	Ser-54[a,c]	fvhsqrresflyrs	P14270 (rat)	[8]
cGMP inhibited phosphodiesterase PDE3B	Ser-302[a,c]	sgkmfrrpslpcis	Q63085 (rat)	[8]
D1 dopamine receptor	Thr-268[c]	fkmsfkretkvlkt	P18901 (rat)	[8]
Metabotropic glutamate receptor subunit mGluR2	Ser-843[a,c]	fgsaaprasanlgq	P31421 (rat)	[8]
Phosphodiesterase 4D5 (PDE4D5)	Ser-126 [ac]	fvhsqrresflyrs	AAC00069 (human)	[16]
cGMP signaling				
Phosducin	Ser-73[a]	kermsrkmsiqeye	P20942 (rat)	[8]
Phosphoinositide and calcium signaling				
Elongation factor-2 kinase	Ser-499[c]	srlhlprpsavale	P70531 (rat)	[8]
Inositol 1,4,5-trisphosphate Type I receptor	Ser-1589[a]	arnaarrdsvlaas	P29994 (rat)	[8]
	Ser-1755[a]	irpsgrresltsfg		
Phospholipase C-γl	Ser-1248[a]	fhvraregsfeary	P10686 (rat)	[8]
Phospholipase C-β3	Ser1105[a,c]	ildrkrhnsiseak	Q01970 (human)	[8]
Thromboxane A_2 receptor TPα	Ser-329[c]	prlstrprslslqp	NP 001051 (human)	[8]
CaM-kinase kinase α	Thr-108[c]	sprawrrptieshh	BAA75246 (rat)	[17]
	Ser-458[c]	vksmlrkrsfgnpf		
Rho signaling				
RhoA small GTP-binding protein	Ser-188[a,c]	qarrgkkksgclvl	P06749 (human)	[8]
T cell receptor signaling				
COOH-terminal Src kinase (Csk)	Ser-364[a,c]	ealrekkfstksdv	P41240 (human)	[8]
Mitogen-activated protein kinase signaling				
Hematopoeietic protein tyrosine phosphatase (hePTP)	Ser-23[a,c]	vrlqerrgsnvalm	P35236 (human)	[8]
Mammalian STE20-like kinase 3 b isoform (MST3b)	Thr-18[c]	lalnkrratlphpg	AAD42039 (human)	[8]
v-Mos	Ser-56[a,c]	psvdsrscsiplva	P00538 (maloney murine sarcoma virus)	[8]
	Ser-102[a,c]	vclmhrlgsggfgs		
	Ser-263[a,c]	qdlrgrqaspphig		
GTPase activating protein specific for Rap1 (rap1GAP)	Ser-490[a,c]	gksptrkksgpfgs	P47736 (human)	[8]
	Ser-499[a,c]	gpfgsrrssaigie		
Guanine nucleotide exchange factor Ras-GRF1	Ser-916[a,c]	nkevfrrmslantg	P27671 (mouse)	[8]
Protein tyrosine phosphatase-SL (PTP-SL)	Ser-231[c]	iglqerrgsnvslt	NP 035347 (human)	[8]
Raf-1 serine/threonine protein kinase	Ser-43[a,c]	qfgyqrrasddgkl	P11345 (rat)	[8]
Rap1b low molecular weight GTP/GDP-bindingprotein	Ser-179[c]	vpgkarkksscqll	P09526 (human)	[8]
Striatal-enriched protein tyrosine phosphatase, 61 kDa (STEP)	Ser-160[a,c]	lppedrrqsvsrqp	P54830 (mouse)	[8]
$STEP_{61}$ and 46 kDa $STEP_{46}$ splice variant	Ser221[a,c,e]	mglqerrgsnvslt		

continues

Table II *continued*

Substrate	*In vivo/ in situ* site	Sequence	Accession no./species	Ref
Modulators of protein phosphatase 1				
Glycogen binding (G) subunit of protein phosphatase 1	Ser-46[a]	spqpsrrgsdssed	NP 002702 (human)	[8]
	Ser-65[a]	pssgtrrvsfadsf		
Inhibitor-1of (I-1) protein phosphatase I	Thr-35[a]	eqirrrrptpatlv	P01099 (rabbit)	[8]
Dopamine and cAMP-regulated phosphoprotein, 32 kDa (DARPP-32)	Thr-34[a,b]	emirrrrptpamlf	P07516 (bovine)	[8]
Transcriptional regulation				
Cyclic AMP response element binding protein (CREB)	Ser133[a,b,c]	reilsrrpsyrkil	P15337 (rat)	[8]
Cyclic AMP responsive element modulator (CREMτ)	Ser-117[c]	reilsrrpsyrkil	P27699 (mouse)	[8]
Nuclear factor of activated T cells 3 (NFAT3)	Ser-272[a,c]	spcgkrrysssgtp	Q14934 (human)	[8]
	Ser-289[a,c]	spalsrrgslgeeg		
Nuclear factor κB (NF-κB)	Ser-276[a,c]	vsmqlrrpsdrels	Q04207 (mouse)	[8]
Retinoic acid receptor-α (RARα)	Ser-369[a,c]	vyvrkrrpsrphmf	P11416 (mouse)	[8]
Retinoid X receptor-α (RXRα)	Ser-27[c]	ltsptgrgsmaaps	P19793 (human)	[8]
Sex determining region of Y gene product (SRY protein)	Ser-32[c]	nipalrrsssflct	Q05066 (human)	[8]
SRY-box related transcription factor SOX9	Ser-64[c]	gepdlkkeseedkf	P48436 (human)	[8]
	Ser-181[b,c]	kyqprrrksvkngq		
Steroidogenic factor-1 (SF-1)	Ser-430[c]	clvevralsmqake	P33242 (mouse)	[8]
Thyroid hormone receptor α1	Ser-28[a,c]	ldgkrkrkssqclv	P04625 (chick)	[8]
	Ser-29[a,c]	dgkrkrkssqclvk		
Vasoactive intestinal polypeptide receptor transcriptional	Ser-245[c]	ktkkarkdseeges	AAC40192 (rat)	[8]
repressor protein (VIPR-RP)	Ser-361[c]	kgsptkresvsped		
Class II transactivator (CIITA)	Ser-834 [c]	vqelpgrlsflgtr	AAA88861 (human)	[18]
	Ser1050 [c]	laasllrlslynnc		
Thyroid transcription factor (TTF1)	Ser-337 [c]	pdlahhaaspaalq	P23441 (rat)	[19]
Histones				
Histone H1c	Ser-37[a]	pagvrrkasgppvs	P15864 (mouse)	[8]
Histone H3	Ser-10[a,b,c]	rtkqtarkstggka	P16106 (human)	[8]
Apoptosis and cell survival				
Bcl-2/Bcl-X_L-antagonist, causing cell death (BAD)	Ser155[a,b,c]	ygrelrrmsdefeg	Q61337 (mouse)	[8]
Glycogen synthase kinase 3α (GSK-3α)	Ser-21[b]	gsgrartssfaepg	P18265 (rat)	[8]
Glycogen synthase kinase-3β (GSK-3β)	Ser-9[b,c]	msgrprttsfaesc	P18266 (rat)	[8]
Interleukin receptor-3 β_c chain	Ser-585[b,c]	ylgpphsrslpdil	NP_000386 (human)	[8]
Ligand-gated ion channels				
$GABA_A$ receptor β1 subunit	Ser-409[c]	kgrirrrasqlkvk	P50571 (mouse)	[8]
$GABA_A$ receptor β3 subunit	Ser-408[c]	Kthlrrrsqlkik	P15433 (mouse)	[8]
	Ser-409[c]	kkthlrrrsqlki		
Glutamate receptor GluR1 subunit (AMPA receptor)	Ser845[a,b,c]	rtstlprnsgagas	P19490 (rat)	[8]
Glutamate receptor GluR4 subunit (AMPA receptor)	Ser-842[a,c]	airnkarlsitgsv	P48058 (human)	[8]
Glutamate receptor GluR6 subunit (kainate receptor)	Ser-684[c]	afmssrrqsvlvks	P42260 (rat)	[8]
Glutamate receptor NR1A subunit (NMDA receptor)	Ser897[a,b,c]	ssfkrrrsskdtst	P35439 (rat)	[8]
Nicotinic acetylcholine receptor δ subunit	Ser-361[a,c]	ndlklrrsssvgyi	P02718	[8]
	Ser-362[a,c]	dlklrrsssvgyis	(*T. californica*)	
P_{2X2} purinoreceptor	Ser-431[c]	avqsprpcsisalt	P49653 (rat)	[8]
Sodium ion movement				
Na^+ H^+ exchanger 3 (NHE3)	Ser-552[a,c]	vaegerrgslafir	P26433 (rat)	[8]
	Ser-605[a,c]	qsleqrrrsirdte		

Table II *continued*

Substrate	*In vivo*/ *in situ* site	Sequence	Accession no./species	Ref
Na^+,K^+ ATPase α1 subunit	Ser943[a,b,c]	vicktrrnsvfqqg	P06685 (rat)	[8]
Serum and glucocorticoid regulated kinase (Sgk)	Thr-369[c]	ddlinkkitppfnp	O00141 (human)	[8]
Voltage sensitive Na^+ channel (Rat brain type IIA) α subunit	Ser-573[a,c]	slfsprrnsraslf	P04775 (rat)	[8]
	Ser-610[a,c]	edndsrrdslfvph		
	Ser-623[a,c]	hrhgerrpsnvsqa		
	Ser-687[a,c]	teirkrrsssyhvs		
Voltage-sensitive Na^+ channel (cardiac type H1) α subunit	Ser526[a,b,c]	rtsmrprssrgsif	P15389 (rat)	[8]
	Ser529[a,b,c]	mrprssrgsiftfr		
Chloride conductance				
Cystic fibrosis transmembrane conductance regulator (CFTR)	Ser-660[a,c]	qfsaerrnsiltet	P13569 (human)	[8]
	Ser-700[a]	efgekrknsilnpi		
	Ser-737[c]	deplerrlslvpds		
	Ser-795[c]	ttastrkvslapqa		
	Ser-813[c]	idiysrrlsqetgl		
Phospholemman	Ser-68[a,c]	frssirrlstrrr	O08589 (rat)	[8]
Potassium channels				
Shaker K^+ channel	Ser-507[c]	tlgqhmkksslses	P08511 (*Drosophila*)	[8]
	Ser-508[c]	lgqhmkkslsess		
Slo K_{Ca} channel splice variant A1C2E1G3I0	Ser-942[b,c]	pivlqrrgsvygan	JH0697[d] (*Drosophila*)	[8]
h*Slo* BK_{Ca} α subunit of large conductance Ca^{2+}-dep. K^+ channel (maxi-K)	Ser-869[c]	vhgmlrqpsittgv	NP 002238 (human)	[8]
Kv1.1 α subunit of the *Shaker* RCK1 Voltage-gated K^+ channel	Ser-446[c]	dsdlsrrssstisk	P10499 (rat)	[8]
Kvβ1.3 subunit of the Kv1.5 K^+ channel Ik_{ur}	Ser-24[c]	entklrrqsgfsva	AAC41926.1 (human)	[8]
Kv4.2 α subunit of the *Shal*-type K^+ channel	Thr-38[a,b]	pprqerkrtqdali	NP 062671 (mouse)	[8]
	Ser-552[a,b]	nvsgshrgsvqels		
Kir 1.1 Renal outer medullary K^+ channel 1, 2 (ROMK1, ROMK2)	Ser-25[a,c]	srqrarlvskegrc	P35560 (rat)	[8]
	Ser-200[a,c]	irvanlrkslligs		
	Ser-294[a,c]	satcqvrtsyvpee		
Kir2.1	Ser-425[c]	eprplrresei	Q64273 (rat)	[8]
Kir2.3 Inward rectifier K^+ channel (IRK)	Ser-440[a,c]	dnisyrresai	P48050 (human)	[8]
Kir6.2 subunit of the ATP-sensitive K^+ channel (K_{ATP})	Ser-372[c]	argplrkrsvpmak	Q14654 (human)	[8]
	Thr-224[c]	hmqvvrkttspege		
SUR1 subunit of the ATP-sensitive K^+ channel (K_{ATP})	Ser-1571[c]	ekllsrkdsvfasf	Q09428 (human)	[8]
Water homeostasis				
Aquaporin-2	Ser-256[b,c]	erevrrrqsvelhs	P34080 (rat)	[8]
Other transporters				
P-glycoprotein mdr1b	Ser-665[a,c]	sksplirrsiyrsv	P06795 (mouse)	[8]
	Ser-681[a,c]	kqdqerrlsmkeav		
Steroidogenic acute regulatory protein (StAR)	Ser-57[c]	inqvrrrssllgsr	P49675 (human)	[8]
	Ser-195[c]	vrcakrrgstcvla		
Extracellular proteins				
Atrial natriuretic peptide	Ser-104[a]	gprslrrsscfggr	P01161 (rat)	[8]
Vitronectin	Ser-378[a]	rnqnsrrpsratwl	P04004 (human)	[8]
Trafficking and motility				
Actin bundling protein L-plastin	Ser-5[a,c]	margsvsdeemmel	P13796 (human)	[8]
Low-density lipoprotein receptor-related protein (LRP)	Ser-4520[c]	mgghgsrhslastd	Q07954 human	[8]
Myosin light-chain kinase (MLCK) and telokin splice variant	Ser-1005[a,c,f]	sglsgrksstgspt	P29294 (rabbit)	[8]

continues

Table II *continued*

Substrate	*In vivo*/ *in situ* site	Sequence	Accession no./species	Ref
Protein tyrosine phosphatase-PEST (PTP-PEST)	Ser-39[a]	dfmrlrrlstkyrt	NP 002826 (human)	[8]
	Ser-435[a]	dkklernlsfeikk		
Small heat shock-related protein HSP20	Ser-16[a,b]	qpswlrrasaplpg	O14558 (human)	[8]
Synapsin I	Ser-9[a]	mnylrrrlsdsnfm	P09951 (rat)	[8]
Vasodilator-stimulated phosphoprotein (VASP)	Ser-157[a,c]	sehierrvsnaggp	P50552 (human)	[8]
	Ser-239[a,b,c]	agaklrkvskqeea		
	Thr-278[a,c]	mlarrrkatqvgek		
Snapin	Ser-50[c]	shvhavresqvelr	XP_057189(human)	[20]
Sso1 t-SNARE	Ser-79[c]	eqashlrhsldnfv	NP_015092 (yeast)	[21]
Cysteine string protein (csp)	Ser-10[c]	dcqrqrslstsges	Q29455(bovine)	[22]
Striated muscle contraction				
Myosin-binding protein-C cardiac isoform	Ser-275[a,c]	llsafrrtslaggg	Q14896 (human)	[8]
	Ser-284[a,c]	lagggrrisdshed		
	Ser-304[a,c]	ssllkkrdsfrtpr		
Phospholamban	Ser-16[a,b]	trsairrastiemp	P26678 (human)	[8]
Ryanodine receptor type 2 (sarcoplasmic reticulum Ca^{2+} release channel)	Ser-2809[c]	lynrtrrisqtsqv	P30957 (rabbit)	[8]
Troponin I	Ser-23[a]	apapirrrrsnyra	P19429 (human)	[8]
	Ser-24[a]	papirrrrssnyray		
Voltage-sensitive L-type Ca^{2+} channel (skeletal muscle) $\alpha 1$	Ser-1757[a,c]	pergqrrtsltgsl	P07293 (rabbit)	[8]
Subunit	Ser-1854[a,c]	pgslsrrrslgsld		
Voltage-sensitive L-type Ca^{2+} channel (cardiac) $\alpha 1$ subunit	Ser-1928[a,b,c]	saslgrrasfhlec	P15381 (rabbit)	[8]
Voltage-sensitive L-type Ca^{2+} channel (cardiac) β_{2a} subunit	Ser-459[a,c]	drsaprsasqaeee	A42044 (rat)	[8]
	Ser-478[a,c]	vkksqhrsssathq		
	Ser-479[a,c]	kksqhrssssathqn		
Metabolism and respiration				
ATP citrate lyase	Ser-454[a]	tpapsrtasfsesr	P16638 (rat)	[8]
Cytochrome P450 CYP2E1	Ser-129[a,c]	twkdvrrfslsilr	P05182 (rat)	[8]
Glycogen synthase (muscle type)	Ser-7[a,c]	mplsrtlsvsslpg	AAB69872 (rabbit)	[8]
	Ser-697[a,c]	apewprrasctsss		
	Ser-710[a,c]	sssggskrsnsvdt		
Hormone-sensitive lipase	Ser-563[a,c]	rltesmrrsvseaa	P15304 (rat)	[8]
	Ser-659[a,c]	pdgfhprrrsqgvl		
	Ser-660[a,c]	dgfhprrrsqgvlh		
Phenylalanine hydroxylase	Ser-16[a,b]	npglgrklsdfgqe	P00439 (human)	[8]
Phosphorylase kinase α subunit (muscle type)	Ser-1018[a]	kqvefrrlsistes	P18688 (rabbit)	[8]
Phosphorylase kinase β subunit (muscle type)	Ser-26[a]	rartkrsgsvyepl	P12798 (rabbit)	[8]
6-phosphofructo-2-kinase-fructose-2,6-bisphosphatase liver isozyme 1	Ser-32[a]	svlqrrrqssipqf	P07953 (rat)	[8]
6-phosphofructo-1-kinase, isozyme A (muscle type)	Ser-376[a]	eamklrgrsfmnnw	P00511 (rabbit)	[8]
Pyruvate kinase (liver type)	Ser-43[a]	pagylrrasvaqlt	P12928 (rat)	[8]
Tyrosine hydroxylase	Ser-40[a,b,c]	prfigrrqslieda	P04177 (rat)	[8]
Nuclear-encoded subunit of complex I (NDUFS4)	Ser-131[a]	anfswnkrtrvstk	Q02375 (bovine)	[23]
Micellaneous				
cAMP-regulated phosphoprotein 16/19 kDa (ARPP16/19)	Ser-104[a,b,g]	qdlpqrkpslvask	P56211 (human)	[8]
cAMP-regulated phosphoprotein, 21 kDa (ARPP-21)	Ser-55[a,b]	aqnqerrksksgag	A34957 (bovine)	[8]
Serine/threonine protein kinase LKB1	Ser-431[b,c]	ssnkirrlsackqq	NP_035622 (mouse)	[8]

Table II *continued*

Substrate	*In vivo*/ *in situ* site	Sequence	Accession no./species	Ref
Phogrin	Ser-680[c]	gphtsrinsvssql	CAA90600 (rat)	[24]
	Thr-699[c]	pspsarsstsswse		
rabphilin	Ser-234[a]	hgpptrrasearm	P47709 (rat)	[25]

[a] Direct sequencing and/or phosphopeptide mapping.
[b] Phospho/dephospho-specific antibodies.
[c] Site-directed mutagenesis.
[d] Ser-952 in this splice variant.
[e] $STEP_{61}$ Ser-221 is equivalent to $STEP_{46}$ Ser-49.
[f] MLCK Ser-1005 is equivalent to telokin Ser-13.
[g] ARPP-19 Ser-104 is equivalent to ARPP-16 Ser-88.

Table III Physiological Substrates of PKG

Substrate	*In vivo*/ *in situ* site	Sequence	Accession no./species	Ref
Autophosphorylation				
Autophosphorylation	Ser-63	atqqaqkqsastlq	P14619 (human)	[26]
	Ser-79	prtkrqaisaepta		
Regulation of smooth muscle tone				
Cardiac Troponin I	Ser-23	apapirrrssnyray	P19429 (human)	[27]
(cTN1)	Ser-24	apapirrrssnyray		[28]
(CRP2) Cystein rich Protein 2	Ser-104	vrteerktsgppkgp	P36201 (rat)	[29,30]
Hsp20 (Heat-shock 20 kD like protein P20)	Ser-16	qpswlrrasaplpg	P97541 (rat)	[31]
Regulation of smooth muscle tone (by regulation of intracell. Ca level)				
Cardiac phospholamban	Ser-16	trsairrastiemp	P26678 (human)	[32]
L-type Ca2+ channel α1c subunit	Ser-533	hriskskfsrywrr	P15381 (rabbit)	[33]
Calcium-acivated maxi K+ channel (BK_{Ca}) (α-subunit)	Ser-1072[a]	sqssskkssshvs	AAA84000 (canine)	[34,35]
Ins (1,4,5) P3 receptor type I	Ser-1756	irpsgrresltsfg	P29994 (rat)	[36]
Platelet Aggregation				
Heat shock protein 27 (Hsp27)	Ser-15	pfsllrqpswdpfr	XP_004991 (homo sapiens)	[37]
	Ser-78	apayssralsrqlssg		
	Ser-82	sralsrqlssgvsei		
	Thr-143	srcftrkytlppgv		
VASP (vasodilator stimulated phosphoprotein)	Ser-157	sehierrvsnaggp	CAA86523 (human)	[38,39]
	Ser-239	agaklrkvskqeea		
	Thr-278	mlarrrkatqvgek		
Neuronal function				
G-septin	Ser-91	ksqvsrkasswnre	AAD21035 (rat)	[40,41]
$GABA_A$Receptor ß2 subunit	Ser-410[a]	ksrlrrrasqlkit	P15432 (mouse)	[42]
$GABA_A$Receptor ß3-subunit	Ser-409	kthlrrrssqlkik	1095220 (mouse)	[42]
G-substrate	Thr-68	qkkprrkdtpalhi	AAD13030 (human)	[43]
	Thr-119	qkkprrkdtpalhm		
Dopamin/DARPP-32	Thr-34	emirrrrptpamlf	P07516 (bovine)	[44]
Thromboxane receptor alpha (TPα)	Ser-331[a]	lstrprslslqpql	NP_001051 (human)	[45,46]
Nucleus				
Splicing factor SF1	Ser-20	fpskkrkrsrwnqd	CAA03883 (human)	[47]

continues

Table III *continued*

Substrate	*In vivo*/ *in situ* site	Sequence	Accession no./species	Ref
Histone H2B	Ser-32	kdgkkrkrsrkesy	XP_059791(human)	[48]
germ cell development				
GKAP42	Ser-106[a]	spnpaqkesreenw	BAA92254(mouse)	[49]
Metabolic enzymes				
Tyrosine Hydroxylase	Ser-40	prfigrrqslieda	P04177(rat)	[50]
Nitric-oxide synthase NOS-III	Ser-633	swrrkrkessntds	P29474 (human)	[51]
	Ser-1177	vtsrirtqsfslqe		
6-phospho-fructo-2-kinase	Ser-32	svlqrrrgssipqf	P07953(rat)	[52]
Regulation of other signalling pathways				
CGMP-binding cGMP-specific hosphodiesterase (CGB-PDE)	Ser-92	pgtptrkisasefd	Q28156 (bovine)	[53]
c-Raf1	Ser-43[a]	qfgyqrrasddgkl	P04049 (human)	[54]
PLC-beta3	Ser-26[a]	vvktlrrgskfikw	P51432 (mouse)	[55]
	Ser-1105[a]	ildrkrnnsiseak		
HIV-1 replication and infectivity				
Vif (one of the HIV-1 Proteins)	Ser-144	qaghnkvgslqyla	AAA44202 (HIV type I)	[56]
	Thr-188	tkghrgshtmngh		
Filamentprotein				
Vimetin	Ser-26[b]	pgtasrpsstrsyv	P48616 (bovine)	[57,58]

[a] Site-directed mutagenesis.
[b] Potential phosphorylation site because the serine residue meets the consensus sequence

With the present level of available technologies such as deletion and mutation mapping of the site and mass spectrometry, current consensus is that the primary evidence to consider is that *in vivo* phosphorylation occurs in response to elevated cAMP or cGMP (criteria 3–5), and this takes priority over *in vitro* mapping of PKA and PKG sites (typically the method of use in older literature) where a pitfall is that less stringent phosphorylations may occur. However, application of these criteria excludes phosphorylations occuring *in vivo* that are either silent (that is, no functional change) or whose function has not yet been mapped. Conversely, substrates implicated in physiological pathways but where the precise mechanism or site has not yet been identified are also excluded.

Here we present a comprehensive list of PKA substrates that meet the above eligibility criteria identified through review of the literature (Table II). This list is based on a recently published review by Shabb [8], and has been revised by adding new published data, bringing the number of identified and listed physiological substrates of PKA that meet the eligibility criteria to 116 and the number of analyzed motifs to 162. Furthermore, we present a corresponding list of 29 PKG substrates, representing 38 analyzed motifs, that have been identified by systematic literature search and application of the same set of criteria (Table III). It is interesting that 13 of those motifs are also phosphorylated by PKA (Tables III and II), indicating at least 30 percent overlap in substrates. During these searches we also revealed a number of potential substrates that do not fulfil a convincing combination of the general criteria yet. Furthermore, the discrepancy between the identified substrates that meet the criteria (Tables II and III) and the estimated number of substrates (Table I) indicates that more than 99 percent of human PKA and PKG substrates are still unidentified, and this constitutes a major task to address in the future.

Concluding Remarks

The specificity of a substrate is determined not only by the primary sequence, but also by several other factors that affect the degree of phosphorylation of a given target. The tertiary structure of the substrate affects function and kinetics of the kinase, such as for example the catalytic subunit of cAMP-kinase that in part acquires its substrate specificity from the conserved F at position $P11^{-1}$[12]. The organization of the microenvironment around a phosphorylation event has a clear impact. In that respect, anchoring proteins (AKAPs, GKAPs) play an important role by locating PKA and PKG in close vicinity to their substrates and demonstrate how low-affinity substrates may become physiologically relevant.

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CHAPTER 204

Effects of cGMP-Dependent Protein Kinase Knockouts

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Both mammalian cGMP-dependent protein kinase genes, cGKI and cGKII, have been inactivated in mice. The major phenotypes observed in cGKI knockout mice are decreased life span, impaired relaxation of vascular, visceral, and penile smooth muscle, disturbed platelet function, and altered neuronal functions. cGKII knockout mice have a normal life span, decreased longitudinal bone growth, decreased intestinal chloride secretion, and altered renin secretion.

Cyclic GMP-Dependent Protein Kinases: Genes and Knockouts

Cyclic GMP-dependent protein kinases (cGKs) are serine/threonine kinases which are activated by the second messenger cGMP. Two cGK genes, cGKI and cGKII, have been identified in mammals. The amino terminus (approximately amino acids 1–100) of cGKI is encoded by two alternatively used exons, resulting in the production of two cGKI isoforms, cGKIα and cGKIβ. To generate cGKI and cGKII gene targeted mouse lines, the exon encoding part of the ATP-binding site in the catalytic domain (cGKI) or the first part of the cGMP-binding pocket (cGKII) was destroyed by homologous recombination in embryonic stem cells. These strategies resulted in loss-of-function mutants of the cGKII gene [1] and of the cGKI gene with complete inactivation of both the Iα and Iβ isozyme [2]. Since cGKI-null mutants have a decreased viability (most animals die until six weeks of age), a conditional cGKI mouse line was generated in which the ATP-binding site encoding exon was flanked by loxP sequences allowing for Cre-mediated tissue-specific inactivation of the cGKI gene [3].

Vasorelaxation and Hypertension

Nitric oxide (NO) and atrial natriuretic peptide (ANP) stimulate cGMP synthesis and relax small arteries and arterioles resulting in a decreased blood pressure. Targeted inactivation of the NO-synthase III (NOS III), ANP, or ANP receptor gene causes hypertension (reviewed in reference [4]). Juvenile (4 to 5 weeks old) cGKI knockout mice have an elevated blood pressure [2] indicating that the anti-hypertensive effects of NO and ANP are at least in part mediated by activation of cGKI. Potential *in vivo* targets for cGKI are the Ca^{2+}-activated K^+ (BK_{Ca}) channel [5] and IRAG [6], a protein involved in the intracellular calcium release mechanism. Phosphorylation of these two proteins could reduce the cytosolic calcium concentration, thereby leading to vasorelaxation. Vascular smooth muscle cells (VSMCs) isolated from wild-type mice endogenously express cGKIα, cGKIβ, and IRAG. NO/cGMP inhibited noradrenaline-induced Ca^{2+}-transients in wild-type but not in cGKI-deficient VSMCs. Interestingly, the defective Ca^{2+} regulation in cGKI-deficient VSMCs can be rescued by transfection of the cGKIα isozyme, but not the β isozyme [7]. These results suggest that cGKIα relaxed smooth muscle by decreasing the cytosolic Ca^{2+} level. The role of cGKIß in VSMCs is unclear at present, but may be more related to the cGKI effects on smooth muscle proliferation, differentiation, and gene expression [8]. The results described above do not preclude the possibility that cGKI decreases vessel tone by additional Ca^{2+}-independent mechanisms including the activation of myosin phosphatase [9], of phosphorylation of RhoA [10], and of telokin [11] resulting in dephosphorylation of the myosin light chains without affecting the cytosolic calcium level. Blood pressure

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may be regulated also by cGKII via inhibition of renin secretion [12].

The interpretation of the pathophysiology of the conventional cGKI knockout animals was complicated by the finding that 7-week-old, cGKI-null mutants had a normal or only slightly elevated blood pressure [2] indicating that the lack of cGKI can be bypassed in older animals. However, cGKI-null mutants develop multiple phenotypes with increasing age including infections and inflammation [13], which are known to induce massive NO synthesis. High concentrations of NO can increase cGMP levels to extreme values in vascular smooth muscle [5]. Thus, it is tempting to speculate that the apparent "normalization" of blood pressure in older cGKI-null mutants may be due to crossactivation of cAMP-dependent protein kinase by the high cGMP levels that are potentially generated in these diseased mice [5]. Furthermore, cGMP levels should be increased in cGKI knockout animals since the cGMP hydrolysing phosphodiesterase 5 (PDE 5) is not phosphorylated in cGKI-deficient VSMCs [14]. It has been suggested that *in vivo* activation of PDE 5 requires phosphorylation by cGK [15].

Further analysis of the cGKI-null mutants was limited by their low viability and by the fact that the cGKI gene was inactivated in the germline and thus in every cell of these mice. Therefore, it was difficult to study the role of cGKI in adults and whether the age-related hypertension of these mice reflected a function of cGKI in VSMCs, endothelial cells, or other cell types such as cardiomyocytes. To overcome these limitations, a mouse line has been generated which allows the conditional inactivation of the cGKI gene in somatic cells [3]. Cardiomyocyte-specific cGKI mutants are fully viable and can be studied throughout adulthood. The combined analysis of conventional and cardiomyocyte-specific cGKI knockout mice demonstrated that cGKI mediates the negative inotropic effect of cGMP in the juvenile as well as in the adult murine heart [3]. In line with results obtained in older NOS III knockout mice [16,17], the NO/cGMP/cGKI pathway does not appear to be involved in the negative inotropic action of acetylcholine [3].

Platelet Function

NO is of major importance for the homeostasis of platelet-endothelium and platelet-platelet interactions by inhibiting the adhesion of platelets to injured endothelium, platelet activation, and aggregation [18]. *In vitro* and *in vivo* studies with cGKI-deficient platelets proved that these effects are mediated by activation of cGKI [19]. Platelet adhesion and aggregation during ischemia/reperfusion of the microcirculation was analyzed by intravital video microscopy in wild-type mice infused with cGKI-deficient platelets and cGKI-deficient mice infused with wild-type platelets. These experiments clearly showed that platelet cGKI but not endothelial or smooth muscle cGKI is essential to prevent intravascular adhesion and aggregation of platelets after ischemia, probably by inhibiting the activation of the platelet fibrinogen receptor, glycoprotein IIb–IIIa [19].

Gastrointestinal and Urogenital Function

Both cGKI and cGKII knockout mice show severe gastrointestinal malfunctions. cGKI-deficient mice have disturbed bowel movement leading to delayed passage of food [2]. Apparently, NO-dependent relaxation of stomach smooth muscle is disturbed leading to an increased tonus of the pylorus [2,20]. At present it is not clear whether or not the lack of cGKI in a subpopulation of the interstitial cells of Cajal [21] contributes to the motility defect. Deletion of the cGKII gene abolished CFTR-mediated intestinal water secretion after stimulation of particulate guanylyl cyclase by guanylin or the heat-stable toxin from *Escherichia coli* [1]. This disturbance is confined to the small intestine. Ion transport was normal in the colon of cGKII-deficient mice [22].

Deletion of the cGKI gene impaired the NO/cGMP-induced relaxation of penile smooth muscle leading to erectile dysfunction, but did not affect the motility and fertility of sperm [23]. Furthermore, the NO/cGMP-dependent relaxation of urinary tract smooth muscle is abolished in cGKI-null mutants [24].

Nervous System

Over the past few years a number of neuronal functions of cGKs have been identified. All three cGKs are expressed in specific brain regions of mammals. Analysis of naturally occurring *Drosophila* variants in food-searching behavior, combined with the introduction of transgenes and transposable P elements into the *Drosophila* genome, led to the identification of the *foraging* gene as the *dg2* gene, which encodes cGKII [25]. Behavioral analysis of the cGKII knockout mice revealed increased anxiety and increased consumption of alcohol at first contact [26]. cGKII is expressed in the amygdala and basal forebrain, structures thought to be involved in anxiety and addiction.

cGKIα is expressed in dorsal root ganglia and cerebellar Purkinje cells, whereas cGKIβ predominates in the hippocampus and olfactory bulb. Deletion of the cGKI gene led to impaired pathfinding of sensory neurons in the spinal cord and to a substantial reduction of nociceptive flexion reflexes [27], suggesting that cGKIα is required for the correct guidance of sensory neurons during development. The production of the cGKI and cGKII knockout mouse lines has also allowed to test whether or not these protein kinases are involved in hippocampal long-term potentiation (LTP) [28,29] and cerebellar long-term depression (LTD) [30]. These analyses showed that hippocampal LTP was not affected in juvenile (4 to 5 weeks old) conventional knockout mice lacking cGKI, cGKII, or both enzymes [31]. However, LTP was impaired partially in adult (at least 3 months old) mutants, in which the cGKI gene was inactivated specifically in the hippocampus. In agreement with the conventional cGKI knockout mice [31], LTP was unchanged in juvenile hippocampus-specific cGKI mutants (TK, RF, and FH, unpublished data). These phenotypes suggest that cGKs may regulate discrete neural functions in an age-dependent manner.

Preliminary experiments with Purkinje cell-specific cGKI mutants support the hypothesis that cGKI is an essential part in the regulation of LTD in the cerebellar Purkinje cells.

Bone

The particulate guanylyl cyclases, GC-A and GC-B, which generate cGMP upon binding of peptide ligands (ANP, BNP, and CNP) are expressed abundantly in mouse tibial epiphysis and vertebrae [32]. Cultivation of mouse tibia in the presence of BNP induced a significant increase in total bone length. Transgenic mice overexpressing BNP exhibited skeletal overgrowth that was restricted to bones with endochondral ossification [32]. cGKI and cGKII are expressed in the growth zones of bones [1]. The deletion of cGKI had no apparent effect on the growth of the skeleton. In contrast, cGKII-deficient mice are dwarfs, having limbs 16–30% shorter than normal [1]. Targeted expression of CNP in the growth plate chondrocytes failed to rescue the skeletal defect of the cGKII knockout mice, suggesting that cGKII plays a critical role in the CNP-mediated endochondral ossification [33].

Outlook

Taken together, the analysis of cGK-deficient mice has demonstrated the physiological relevance of these protein kinases as major mediators of cGMP signaling in diverse organs and tissues. cGKI plays an important role for cardiovascular and gastrointestinal homeostasis and has discrete functions in the central and peripheral nervous system. cGKII regulates longitudinal bone growth, intestinal ion transport, and renin release in the kidney, and it may be involved in the generation of complex behaviors like anxiety and addiction. In the future, the combined analysis of conventional and conditional cGK knockout mice and of cGK-deficient primary cells will further advance our understanding of the specific functions of cGKIα, cGKIβ, and cGKII in the mammalian body in health and disease. These studies may also help to develop new strategies for the treatment of cardiovascular, gastrointestinal, skeletal, and neuronal disorders.

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CHAPTER 205

Cyclic Nucleotide-Regulated Cation Channels

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Introduction

Cyclic nucleotides exert their physiological effects by binding to four major classes of cellular receptors: cAMP- and cGMP-dependent protein kinases [1], cyclic GMP-regulated phosphodiesterases [2], cAMP-binding guanine nucleotide exchange factors [3], and cyclic nucleotide-regulated cation channels. Cyclic nucleotide-regulated cation channels are unique among these receptors because their activation is directly coupled to the influx of extracellular cations into the cytoplasm and to the depolarization of the plasma membrane. Two families of channels regulated by cyclic nucleotides have been identified, the cyclic nucleotide-gated (CNG) channels and the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels [4–7]. The two channel classes differ from each other with regard to their mode of activation. CNG channels are opened by direct binding of cAMP or cGMP. In contrast, HCN channels are principally operated by voltage. These channels open at hyperpolarized membrane potentials and close on depolarization. Apart from their voltage sensitivity, HCN channels are also activated directly by cyclic nucleotides, which act by increasing the channel open probability.

General Features of Cyclic Nucleotide-Regulated Cation Channels

Structurally, both CNG and HCN channels are members of the superfamily of voltage-gated cation channels. Like other subunits encoded by this large gene family, CNG and HCN channel subunits are believed to assemble to tetrameric complexes. The proposed structure and the phylogenetic relationship of mammalian CNG and HCN channel subunits is shown in Fig. 1 (see also Table I for recent nomenclature). The transmembrane channel core consists of six α-helical segments (S1–S6) and an ion-conducting pore loop between the S5 and S6. The amino- and carboxy-termini are localized in the cytosol. CNG and HCN channels contain a positively charged S4 helix carrying three to nine regularly spaced arginine or lysine residues at every third position. In HCN channels, as in most other members of the channel superfamily, the S4 helix functions as "voltage-sensor" conferring voltage-dependent gating [8,9]. In CNG channels, which are not gated by voltage, the specific role of S4 is not known.

CNG and HCN channels reveal different ion selectivities. CNG channels conduct both Ca^{2+} and monovalent cations with permeability ratios P_{Ca}/P_{Na} ranging from about 2 to 25 depending on the respective channel type and the cyclic nucleotide concentration [10,11]. By providing an entry pathway for Ca^{2+}, CNG channels control a variety of cellular processes that are triggered by this cation. In contrast, HCN channels are not permeable to Ca^{2+}. These channels pass Na^+ and K^+ ion with a relative permeability ratio P_{Na}/P_K of about 0.15 to 0.25 [12–14].

In the carboxy-terminus, CNG and HCN channels contain a cyclic nucleotide-binding domain (CNBD) that has significant sequence similarity to the CNBDs of most other

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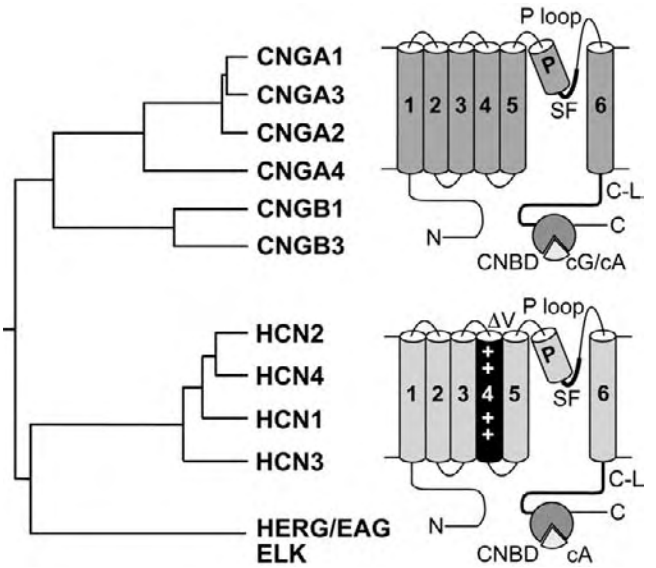


Figure 1 Phylogenetic tree and structural model of cyclic nucleotide-regulated cation channels. The CNG channel family comprises six members, which are classified into α subunits (CNGA1-4) and β subunits (CNGB1 and CNGB3). A "CNGB2" subunit does not exist. The HCN channel family comprises four members (HCN1-4). The channels are distantly related to EAG/HERG and ELK K^+ channels [49]. CNG and HCN channels share a common transmembrane topology, consisting of six transmembrane segments (1–6), a pore loop, and a cyclic nucleotide-binding domain (CNBD). The pore loop comprises the pore helix (P) and an uncoiled strand of 4 to 5 amino acid residues forming the selectivity filter (SF). CNG channels are activated *in vivo* by binding of either cAMP (cA) or cGMP (cG), depending on the channel type. HCN channels activate on membrane hyperpolarization (ΔV) and are enhanced by binding of cAMP. The positively charged amino acid residues in the S4 segment of HCN channels are indicated by "+" symbols. CL, C-linker involved in activation gating of CNG and HCN channels.

Table I Adopted Nomenclature for Cyclic Nucleotide-Regulated Ion Channel Subunits [5,48]

Adopted nomenclature	Previous designations
CNGA1	CNG1/CNGα1/RCNC1
CNGA2	CNG2/CNGα3/OCNC1
CNGA3	CNG3/CNGα2/CCNC1
CNGA4	CNG5/CNGα4/OCNC2/CNGB2
CNGB1	CNG4/CNGβ1/RCNC2
CNGB3	CNG6/CNGβ2/CCNC2
HCN1	HAC2/BCNG1
HCN2	HAC1/BCNG2
HCN3	HAC3/BCNG4
HCN4	HAC4/BCNG3

types of cyclic nucleotide receptors (Fig. 2). In CNG channels, the binding of cyclic nucleotides to the CNBD initiates a sequence of allosteric transitions that lead to the opening of the ion-conducting pore [15]. In HCN channels, the binding of cyclic nucleotides is not required for activation. However, cyclic nucleotides shift the voltage-dependence of channel activation to more positive membrane potentials and thereby facilitate voltage-dependent channel activation [12–14]. Despite the fact that the CNBDs of HCN and CNG channels show significant sequence homology, the two channel classes reveal different selectivities for cyclic nucleotides. HCN channels display an approximately ten-fold higher affinitiy for cAMP than for cGMP whereas CNG channels select cGMP over cAMP [4,5].

CNG Channels

CNG channels are expressed in retinal photoreceptors and olfactory neurons and play a key role in visual and olfactory signal transduction [16]. CNG channels are also found at low density in some other cell types and tissues such as brain, testis, and kidney (for recent compilation of tissue expression see [17]). Although the function of CNG channels in sensory neurons has been unequivocally demonstrated, the role of these channels in other cell types remains to be established. Based on phylogenetic relationship, the six CNG channels identified in mammals are divided in two subfamilies, the α subunits (CNGA1-4) and the β subunits (CNGB1 and CNGB3) (Fig. 1). CNG channel α subunits (with the only exception of CNGA4) form functional homomeric channels in various heterologous expression systems. In contrast, β subunits do not give rise to functional channels when expressed alone. However, together with CNGA1-3 they confer novel properties (e.g. single channel flickering, increased sensitivity for cAMP and L-cis diltiazem) that are characteristic of native CNG channels [4]. The physiological role and subunit composition are known for three native CNG channels: the rod and cone photoreceptor channel and the olfactory channel. The CNG channel of rod photoreceptors consists of the CNGA1 subunit [18] and a long isoform of the CNGB1 subunit (CNGB1a [19]). The cone photoreceptor channel consists of the CNGA3 [20] and the CNGB3 [21] subunit. CNG channels control the membrane potential and the calcium concentration of photoreceptors. In the dark both channels are maintained in the open state by a high concentration of cGMP. The resulting influx of Na^+ and Ca^{2+} ("dark current") depolarizes the photoreceptor and promotes synaptic transmission. Light-induced hydrolysis of cGMP leads to the closure of the CNG channel. As a result the photoreceptor hyperpolarizes and shuts off synaptic glutamate release. Mutations in CNG channel genes have been linked to retinal diseases. Mutations in the CNGA1 [22] and CNGB1 [23] subunits have been identified in the genome of patients suffering from retinitis pigmentosa. The functional loss of either the CNGA3 [24,25] or the CNGB3 [26] subunit causes total color blindness (achromatopsia) and degeneration of cone photoreceptors.

The CNG channel expressed in cilia of olfactory neurons consists of three different subunits: CNGA2 [27,28], CNGA4 [29,30], and a short isoform of the CNGB1 subunit (CNGB1b) [31,32]. The channel is activated *in vivo* by cAMP, which is synthesized in response to the binding of

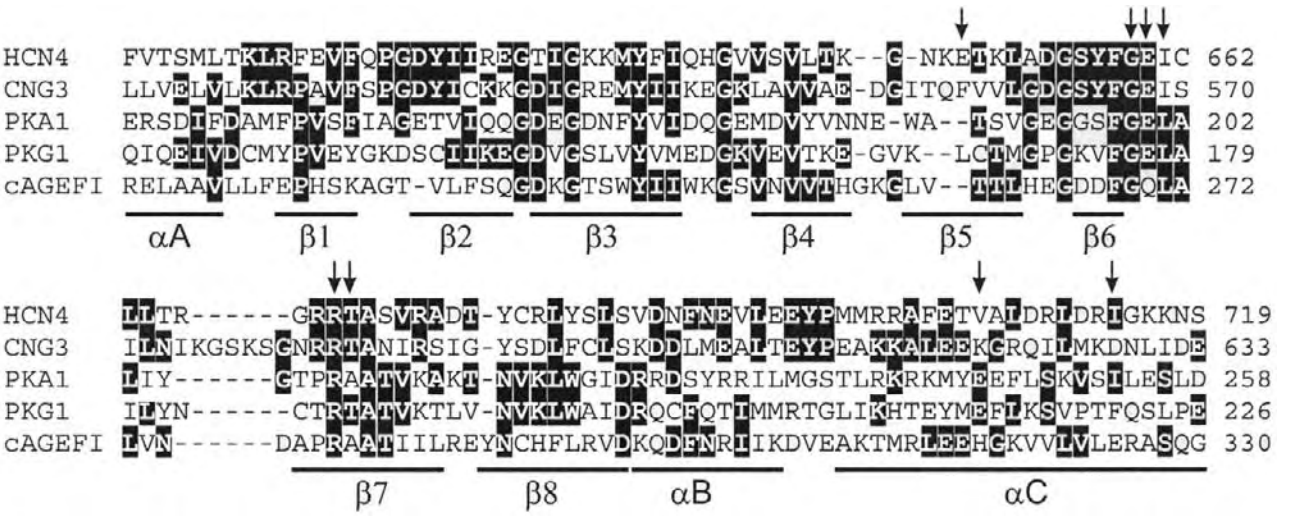


Figure 2 Amino acid squences of CNBDs of cyclic nucleotide-binding proteins. Residues identical in at least two sequences are highlighted by a black background. Amino acids that are supposed to interact with cyclic nucleotides in CNG channels are indicated by arrows [50]. HCN4 (human HCN4, accession NP_005468), CNG3 (bovine CNG3, accession Q16281), PKA1 (cAMP binding site 1 of bovine cAMP-dependent protein kinase, α-catalytic subunit, accession P00517), PKG1 (cGMP-binding site 1 of bovine cGMP-dependent protein kinase type I α, accession P00516), cAGEF1 (human cAMP-GEF1, accession NP_006096).

odorants to their cognate receptors. The olfactory CNG channel is thought to conduct almost exclusively Ca^{2+} under physiological ionic conditions [33]. The resulting increase in cellular Ca^{2+} activates a Ca^{2+}-activated Cl^- channel, which further depolarizes the cell membrane. Ca^{2+} is not only a permeating ion of the olfactory CNG channel, it also represents an important modulator of this channel. By forming a complex with calmodulin that binds to the CNGA2 subunit, Ca^{2+} decreases sensitivity of the CNG channel to cAMP [34]. The resulting inhibition of channel activity is the principal mechanism underlying odorant adaptation [35].

HCN Channels

A cation current that is slowly activated by membrane hyperpolarization (termed I_h, I_f, or I_q) is found in a variety of excitable cells, including neurons, cardiac pacemaker cells, and photoreceptors [36]. The best understood function of I_h is to control heart rate and rhythm by acting as "pacemaker current" in the sinoatrial (SA) node [37]. I_h is activated during the membrane hyperpolarization following the termination of an action potential and provides an inward Na^+ current that slowly depolarizes the plasma membrane. Sympathetic stimulation of SA node cells raises cAMP levels and increases I_h by a positive shift of the current activation curve, thus accelerating diastolic depolarization and heart rate. Stimulation of muscarinic receptors slows down heart rate by the opposite action. In the brain I_h fulfills diverse functions: it controls the activity of spontaneously spiking neurons ("neuronal pacemaking" [36]), it is involved in the determination of resting potential [36], in photoreceptors it provides rebound depolarizations in response to pronounced hyperpolarizations [36], it is involved in the transduction of sour taste [38], and it is involved in the control of synaptic plasticity [39].

HCN channels represent the molecular correlate of the I_h current [5–7]. In mammals, the HCN channel family comprises four members (HCN1-4) that share about 60 percent sequence identity to each other and about 25 percent sequence identity to CNG channels. The highest degree of sequence homology between HCN and CNG channels is found in the CNBD. When expressed in heterologous systems all four HCN channels generate currents displaying the typical features of native I_h: (1) activation by membrane hyperpolarization, (2) permeation of Na^+ and K^+, (3) positive shift of the voltage-dependence of channel activation by direct binding of cAMP, and (4) channel blockade by extracellular Cs^+. HCN1–4 mainly differ from each other with regard to their speed of activation and the extent by which they are modulated by cAMP. HCN1 is the fastest channel, followed by HCN2, HCN3, and HCN4. Unlike HCN2 and HCN4, whose activation curves are shifted by about +15 mV by cAMP, HCN1 is only weakly affected by cAMP (shift of less than +5 mV). Site-directed mutagenesis experiments have provided initial insight into the complex mechanism underlying dual HCN channel activation by voltage and cAMP. Like in other voltage-gated cation channels, activation of HCN channels is initiated by the movement of the positively charged S4 helix in the electric field [8,9]. The resulting conformational change in the channel protein is allosterically coupled by other channel domains to the opening of the ion-conducting pore. Major determinants affecting channel activation are the intracellular S4–S5 loop [40], the S1 segment [41], and the extracellular S1–S2 loop [41]. The CNBD fulfills the role of an auto-inhibitory channel domain. In the absence of cAMP the cytoplasmic carboxy-terminus inhibits HCN channel gating by interacting with the channel

core and thereby shifting the activation curve to more hyperpolarizing voltages [42]. Binding of cAMP to the CNBD relieves this inhibition. Differences in the magnitude of the response to cAMP among the four HCN channel isoforms are largely due to differences in the exent to which the CNBD inhibits basal gating. It remains to be determined whether the inhibitory effect of the CNBD is conferred by a direct physical interaction with the channel core domain or by some indirect pathway. There is initial evidence that the so-called C-linker, a peptide of about 80 amino acids that connects the last transmembrane helix (S6) to the CNBD, plays an important role in this process [43] (Fig. 1). The C-linker was also shown to play a key role in the gating of CNG channels, suggesting that the functional role of this domain has been conserved during channel evolution [15].

HCN channels are found in neurons and heart cells. In SA node cells, HCN4 represents the predominantly expressed HCN channel isoform [44,45]. In mouse brain all four HCN isoforms have been detected [46,47]. The expression levels and the regional distribution of the HCN channel mRNAs vary profoundly between the respective channel types. HCN2 is the most abundant neuronal channel and is found almost ubiquitously in the brain. In contrast, HCN1 and HCN4 are enriched in specific regions of the brain such as thalamus (HCN4) or hippocampus (HCN1). HCN3 is uniformly expressed throughout the brain, however at very low levels. HCN channels have also been detected in the retina and some peripheral neurons such as dorsal root ganglion neurons [45].

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CHAPTER 206

Epacs, cAMP-Binding Guanine Nucleotide Exchange Factors for Rap1 and Rap2

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Introduction

In 1998 two independent groups identified a novel family of cAMP-binding proteins, Epacs or cAMP-GEFs [1,2]. These highly conserved and rather ubiquitously expressed proteins serve as guanine nucleotide exchange factors (GEFs) for the small GTPases Rap1 and Rap2 and as such induce signal transduction pathways independent from protein kinase A (PKA). One process among others that is controlled by Rap is integrin-mediated cell adhesion [3].

The Epac Family

In humans the Epac (exchange protein directly activated by cAMP) family consist of two members, Epac1 and Epac2 (Fig. 1). Epac1 has in its N-terminal part a DEP (dishevelled, egl-10, pleckstrin) domain, responsible for membrane localization, followed by a cAMP-binding site that closely resembles the cAMP-binding domains in PKA and cyclic nucleotide gated ion channels (Fig. 2). The C-terminal part consists of the REM domain and the catalytic domain. These domains closely resemble similar domains in GEFs for other members of the Ras family, such as Sos and RasGRP. The CDC25-homology domain mediates the exchange of guanine nucleotides bound to the target GTPase in the case of

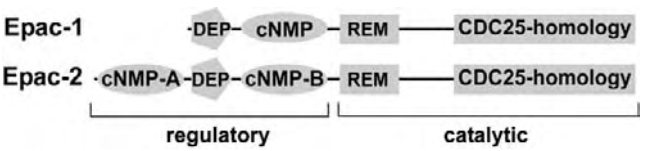


Figure 1 Domain organization of Epac. The regulatory and the catalytic part of the protein are indicated. Dishevelled, egl-10, pleckstrin (DEP) cyclic nucleotide binding domain (cNMP), Ras exchange motif (REM), CDC25 homology domain (CDC25 homology).

Epac Rap1 and Rap2. The REM domain is responsible for the stabilization of the CDC-25 homology domain [4] but is not directly involved in the catalysis of nucleotide exchange. Epac2 has in addition a second N-terminal cAMP domain.

The cAMP-Binding Domain of Epac Closely Resembles Those of PKA and Channels

Sequence comparison between various cAMP-binding sites as well as the crystal structure of cAMP-binding sites of PKA in the presence of cAMP identifies various consensus sequences specific for cAMP–binding, including the phosphate binding cassette (PBC) [5]. These motifs are all present in the cNMP-binding domain of Epac1, as well as in the second cNMP-binding domain of Epac2 (Fig. 2)

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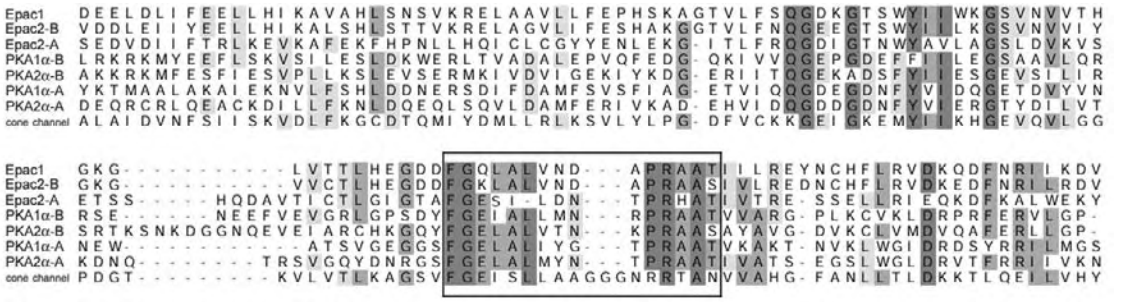


Figure 2 The phosphate binding cassette (PBC) is clearly conserved in Epac. The PBC is marked by a box. All sequences shown are human.

Epac Is Conserved Through Evolution

In higher organisms two Epac proteins, Epac1 and Epac2 (Fig. 1), are found, which show strong sequence homology not only among species (human Epac2 and mouse Epac2) but also between each other (human Epac1 and human Epac2) (Fig. 3). The major difference between Epac1 and Epac2 is an additional N-terminal cNMP-binding domain. In lower organisms only one Epac is present that is more related to Epac2, due to its additional putative cNMP-binding domain. However, this domain is less conserved and lacks PBC, which makes cAMP binding to these domains highly unlikely. Note also that sequence conservation is strongest in the CDC25 homology domain, which mediates the catalytic activity, and in the more C-terminal cNMP binding domain, which is the (main) regulatory element. A minor role in regulation for the first cNMP-binding domain fits the biochemical data (see below).

Properties of Epac

In vitro experiments have shown that in the absence of cAMP, Epac is unable to increase intrinsic exchange of nucleotide on the small GTPase Rap1. However, cAMP accelerates the exchange activity by at least two orders of magnitude, proving the responsiveness of Epac to cAMP. By using isothermal titration calorimetry, the affinity of cAMP has been determined for each of the single domains of Epac1 and Epac2, as well as for the whole regulatory domain of Epac2 (Table I). These data indicate that there is no significant cooperativity in cAMP binding to the two cAMP-binding sites in the regulatory part of Epac2 [6]. In addition, deletion of the first cAMP-binding domain from Epac2 does not abolish the requirement of cAMP for activity [6]. The second cAMP-binding domain, which is conserved in the domain structure of Epac1 and Epac2, is therefore sufficient to maintain the inactive state of Epac2 in the absence of cAMP. This is in agreement with the relative low conservation of the first cAMP domain between species (see above).

The value of half maximal activity (IC_{50}-value) of Epac was determined by measuring the concentration dependency of cAMP-induced activation of Epac. For Epac1 an IC_{50} value of 40 μM is found (H. Rehman, unpublished data). This value reflects the affinity of cAMP for full-length Epac, and is clearly lower than the affinity of cAMP for the separate regulatory domain. This implies that the presence of the catalytic part of the protein reduces the cAMP affinity. Apparently, a conformational change induced by cAMP binding "consumes" parts of the binding energy.

Expression and Subcellular Localization of Epacs

Epac1 is expressed in a large number of tissues tested but most notably in kidney, ovary, thyroid, heart, and brain, where it is confined to the septum and the thalamus of the neonatal brain. Epac2 is predominantly expressed in the cerebral cortex, the hippocampus, the habenula, and the cerebellum of the brain and in the adrenal gland [2]. Subcellularly, Epac 1 is attached to membranes through its DEP domain [6], and an Epac-GFP fusion protein is located predominantly in the preinuclear region, where Rap1 and Rap2 are also located (F. J. Zwartkruis, unpublished observation).

Cellular Function of Epacs

Epacs mediate the activation of Rap1 and Rap2 by receptors that elevate the levels of cAMP. However, the biological effects are still unclear. A number of reports indicate cAMP-mediated but PKA-independent events that may be mediated by Epac. For instance, the following events have been noted: (1) cAMP-induced translocation of CFTR-like chloride channels in renal cells [7], (2) cAMP regulation of Glut-1 translocation to plasma membrane through PI3-kinase-dependent and PKA-independent signaling pathways [8], (3) cAMP-induced calcium influx in PBL cells [9], (4) cAMP-induced activation of PKB in thryroid cells [10], and (5) cAMP-induced transcription in glioma cells [11].

cAMP-induced Epac-mediated regulated secretion in pancreatic B-cells [12,13] is the only effect directly assigned to Epacs, but a role for Rap in this process was not established.

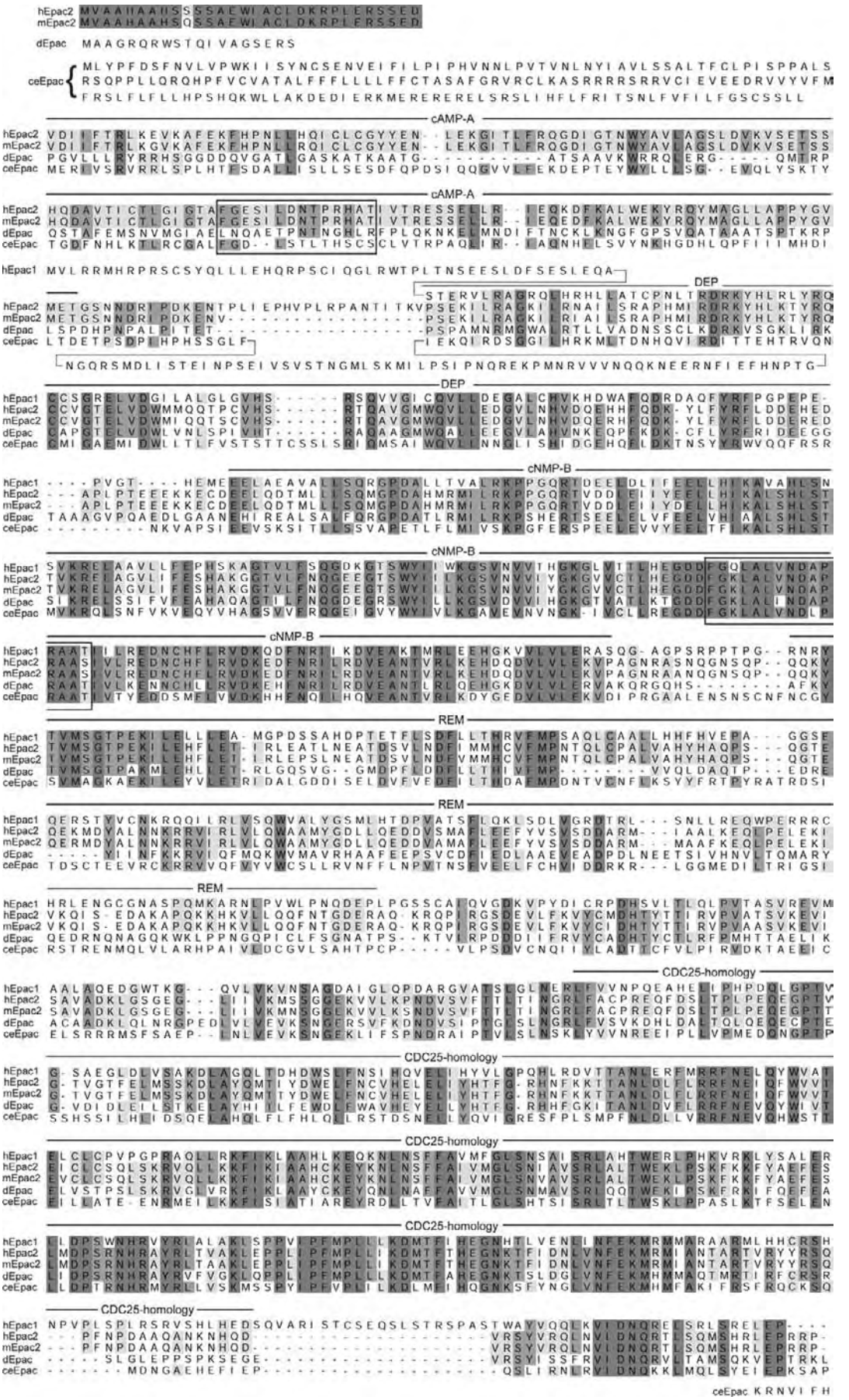


Figure 3 Sequence alignment of Epac from various species. The variable N-terminus is excluded form the alignment and given as sequence only. Domains are indicated according to Fig. 1 by bars over the sequence. The conserved phosphate binding cassette (PBC) is highlighted by boxes in both cNMP binding domains (h, *homo sapiens*; m, *mus musculus*; d, *Drosophila melanogaster*; ce, *Caenorhabditis elegans*).

Tabel I Affinities of cAMP Binding Domains

	Epac2		
Epac1	cAMP-A	cAMP-B	RD
4 µM	90 µM	1 µM	80 µM ; 0.5 µM

Rap1 has been implicated in a variety of cellular functions, most notably in the regulation of ERK activity. However, these effects are mediated by PKA and Epacs are not involved. Whether this implies that a different pool of Rap1 is activated by PKA or that Rap1 is not involved in this process at all is still a matter of debate, although we favor the second alternative.

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CHAPTER 207

Cyclic Nucleotide-Binding Phosphodiesterase and Cyclase GAF Domains

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Introduction

The GAF domains are one of the largest and most widespread of the small-molecule binding domains [1]. They are distantly related to PAS domains, another superfamily with a similar fold [1]. The acronym GAF was coined from the first three protein families in which they were identified: cyclic c*G*MP-specific and -regulated cyclic nucleotide phosphodiesterases, Anabaena adenylyl cyclase, and E. coli transcription factor *F*hlA [2]. Over 890 GAF domain containing proteins have now been identified in a non-redundant database [3]. They are involved in many signal transduction pathways, protein regulation, and sensory systems [1,2]. This review will focus on the structure and function of cyclic nucleotide binding GAF domains found in PDEs and also include a short discussion of several others to which they are related.

cAMP and cGMP are used as second messengers in many cellular signal transduction pathways. The intracellular levels of cyclic nucleotides are determined by the relative activities of adenylyl and guanylyl cyclases, the enzymes that catalyze their synthesis, and cyclic nucleotide phosphodiesterases (PDEs), which hydrolyze 3′,5′ cyclic nucleotides to their respective inactive 5′-nucleotide monophosphates. Eleven PDE families have been identified in mammalian tissues [4]. PDEs are nearly all homodimers with monomer molecular weights ranging from 50 to 135 kDa. The PDEs constitute the major pathway for the elimination of the cyclic nucleotide signal from the cells to precisely maintain their levels within a narrow range.

PDEs are multidomain proteins with a C-terminal conserved catalytic domain and one or more N-terminal regulatory segments. The regulatory segment from five of the PDE families (PDE2, 5, 6, 10, 11) contain one or two GAF domain modules. For the first three families, cGMP-binding to at least one GAF domain in the regulatory segment modifies the PDE's catalytic activity. In PDE2, cGMP bound to the GAF domain stimulates the Vmax by more thanten-fold [5–8]. For PDE5A, cGMP binding to GAF A stimulates the phosphorylation by PKG of a Ser just N-terminal to GAF A [9] and increases the catalytic activity.

In rod and cone photoreceptor cells, the catalytic activity of PDE6 is inhibited by binding of a small 9 kDa inhibitory gamma subunit whose affinity is regulated by binding of cGMP to the regulatory segment. The apparent binding affinity of frog rod Pγ for PDE6 was 28 pM in the presence of cGMP and 16-fold weaker with the GAF sites unoccupied [10]. Unlike PDE2A and 5A, there is no evidence for this enzyme showing direct cooperativity between the GAF domains and catalytic sites. cGMP binding by GAF A does not act allosterically on the catalytic domain. Instead, it binds the central polycationic region of Pγ and increases the affinity of

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the C-terminal region for the catalytic site [11]. In turn, binding or dissociation of cGMP to GAF A may also influence the interaction with transducin or its cognate GAP protein, RGS9. A photoactive peptide containing the polycationic region of Pγ cross-linked to two residues in GAF A [12]. There is circumstantial evidence for gamma-like subunits associated with PDE5A in lung [13].

Two more recently discovered and less well-characterized PDE families, PDE10A and PDE11A, are homologous to PDE 2, 5, and 6 in their regulatory segment and contain GAF domains; however, careful binding studies have not yet been reported. A preliminary study suggests that PDE10A lacks a high-affinity cGMP binding site but may contain a low affinity site [14]. One unique structural feature of this family is the presence of splice variants that contain truncated GAF domains in the N-terminal region. These tissue-specific splice variants have been postulated mostly from Northern blot or RT-PCR data, and their existence as active proteins in the cell is not yet fully confirmed [15–18]. However, a genomics study of the PDE11A gene shows separate promoters for both splice variants with an incomplete GAF A (PDE11A3) or GAF B domain (PDE11A1) [19]. It is interesting that a similar series of structures have been seen in a PDE family from Trypanosomes [20,21]. The initial studies of PDE11A1 suggest that neither cAMP nor cGMP are allosteric effectors [18]. However, the splice variant tested lacked the GAF A of the full-length enzyme (PDE11A4) and has an incomplete GAF B. PDE11A4, with both GAF A and B, had no significant cGMP or cAMP binding activity in another preliminary study [17]. A "half-a-PAS" splice variant has been reported for PDE8A, the only PDE to contain a PAS domain [22].

Atomic Structure

The first atomic structure of a GAF domain came recently from a structural genomics project for signal transduction domains. A hypothetical GAF protein, YKG9, from the yeast genome was characterized at high resolution. However, yeast do not make cGMP and this protein did not bind the nucleotide [23]. The first atomic structure of a PDE GAF domain, in this case with bound cGMP, was recently determined (Martinez *et al.*, PNAS). The dimeric regulatory segment of mouse PDE2A, containing both the GAF A and B domains, was determined at 2.9 Å resolution. GAF A and B have very similar folds to YKG9. Although both the catalytic and GAF domains of PDEs bind cyclic nucleotides, and some homology exists, their folding structures are quite different and they appear to be examples of convergent evolution. For example, the catalytic domain of PDE4 is a bundle of 17 alpha helices in three subdomains [24], whereas the GAF domain is a beta sheet packed on the back side with two to four alpha helices and on the other side with a mixture of short alpha helices and loops that form the sides of the ligand-binding pocket [23]. In addition, the zinc-binding motif of the catalytic domain [25] is not present in the GAF domain.

These two new GAF domain structures from PDE2A can now be compared with that of YKG9. The overall folds of the three GAF domains with known structure are very similar, with the yeast variant having an additional N-terminal helix compared to the PDE2A GAF A. Yeast YKG9 (PDB 1F5M, monomer A) is distantly related to PDE2A GAF A with 136 Cα atoms superimposing within 4.0 Å and a sequence identity of 10 percent, and to GAF B with 130 equivalent Cα atoms that superimpose within an r.m.s deviation of 3.3 Å and a sequence identity of 16 percent.

In PDE2A, GAF A and B are connected through short linkers of several residues to a 32 residue long connecting helix of nine turns (Fig. 1). Unexpectedly, GAF A is engaged in numerous inter-subunit interactions, whereas the GAF B domains are well separated. The first five turns of the connecting helix also provide inter-subunit contacts but afterwards diverge. This occurs after C386, a residue that appears (at this somewhat limited resolution) to form a disulfide bond. A C386S mutant has an essentially identical structure. Therefore, the presence or absence of this disulfide bond has little effect on conformation. Whether this disulfide is integral to the activation or positive cooperativity of PDE2A remains to be determined. GAF A has a surprising, completely different dimer interface than the YKG9 dimer, with contacts from a completely different set of secondary structure elements. Recently, the overall shapes of the bovine PDE6A/B and PDE5A have been visualized at 28 Å resolution from uranyl-stained electron microscopy images [26]. These data show that both PDEs are dimers with the N-terminal regions interacting, which is clearly consistent with the dimerization interface between GAF A domains elucidated at higher resolution in PDE2A. An intriguing finding is that the dimerizing connecting helices that separate GAF A and B in PDE2A appear in the micrographs to be holes surrounded by connections of electron density. Although this could be an artifact of the low resolution and/or the uranyl acetate negative stain, the sequence of the PDE2A connecting helix is poorly conserved among the GAF PDEs.

In the 2.9 Å resolution crystal structure of PDE2A, electron density was found for a molecule of cGMP bound to GAF B. The binding site corresponds to the PAS pocket, showing the conservation of ligand binding function. It is interesting that the cGMP is completely buried, along with three bound waters. This strongly suggests that the protein must also exist in another more open state in which the initial binding occurs. The most distinctive feature of cGMP, its 3′,5′ cyclic phosphate group, has no side chain contacts. Two backbone amides (I458, Y481, A459) and a water make hydrogen bonds to the exocylic oxygens of the phosphate. In addition, the positive end of the helix dipole of helix α3 points directly at the negatively charged phosphate group. The guanine ring and ribose group make a total of six polar contacts and two hydrophobic contacts. One residue, D439, makes both a side chain contact (to the N2 nitrogen) and a backbone amide contact (to the O6 carbonyl). We believe this residue is a main specificity determinant for cGMP over cAMP.

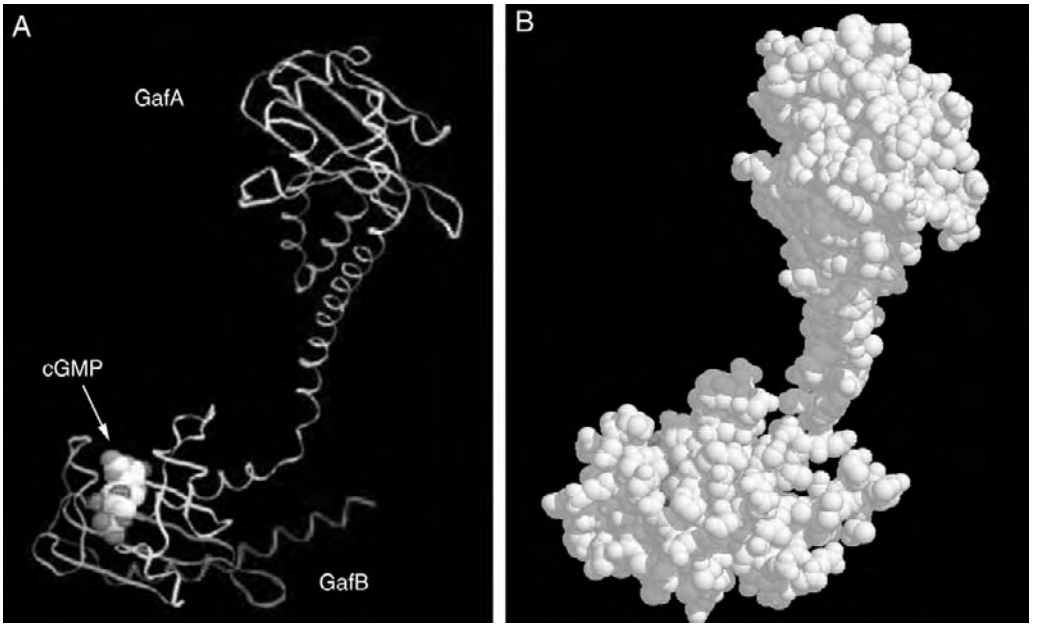


Figure 1 Structure of the PDE2A GAF A/GAF B monomer plus cGMP. Shown on the left is a ribbon structure of the monomer with a space-filling model of cGMP bound to GAF B. Shown on the right is a full space-filling model of both. Notice that the cGMP is almost completely buried. Less than 1 percent of the molecule is exposed to solvent. Presumably therefore the protein must go through some other conformation in order to bind the cyclic nucleotide.

Aside from this work, published cGMP binding to individual PDE GAF domains have only been done for PDE5A, which shows binding to the GAF A domain [23,27]. A comparison of the 2A GAF B structure to the 5A GAF A sequence suggests that the binding motif is SX(13-18)FDX(18-22) IAX(21)[Y/N]X(2)VDX(2)TX(3)TX(19)[E/Q]. The [Y/N] and IA are backbone contacts not expected to be conserved. It is a theoretical possibility that PDE2A GAF A and PDE5A GAF B may be able to form additional cGMP binding sites, but the poor conservation of the motif and biochemical data suggest otherwise. Given the variety of ligands bound by PAS and GAF domains, it raises the intriguing possibility of unknown small molecule ligands regulating GAF PDEs. PDE6 is the PDE whose stoichiometry of cGMP is best documented, one 1 cG/monomer. The binding motif is best conserved in GAF A.

Although the YKG9 structure has no bound ligand and does not bind cGMP [23], it does contain a buried pocket with a volume of 590 $Å^3$ corresponding to the location of the cGMP-binding site of our GAF domain. A structural superposition of YKG9 onto GAF B shows approximately 11 ordered waters, which are scattered about the volume occupied by the cGMP molecule in PDE2A GAF B. It is of possible significance that the YKG9 GAF pocket contains a buried acidic cluster (E132, D149, and D151). This may imply that YKG9 could bind an as yet unknown, positively charged ligand. One of these residues (D149) superimposes with E512 in PDE2A GAF B, the only charged residue buried in that GAF pocket.

The PDE2A GAF A+B structure does not suggest an obvious explanation for the activation mechanism of PDE2A. In a recent paper, a cAMP-activated cyanobacterial adenylyl cyclase was enzymologically characterized and shown to contain GAF A and GAF B domains, like PDE2A. Binding of cAMP but not cGMP to this domain activated the cyclase. However, the organization of the cyclase differed in that it also contains a PAS domain interspersed in between GAF B and the cyclase domain [28]. Although the overall sequence homology of the cyanobacterial and mammalian GAF domains is not particularly high, it appears that the key residues necessary for binding in PDE2A GAF B are present (Fig. 2). Substitution of the mammalian PDE2 GAF A/GAF B domain cassette into the cyanobacterial cyclase amazingly allowed a fully functional cyclase to be formed, but one that now could be activated by cGMP. These data strongly suggest that the GAF domains function as a general, highly conserved, cyclic nucleotide switch for activating adjacent catalytic domains. In this case two organisms that are over two billion years removed from each other in evolution can utilize the other's GAF domain structural motif. The intervening PAS domain in the adenylyl cyclase apparently does not block this mechanism. This result argues against an intramolecular association between the GAF A and B assembly and the catalytic domain of either enzyme. More likely, binding of cGMP creates a conformational change in GAF B that is transmitted mechanically to the catalytic domain. This may involve a loop that blocks the active site. Alternatively, if the catalytic domains contact each other, the active site may be partially occluded in the inactive form by the opposite domain. A third possibility is conformational change within the active site itself.

In nearly all PDE GAF domains, as well as in the adenylyl cyclase cyaB1 of Anabaena, there is a conserved

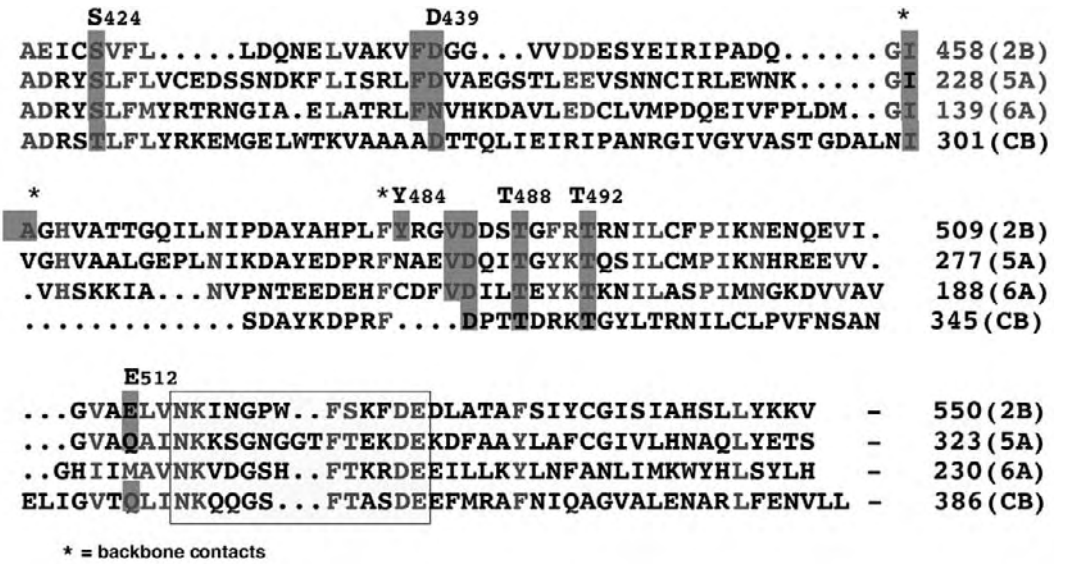


Figure 2 Comparison of cNMP binding GAF domain sequences. Clustal W sequence alignment of the four GAF domains known to bind cyclic nucleotide. Gaps were further aligned by eye. 2B, mouse PDE*2*A GAF *B*; 5A, human PDE*5*A GAF *A*; 6A, human PDE*6*A GAF A; and CB, Anabaena Adenylyl *C*yclase GAF **B**. Notice that in this alignment nearly all of the major cGMP contact points (shading) present in the PDE2A GAF B crystal structure are present in all of the other GAF domains that bind cyclic nucleotide. Backbone contacts are shown by asterisks. The original GAF domain signature sequence NKxxxFxxDE sequence found in the PDE GAF domains is also present in the Anabaena cyclase.

N[KR]X_nFX_3DE motif (Fig. 2) [29]. This motif straddles a loop on the other side of the beta sheet and approximately 17 Å from the cGMP binding pocket in PDE2A GAF B. Nevertheless, point mutations in the N[KR]X_nFX_3DE motif of GAF A, but not GAF B, of PDE5 greatly weaken cGMP binding [30]. In the structure of both GAF A and GAF B of PDE2, a salt bridge appears to be present between the K and D residues. This suggests that this motif may be important for conformation, or for a conformational change upon cGMP binding. Similar mutations in the Asp of this motif in GAF B but not GAF A of Anabaena AC disabled cAMP-dependent activation of cyclase activity [28].

The first crystal structures of GAF domains have solved many questions regarding regulation of the proteins but have also raised many questions. There may well be other ligands for each of the non-cGMP binding GAF domains in PDE2, PDE5, PDE6, and possibly the poorly characterized PDE10 and PDE11. What is the role of the GAF domains that don't bind cGMP? Do they serve only as dimerization domains for the holoenzyme? Is the common regulatory mechanism hinted at by the Anabaena cyclase/ratPDE2 chimera also conserved among the five GAF PDE families, yet affects the catalytic domain of three different PDE in such different ways? Ultimately, crystal or NMR structures will be needed for the various PDE domains with and without bound ligands to visualize the conformational changes that occur upon binding. Structures of the full-length holoenzymes and possibly also chimeras may also be required. Since these small molecule-binding GAF domains are intimately involved in regulation of so many different enzymes, they are also likely to become good targets for drug development.

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CHAPTER 208

cAMP Signaling in Bacteria

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Introduction and Significance

Our understanding of cyclic AMP (cAMP) signaling in bacteria has resulted primarily from studying the *Escherichia coli* catabolite gene activator protein (CAP), also known as the cAMP receptor protein (CRP). CAP is a 45 kDa homodimer that positively regulates the expression of over 150 genes [1]. Transcriptional activation of RNA polymerase by CAP requires that CAP bind cAMP, undergo an allosteric conformational change, and bind to a specific DNA sequence near the polymerase binding site.

Kolb *et al.* [2] write that "[t]he title of paradigm has been awarded to CRP on many occasions and for many of its functions." This is well-deserved. CAP is one of the most studied transcriptional regulators [3]. The conformational change of CAP upon cAMP binding has become an important paradigm for allostery [4]. The CAP DNA–binding domain contains a helix-turn-helix motif conserved in a large number of DNA-binding proteins; CAP is a founding member of this "First Family" of DNA-binding proteins, which include the homeodomain and the "winged helix" subfamilies [5,6]. The cAMP-binding domain is homologous to the regulatory subunit of cAMP-dependent protein kinases and the cyclic nucleotide binding domains of cyclic nucleotide-gated channels [7,8]. This homology to CAP has furthered our understanding of cyclic nucleotide-gated channels [8,9].

Background and History

In an environment containing glucose and other carbon sources, *E. coli* metabolizes glucose while metabolism of other sources is inhibited. Upon glucose depletion, the enzymes that metabolize the remaining carbon sources are induced. This phenomenon has been called the "glucose effect" or "catabolite or glucose repression" and was studied extensively by Jacques Monod over 60 years ago [10,11]. This effect is mediated by cAMP. *E. coli* growing in glucose-containing media have low cAMP levels; but once glucose is removed, cAMP concentrations increase rapidly [12]. Furthermore, exogenously added cAMP also relieves glucose repression [13,14]. Glucose indirectly inhibits adenylate cyclase, which synthesizes cAMP from ATP [15]. CAP was discovered over thirty years ago and was shown to bind to cAMP and mediate its effects [16,17].

The CAP-cAMP complex structure was originally determined in the absence of the amino acid sequence from a 2.9 Å resolution isomorphous map [18]. The amino acid sequence was subsequently incorporated into the structure [19], which was later refined to 2.5 Å resolution [20] and most recently to 2.1 Å resolution [21]. Each subunit folds into two domains, a large (residues 1–130) N-terminal cAMP-binding domain and a small (residues 140–209) DNA-binding domain. The residues between the two domains form a hinge that accommodates movement of one domain relative to the other.

The cAMP conformation when bound to CAP was a matter of disagreement. In the crystal structure, the cAMP molecules, which are among the best-ordered parts of the structure, are in an *anti* conformation. Just as unambiguously, and approximately when the CAP-cAMP structure was initially solved, a *syn* conformation for the cAMP was inferred from NMR experiments [22]. This apparent paradox was not resolved for 15 years, as discussed below.

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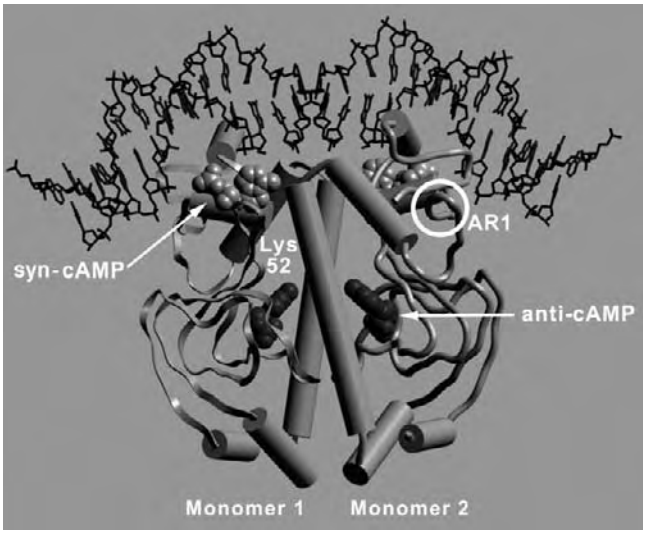


Figure 1 The cAMP-CAP-DNA dimer crystal structure [25]. The activating region 1 and Lys52 are labeled in the respective monomers where each is closer to the viewer. The *anti* and the *syn* cAMP molecules are shown as dark and light gray , respectively.

The crystal structure of CAP-cAMP complexed with a 30 base-pair DNA was solved initially at 3 Å resolution [23] and subsequently refined to 2.5 Å resolution [24]. The DNA in the complex is bent by 90°, which results almost entirely from two 40° kinks. A second CAP-DNA crystal form using a 46 base-pair DNA was solved at 2.2 Å resolution (Fig. 1) [25]. In this crystal form the DNA ends are disordered and unencumbered by crystal packing forces. The second crystal form structure confirms the major results of the original structure.

Transcriptional Regulation by CAP

The architecture of CAP-dependent promoters is amazingly varied. CAP can be either an activator or a repressor, and it can act alone or with other factors. CAP-binding site positions vary considerably among promoters. CAP can stimulate transcription alone when the DNA-binding sites are centered at –41.5, –61.5, and –70.5, as in the *gal*, *lac*, and *malT* promoters, respectively [26]. When the CAP site(s) is (are) further upstream, CAP cooperates with other transcriptional activators, such as MalT and AraC [27,28]. CAP represses transcription of itself and adenylate cyclase [29,30]. An example more reminiscent of eukaryotic systems is a set of CytR-regulated promoters: CytR repression requires two CAP dimers bound exactly 53 base-pairs apart. CytR binds to this nucleoprotein complex, disrupting transcription activation by CAP [31]. It is beyond the scope of this review to discuss the different mechanisms of CAP action at various promoters. However, these few examples offer a sampling of many ways *E. coli* uses CAP in transcription regulation.

Several CAP regions that are involved in transcriptional activation, and that presumably contact RNA polymerase, have been identified (Fig. 1). One such contact site, activating region 1 (AR1), is a surface-exposed loop (residues 158–162). Positive control mutations—that is, mutations that disrupt transcription activation but do not affect DNA-binding—have been found in AR1 [32–34]. An elegant experiment using heterodimers—one monomer has mutations in AR1, while the other has a mutation that alters the DNA sequence it recognizes—explored which activation loop is required at different promoters [35,36]. Zhou *et al.* demonstrated that when CAP is centered at –61.5, a functional AR1 is essential in the downstream subunit but dispensable in the upstream subunit; the reverse is the case when CAP is centered at –41.5. Suppressors of AR1 mutations map to a second surface-exposed loop containing Lys52 [37]. However, suppression only occurs when the CAP site is centered at –41.5. By studying RNA polymerase alpha subunit deletion and point mutants, it has been shown that the C terminus contains a CAP contact site, when CAP binds at –61.5 but not at –41.5 [38]. Promoter architecture thus plays an important role in determining the exact nature of the CAP-polymerase interaction.

CAP Permits Differential Gene Regulation at Different cAMP Concentrations

Bacteria have two major pathways to control transcription of a global network of genes in response to environmental stimuli and signals. One is to use a transcription factor such as CAP that responds to a signal and undergoes a conformational change, allowing it to bind DNA specifically and thereby regulate gene expression. The other pathway is to use alternative sigma factors, which recognize different promoter sequences, thereby providing the cell with an easy way to turn on a gene family in response to a signal [39]. For example, alternative sigma factors are involved in response to heat shock, sporulation, and flagellar synthesis. Why does *E. coli* use binding to CAP, and not the induction of an alternative sigma factor, in response to cAMP? The answer appears to lie in the flexibility of CAP in regulating gene expression, a flexibility that an alternative sigma factor would be incapable of achieving.

The use of an alternative sigma factor is a digital response. By using a different sigma factor, the cell can turn on and off a whole set of genes in unison. This type of response is critical for discrete changes in developmental or environmental states, such as sporulation or heat shock, or when the cell requires a complex cellular machine, such as flagella, where it is most efficient to produce all the components together.

As noted above, *E. coli* employs CAP in multiple ways; accordingly, the response of CAP to cAMP is analog. The regulation of the expression of different operons can be turned on or off at different cAMP concentrations, with different thresholds for a cAMP response. The threshold is set by adjusting the DNA sequence to change the affinity of the DNA for CAP-cAMP, so that different cAMP concentrations lead to differing affinities for various promoters. For example,

the cAMP-CAP affinity for the *lac* promoter is about an order of magnitude higher than for the *gal* promoter [40]. CAP binds to a consensus sequence 450 times better than to the *lac* site, which is one of the strongest *in vivo*; no natural site is even close in binding affinity to that of the consensus site [41]. This is presumably because a consensus site will always be bound at all physiological cAMP concentrations, and hence no regulation would be possible at promoters containing a consensus-binding site [42].

What advantage does setting different cAMP thresholds at different promoters confer on the cell? Some carbon sources cost more energy to metabolize than others. Therefore, the cell will resort to certain metabolites at different levels of glucose depletion and hence different cAMP concentrations. Similarly, in the presence of multiple nonglucose carbon sources, the cell can utilize its metabolites most efficiently.

A Second cAMP-Binding Site in a CAP Monomer

Various experiments have shown that cAMP binding to CAP induces a conformational change in CAP [4]. It has been observed, with several conformational probes, that CAP has a biphasic dependence on cAMP concentration. These probes—which include proteolytic digestion rates, Cys178 modification, tryptophan fluorescence, fluorescence of an extrinsic probe, and DNA affinity—display two cAMP concentration-dependent behaviors. One set of behaviors is seen up to approximately 200 micromolar cAMP concentrations and the other at millimolar concentrations [43–45]. These observations have been explained by the presence of three conformational states: free cAMP, and CAP dimers with one and two molecules bound, respectively. However, the structure of CAP bound to a 46 base-pair DNA revealed a second cAMP molecule bound to each protein monomer (Fig. 1) [25]. This second cAMP molecule was in the *syn* conformation, and resolved the long-standing discrepancy between NMR and crystallographic observations on the cAMP conformation bound to CAP. Crothers and Steitz [46] postulated the existence of a second cAMP-binding site, when they suggested that at the millimolar concentrations in which the NMR experiments were done there was "a second weakly bound and rapidly exchanging cAMP which is in the *syn* conformation." The presence of a second cAMP-binding site in each monomer suggests that the experiments showing the biphasic dependence on cAMP concentration be reinterpreted in terms of a new model involving three conformational states: free CAP, CAP with two cAMP molecules bound to the *anti* binding site, and CAP with two cAMP molecules bound to the *anti* and two to the *syn* binding sites.

Whether the *syn*-cAMP binding site is physiologically relevant remains unresolved. *In vivo*, does the cAMP concentration near CAP reach the millimolar concentrations necessary for physiological relevance? Alternatively, is *syn*-cAMP binding an artifact only observed *in vitro*?

Perspectives and Conclusions

Great strides have been made in understanding the molecular mechanism of action by CAP and its response to cAMP; however, many questions remain. Despite extensive efforts to crystallize apo-CAP, its structure has not been determined. The nature of the conformational changes that occur upon cAMP binding has been the subject of speculation and investigation [4,21,47]. Recent progress has included both the crystal structure solution of a transcription factor in the CAP family, CooA, without its effector CO [48], and a low-resolution NMR apo-CAP structure [49]. However, a high-resolution apo-CAP structure would be invaluable.

Over the past two decades, structural biology has contributed a great deal to understanding transcription and its regulation. RNA polymerase structures have provided a wealth of information about the basic mechanisms of transcription. Meanwhile, progress in solving the structures of a large number of transcription factor DNA-binding domains bound to their specific sites has elucidated how transcriptional regulators bind DNA elements within promoters. Structural biology has only begun to address how transcriptional regulators, once bound to DNA, interact with the basic transcription apparatus. Understanding the molecular mechanism of CAP activation is an attainable goal and will provide a useful model for transcription activation in general. The structures of both RNA polymerase and the entire CAP protein are known, including the CAP regions thought to contact RNA polymerase. In addition, since there is no chromatin in prokaryotes, issues of DNA accessibility and chromatin remodeling are not relevant for transcription activation by CAP. Solving the prokaryotic DNA-CAP-polymerase structure would make an enormous contribution to understanding transcription activation even in eukaryotes.

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CHAPTER 209

Cyclic Nucleotide Signaling in Paramecium

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Introduction

The unicellular protozoan *Paramecium* uses both cyclic nucleotide second messengers, cAMP and cGMP, for signal transduction. Contrary to mammalian cells, in which cyclic nucleotide formation is under hormonal control, the primary input in *Paramecium* appears to be membrane potential and ion currents. Studies showed that regulation of cyclic nucleotide levels takes place at the site of formation, that is, by direct modulation of adenylyl cyclase (AC) and guanylyl cyclase (GC) activities. Molecular analyses revealed that the architecture of the respective cyclase enzymes differs from their mammalian congeners, leading to new concepts for the coupling of signals to second messenger generation. This chapter concentrates on the features of *Paramecium* AC and GC. Events downstream of cyclic nucleotide formation will be discussed only briefly, as they have been investigated to a much lesser extent. See Fig. 1 for a summary.

cAMP Formation and Adenylyl Cyclase

In the 1980s regulation of cAMP levels in *Paramecium* has been investigated by exposing cells to sudden changes of the extracellular ion milieu [1]. Increases in cAMP content are strictly dependent on hyperpolarization, e.g. elicited by a dilution of external K^+. Upon an eight-fold dilution of K^+ cAMP levels increase up to four-fold within 5 to 10 seconds and then decline to a new steady-state level within few minutes. This behavior has the properties of an adaptive response. cAMP formation is strictly coupled to a K^+-conductance by which K^+-ions exit the cell upon dilution [2] because (1) specific K^+-channel blockers such as tetraethylammonium or Cs^+ abolish the cAMP response; (2) the cAMP response correlates with the K^+ concentration that the cells have been adapted to prior to the stimulus; upon dilution from 16 mM K^+ in the equilibration buffer where the K^+ resting conductance is high, the increase in cAMP is maximal whereas upon dilution from 1 mM K^+ in the equilibration medium where the resting conductance is low, the cAMP response is negligible; (3) The mutant *restless* which cannot control its K^+ resting conductance [3], shows an exaggerated cAMP response even when cells are adapted to low external K^+.

Subsequently the stimulation of cAMP formation has been investigated biochemically via purification of *Paramecium* AC [2,4]: *Paramecium* AC activity is exclusively membrane-bound and requires detergents for solubilization. AC activity is high in ciliary membranes (0.5 nmol cAMP/(mg·min)) and increases to 25 μmol/(mg·min) upon purification to homogeneity. Paramecium AC is a 97 kDa protein with ion pore-forming properties: upon reconstitution into black lipid bilayers an intrinsic cation-specific conductance of 320 ± 60 pS has been observed. The pore displays a slight preference for K^+ and is impermeable to tetraethylammonium. The unique bifunctional property of the *Paramecium* enzyme as an ion channel-AC suggests that the AC activity is regulated by an intrinsic K^+-conductance. This AC may be involved in setting the resting membrane potential and thus behaves like a biological ammeter. Such a view is compatible with the observation that cAMP formation upon K^+-dilution is

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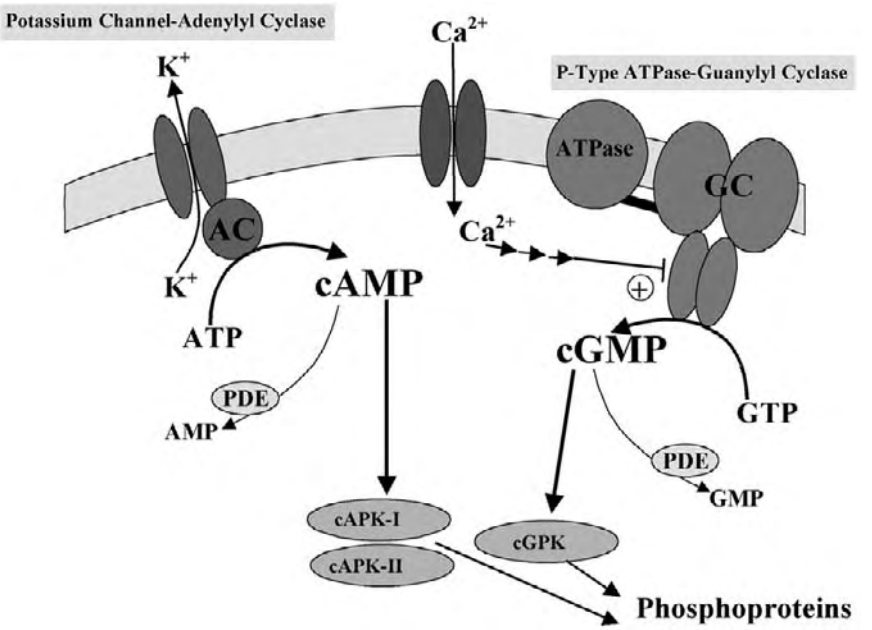


Figure 1 Overview of cyclic nucleotide signaling in *Paramecium.* PDE: cyclic nucleotide phosphodiesterase; cAPK/cGPK: cyclic AMP/GMP-dependent protein kinase; AC/GC: adenylyl/guanylyl cyclase. The light grey ribbon represents the cell membrane with incorporated channels and integral membrane domains. The P-type ATPase-like domain of *Paramecium* GC is connected by a cytosolic peptide linker to the GC-domain. The *Paramecium* AC most likely consists of a K^+-channel and a catalytic domain with a yet unknown multimeric composition.

dependent on the K^+ resting conductance set by adaptation to the respective equlibration buffer (see above).

Recently we cloned a cDNA from *Paramecium* coding for a 98 kDa protein consisting of three domains (unpublished): an N-terminal ion channel domain linked to an AC catalytic domain and a single C-terminal tetratricopeptide unit. Thus the predicted protein correlates with the previous physiological and biochemical data. The functional properties of the protein have yet to be established.

Some chemoattractants cause hyperpolarization and thus elicit a transient cAMP response [5], e.g. L-glutamate. However, hyperpolarization brought about by addition of NH_4^+ or acetate ions has no effect on cAMP levels. Currently, it is unclear whether the response to L-glutamate is via stimulation of AC or via inhibition of cAMP-phosphodiesterase.

Guanylyl Cyclase and cGMP Formation

Paramecium contains a membrane-bound GC activity about 10 percent of which localize to ciliary membranes [6,7]. Soluble, that is cytosolic, GC activity has never been detected. Ca^{2+}-ions play a prominent role in the regulation of the protozoan GC as removal of Ca^{2+} greatly diminishes activity [4,8]. The inhibition is specifically relieved by readdition of Ca^{2+} ions. Sr^{2+} is less effective and Ba^{2+} ions fail to reactivate [8]. The Ca^{2+}-dependence of the GC *in vitro* fits reasonably well the boost-like increase of cGMP levels *in vivo* upon a stimulated Ca^{2+} influx. One route by which Ca^{2+} can enter *Paramecium* is a depolarization-activated, voltage-gated Ca^{2+} channel activated e.g. by an increase of extracellular K^+. Channel inactivation is rapid within a few milliseconds, and the global cGMP content rises only by about 45 percent under these conditions [9]. In contrast, a depolarization caused by Ba^{2+}-addition results in a more sustained Ca^{2+}-entry and a substantial, yet short-lived increase in cGMP [9]. Mutants with defects in the depolarization-activated Ca^{2+} conductance show altered cGMP responses. *Pawn* mutants, which lack the depolarization-gated Ca^{2+}-influx, do not display a significant cGMP increase upon treatment with Ba^{2+} [9,10]. On the other hand, *dancer* cells in which the Ca^{2+} channel is very slowly inactivating after opening, show a sustained cGMP response toward Ba^{2+}-depolarization and, in contrast to the wild-type, cGMP levels in *dancer* cells rise three-fold upon a K^+-depolarization [11]. An alternate route of Ca^{2+} entry is activated by chemical hyperpolarization, e.g. by the drug amiloride, which itself does not affect GC activity *in vitro* [12]. Upon amiloride addition cGMP-levels increase 6 to 30-fold within 10 to 20 sec, dependent on extracellular Ca^{2+}. *Pawn* mutants show an identical cGMP-response to amiloride, suggesting that the hyperpolarization-activated conductance is physically distinct from the depolarization-gated Ca^{2+}-channel opened by Ba^{2+}.

The molecular architecture of *Paramecium* GC has been elucidated via a homology-cloning approach [13]. A 7.2 kb cDNA was obtained coding for a 280 kDa integral membrane protein of two domains. The N-terminal domain of 155 kDa is similar to P-type ion transport ATPases and is

joined by a cytoplasmic linker to the C-terminal 115 kDa guanylyl cyclase domain. The calculated topology of the GC domain is identical with that of mammalian membrane-bound ACs (see previous chapters). It contains two sets of six transmembrane helices, which are linked to the two catalytic subdomains, called C1a and C2. The C1a subdomain contains the crucial amino acids required for substrate recognition and transition state stabilization and the C2 subdomain contains the two metal–cofactor binding aspartates. Thus the functions of C1a and C2 in *Paramecium* GC are inverse compared to mammalian ACs, where C1a binds Mg^{2+} and C2 takes over the other functionalities. The P-type ATPase-like domain contains ten transmembrane helices and harbors the invariant DKTGT(L/I)T signature motif of this class of transporters. However, several crucial deviations from functionally characterized consensus motifs indicate that this domain is not an active transporter but probably has adopted a new, as yet unrecognized function [13].

Due to the unorthodox genetic code in use in *Paramecium* a heterologous expression of the GC requires that the expression cassettes are adjusted to universal codon usage [13]. This way, a specific GC activity of up to 1 nmol cGMP/(mg·min) is detected in Sf9 cells expressing the C-terminal GC-domain. Cells expressing the holoenzyme yield about 0.5 nmol cGMP/(mg·min). A Western blot revealed, however, that in Sf9 cells the holoenzyme is cleaved into two large polypeptides.

The C1a and C2 catalytic subdomains of the *Paramecium* GC can be individually expressed in *E. coli* as soluble proteins, as previously demonstrated for the C1a and C2 domains of the mammalian ACs [14–16]. Alone the affinity-purified subdomains are inactive, yet a mixture of C1a and C2 reconstitutes a robust GC activity [17]. This system was then used for a further characterization of the catalyst. Based on x-ray and mutagenesis data from the mammalian AC/GC enzymes [18–20], Glu1681 and Ser1748 of *Paramecium* C1a were predicted to be key residues for GTP substrate specificity. Indeed mutation of both residues that specify the ATP substrate in the mammalian ACs, i.e. E1681K and S1748D, converts the *Paramecium* GC-C1a/C2 heterodimer into a specific AC [17].

Taken together the *Paramecium* GC is a unique protein assembly wherein an ion pump-like domain is fused to a classical mammalian AC domain in which the substrate specificity was modified during its evolution.

Downstream of Cyclic Nucleotide Formation

Intracellular levels of cyclic nucleotides reflect the balance between synthesis by the cyclases and hydrolysis by phosphodiesterases (PDE). In *Paramecium* PDE activity is high [21]. So far, the regulation of PDE activity has not been investigated in sufficient detail. However, the sharp rise and rapid fall in cGMP levels upon Ba^{2+}-depolarization vividly demonstrates the highly dynamic equilibrium of cGMP-formation and hydrolysis. Indeed it is estimated that the cellular cGMP turns over every two seconds in *Paramecium* [22].

The only known primary effectors for cyclic nucleotides in *Paramecium* are cAMP- and cGMP-dependent protein kinases. Two forms of cAMP-dependent protein kinases, cAPK-I (70 kDa) and cAPK-II (220 kDa), have been partially purified [23–25]. Both forms contain a single catalytic (C) and regulatory subunit (R). They differ in their R-subunits, which are subject to autophosphorylation *in vitro* [26]. The cloned subunit of cAPK-I has up to 38 percent identity to known R-subunits from other unicells and mammalian tissues. In agreement with the molecular weight of the protozoan cAPK-I an N-terminal dimerization region is missing. This explains RC-dimer composition in contrast to the R_2C_2 tetrameric structure in mammals [27]. Further, a cGMP-dependent protein kinase (cGPK) has been partially purified and correlated to a single 77 kDa protein [28]. It appears to autophosphorylate *in vitro* and uses ATP as well as GTP as phosphoryl donors. Numerous proteins appear to be phosphorylated in *Paramecium* in a cGMP and/or cAMP-dependent manner *in vitro* whose identity and function largely remains to be identified [29,30]. Some work has concentrated on a 29 kDa dynein light chain that is a substrate for cAMP-dependent phosphorylation *in vitro* and appears to be a component of the 22S axonemal dynein [31–34]. It has been suggested that this protein may regulate dynein function in *Paramecium* as hyperpolarization increases swimming speed and cAMP formation and because phosphorylation of the 29 kDa protein regulates microtubule translocation *in vitro*. It is interesting that the cAMP response to hyperpolarization is rather transient whereas the increase in swimming speed clearly extends to longer periods of time, demonstrating a lasting signal transduction.

With the advance of several genome projects targeting protozoans of the parvkingdom of Alveolata, including *Tetrahymena* and the apicomplexan parasites *Plasmodium* and *Cryptosporidium*, it is noteworthy that similar cGMP and cAMP signal transduction machineries seem to exist in all members of this parvkingdom, among those several of extremely high pathogenic potential. The studies with *Paramecium* may, therefore, turn out to be a valuable starting point for investigating signal transdcution in this group of protozoans [35].

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CHAPTER 210

Cyclic Nucleotide Signaling in Trypanosomatids

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Introduction

The trypanosomatids (order: kinetoplastida) include the causative agents of human sleeping sickness and of the cattle disease nagana in Sub-Saharan Africa (*Trypanosoma brucei*), a host of diseases of camels, water buffaloes, and horses (*T. evansi*, *T. equinum* and *T. equiperdum*); Chagas disease in South and Middle America (*T. cruzi*); and Kala Azar (*Leishmania donovani*) and a host of other human Leishmanial infections worldwide.

Cyclic nucleotide signaling in parasitic protozoa has lately become an attractive field of research, not at the least because many experimental approaches have been greatly facilitated by the advanced stage of several of the genome projects (e.g. *T. brucei*: http://parsun1.path.cam.ac.uk; *T. cruzi*: http://www.dbbm.fiocruz.br/TcruziDB; *Leishmania major*: http://www.ebi.ac.uk/parasites/leish.html).

Cyclic Nucleotide Signaling, Cell Proliferation, and Differentiation

During *in vitro* differentiation of *T. brucei* from bloodstream forms to the insect stage (procyclics),[1] two peaks of adenylyl cyclase (AC) activity were observed. The first peak occurred 6–10 hr after triggering differentiation, before the first cell division. A second peak was observed when the cells emerged from the first division and before they began to proliferate [1,2]. However, an independent study using a different trypanosome strain found no involvement of cAMP in differentiation *in vitro* [3]. During animal infections, the intracellular cAMP levels of the trypanosomes increased from the early stages of the infection to the peak of parasitaemia and then decreased as differentiation from long slender to short stumpy forms began [4,5].[2,3] These early observations were recently corroborated by *in vitro* experiments [6]. When cultured bloodstream form trypanosomes reached a threshold density, they secreted a low-molecular-mass factor (SIF: stumpy-inducing factor), which induced an increase in intracellular cAMP. This increase was followed by cell cycle arrest, and resulted in a high efficiency of differentiation to stumpy forms. Membrane-permeable cAMP analogs and the PDE inhibitor etazolate mimicked SIF activity, indicating that SIF acts via cAMP and that the elevated intracellular cAMP is a signal for differentiation. Similarly, membrane-permeable cAMP analogs, PDE inhibitors, or the inactivation of specific PDEs by RNA interference were shown to elevate intracellular cAMP and to concomitantly inhibit proliferation of cultured bloodstream forms of *T. brucei* [7,8].

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[1]Procyclics: proliferative form of *T. brucei* adapted to the environment of the tsetse fly midgut.

[2]Long slender forms of *T. brucei*: proliferative form of the trypanosomes in the blood of mammalian hosts.

[3]Short stumpy forms of *T. brucei*: long slender forms eventually differentiate into short stumpy forms, which are pre-adapted to survival in the tsetse fly vector. Short stumpy forms do no longer proliferate and have a limited lifetime in the mammalian bloodstream.

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In *T. cruzi*, increased cAMP was also found to downregulate cell proliferation. High intracellular cAMP levels inhibited DNA, RNA, and protein synthesis [9]. Fetal calf serum, which is mitogenic for *T. cruzi*, was shown to decrease intracellular cAMP [10].

However, increased cAMP appears to play a role in differentiation of *T. cruzi*. Prior to differentiation to metacyclic forms, the intracellular cAMP levels increased four-fold.[4] In agreement with these findings, exogenous cAMP, cAMP analogs, and the PDE inhibitor papaverine induced differentiation *in vitro* [11]. In *T. cruzi*, the steady-state level of the mRNA of an unidentified gene, TC26, was elevated during differentiation to metacyclics or after induction by exogenous cAMP analogs. In a strain of *T. cruzi* that was unable to undergo differentiation, TC26 was not expressed [12]. Recent advances in the *T. cruzi* genome project have demonstrated that TC26 represents a family of repetitive elements.

In *L. donovani*, cAMP and PDE inhibitors blocked the transformation from the intracellular amastigote stage to the extracellular promastigote form.[5,6] In *L. tropica*, caffeine was shown to increase intracellular cAMP levels and to reduce proliferation [13]. Yet binding of a specific component of human serum, C-reactive protein, to the surface of *L. mexicana* promastigotes induced differentiation to the amastigote form, possibly through a cAMP mediated signaling cascade [14].

Individual Components of the Cyclic Nucleotide Signaling Pathways

Cyclases

In striking contrast to the situation in higher organisms, the very small genomes of *T. brucei* and *T. cruzi* (about 40 Mbp) contain a large number of genes for different adenylyl cyclases [15,16]. In *T. brucei*, two types of AC genes can be discerned in terms of their genomic organization. Each telomeric VSG expression site contains a number of expression-site associated genes (ESAGs), one of which is always an AC [17].[7] All other AC genes (GRESAGs, genes related to ESAG4) are scattered throughout the genome and are present either as single copy genes or as small gene families.

All ACs from *T. brucei* [18–21], *T. cruzi* [16], *L. donovani* [22], and *T. equiperdum* [23] for which sequence information is available exhibit a similar predicted structure. A short N-terminal leader sequence is followed by a large extracellular domain that bears no significant similarity to other proteins or among the different trypanosomal ACs. This putative extracellular receptor domain is connected via a single transmembrane helix to the intracellular catalytic domain [24]. The catalytic domains are strongly conserved, not only between kinetoplastid ACs, but also between the kinetoplastid and mammalian ACs [25,26] and are presumably activated by dimerization [22,24,26]. The AC activity of *T. brucei* is insensitive to agents known to activate mammalian cyclases, such as GTP or GTP analogs, forskolin, or cholera and pertussis toxins [1]. The AC activity in bloodstream form *T. brucei* can be transiently activated by Ca^{2+}, whereas no such effect could be detected in procyclic form [27].

The overall structure of the trypanosomal ACs is reminiscent of that of the mammalian membrane-bound receptor guanylyl cyclases [28]. The large number of different trypanosomal ACs, all with different N-terminal (extracellular) domains, and their structural similarity with the receptor guanylyl cyclases suggest that the trypanosomal ACs may serve as extracellular receptors [15]. As trypanosomatids contain neither G-protein-coupled receptors nor heterotrimeric G proteins (see below), the ACs may serve as enzyme-linked receptors in an alternative paradigm for chemical sensing. The functional correlate to the structural similarity between trypanosomal ACs and the metazoan receptor GCs might be reflected by the vertebrate GC-D olfactory neurons. In this subpopulation of the olfactory receptor neurons, membrane-bound receptor GCs serve as olfactory receptors, instead of the G-protein coupled receptors expressed in the majority of olfactory receptor neurons [29].

Cyclic Nucleotide-Specific Phosphodiesterases

In *T. brucei*, PDE activity in lysates of bloodstream forms was first demonstrated over 20 years ago [30]. More recent work has now identified at least two different PDE families in *T. brucei*. TbPDE1 is coded for by a single-copy gene and represents a class I PDE with an unusually high K_m for its specific substrate, cAMP (Kunz *et al.*, submitted). This PDE is not essential for proliferation of *T. brucei* in culture, nor for the infection of the tsetse fly vector [31]. Three members of a second class I family of cAMP-specific PDEs of *T. brucei* have recently been identified and characterized (TbPDE2A [7]; TbPDE2B: [7a] TbPDE2C [8]). They share highly conserved catalytic domains but differ in their N-terminal regulatory regions [15], each of which contain one or two GAF domains [32]. All TbPDE2 family members characterized so far are highly specific for cAMP and exhibit a low K_m, and their activity is not affected by cGMP. Several broad-spectrum PDE inhibitors such as the methyl-xanthines are completely inactive toward the TbPDE2 enzymes. Dipyridamole, trequinsin, sildenafil, and ethaverine inhibit the recombinant enzymes and block

[4]Metacyclic forms: the mammalian-infective forms of *T. brucei* and *T. cruzi,* which are deposited by the respective insect vectors in the blood of the mammalian host.

[5]Amastigote: intracellular, nonflagellated forms of *T. cruzi* and *Leishmania ssp*., which replicate within mammalian host cells.

[6]Promastigote: extracellular, flagellated forms of *T. cruzi* and *Leishmania ssp.*

[7]VSG (variable surface glycoprotein) of *T. brucei*: a tightly packed layer of single type of such a protein covers the entire surface of *T. brucei*. The genome contains several hundred genes for different VSGs. Only one of these is active at any given time, and activation switches stochastically between individual genes with a low frequency.

proliferation of bloodstream trypanosomes in culture [7]. RNA interference experiments confirmed that inactivation of TbPDE2 family members is lethal to bloodstream trypanosomes in culture [8], suggesting that this PDE family might constitute an interesting drug target.

Two kinds of cAMP-specific PDE activities were detected in *T. cruzi*. One enzyme was a soluble, cAMP-specific PDE with a K_m of 40 μM, and it exhibited full activity at pH 8.0 and in the presence of 5 mM Mn^{2+}. The enzymatic activity was not Ca^{2+} dependent, and was not inhibited by theophylline and caffeine [33]. A second PDE activity was purified by affinity chromatography on a brain calmodulin-Sepharose column. Activation required micromolar concentrations of Ca^{2+}, and activity was blocked by EGTA and by calmodulin inhibitors [34].

A cAMP PDE activity was also detected in *L. tropica* and *L. donovani,* which required Mg^{2+} for full activation [35]. A cAMP-specific PDE activity from *L. mexicana* was recently characterized [36]. This PDE activity was found both in the cytoplasm and on the outer cell surface. It was identified as a 60 kDa protein on SDS-PAGE, exhibited an unusually high K_m of 277 μM for cAMP, and required Mg^{2+} for maximal activity. Based on its high K_m, the enzyme might represent a class II PDE, or it may represent the leishmanial homolog of TbPDE1 of *T. brucei.*

Protein Kinase A

In *T. brucei*, three genes were predicted to code for different catalytic subunits of PKA homologs [37]. The single-copy gene of a regulatory subunit of PKA has recently been identified and characterized. Although the overall sequence organization of the protein is well conserved with respect to its mammalian homologs, the PKA holoenzyme from *T. brucei* could only be activated by cGMP but not by cAMP. When expressed as recombinant proteins, both cyclic-nucleotide-binding domains of the regulatory subunit did bind cGMP, and this binding was not competed by cAMP [38]. The K_ds for cGMP binding to both of the domains were in the 10 μM range, and its physiological significance remains to be established.

In *T. cruzi*, both the regulatory and catalytic subunits of PKA have been purified from cultured cells. The putative catalytic subunit appeared to be similar to the bovine heart PKA catalytic subunit and cross-reacted with an antibody against the bovine enzyme. The putative regulatory subunit inhibited the catalytic subunit of bovine heart PKA, and this inhibition could be reversed by cAMP. Reconstitution experiments with the two purified proteins resulted in a holoenzyme with an activity similar to that of bovine heart PKA holoenzyme. The molecular weight of the reconstituted holoenzyme suggested a tetrameric structure [39,40].

A presumptive catalytic subunit of PKA was purified from *L. donovani*. Its activity could be inhibited by the regulatory subunit of bovine heart PKA, and the inhibition could be reversed by cAMP. A heat-stable porcine heart PKA inhibitor also inhibited its activity [41].

Cyclic Nucleotides and Host Parasite Intervention

In the bloodstream of the host, *T. brucei* cells are faced with a massive antibody response. As a potential countermeasure, they have acquired a mechanism for active disaggregation when agglutinated by VSG-specific antibodies [42]. The disaggregation mechanism is energy-dependent, does not result in the proteolysis of the bound antibody, and does not involve the shedding of VSG from the cell surface. The mechanism is modulated by PKA and cAMP. This suggests that a cAMP-signaling cascade might be triggered by antibody binding to the VSG coat. This cascade eventually results in the evasion of *T. brucei* from the onslaught of the host's anti-VSG antibody response.

Infection of mammalian cells by *T. cruzi* involves the mobilization of intracellular Ca^{2+} and the elevation of cAMP in the host cells. Parasites that lack the oligopeptidase B required to trigger the Ca^{2+} release in the host cell are still able to induce the elevation of cAMP in the host cell, and to infect them, albeit more slowly [43]. In addition, cGMP was also shown to play a role in modulating macrophage susceptibility to *T. cruzi* infection. Increasing the intracellular cGMP level in macrophages resulted in a marked increase in the number of parasites associated with the cells and in the percentage of infected cells. Similar pretreatments of the parasites had no effect on the host-parasite interaction [44]. The first step of the invasion of *T. cruzi* into its host cells consists in the adhesion to the host membrane. Attachment of infective trypanosomes to host muscle cell sarcolemma resulted in the rapid inhibition of parasite AC activity and significantly reduced intracellular levels of cAMP in the parasite [45].

L. tropica and *L. donovani* secrete a soluble factor that inhibited mammalian AC activity whereas it had no effect on the AC activity of the parasite [46]. cAMP was reported to modulate the intracellular superoxide dismutase activity in *L. donovani*, resulting in a reduction of parasite survival in the intraphagolysosomal environment [47].

Concluding Remarks

The current state of the study of cyclic nucleotide signaling in trypanosomatids still is very patchy. Nevertheless, the available results already provide a glimpse on the contours of the larger picture. One important conclusion that already can be drawn from the available data is that the trypanosomatids differ widely from their mammalian hosts in some, though not all, aspects of their cyclic nucleotide signaling pathways. Although some enzymes involved in these pathways, such as the PDEs that were investigated in great detail in mammals, can similarly be detected in trypanosomatids, amazing differences are seen with others. No G-protein-coupled receptors, heterotrimeric G proteins, or G-protein-activated adenylyl cylases were found in trypanosomatids. The structure of their adenylyl cyclases, of which they express a large number of different isoenzymes, is entirely

different from that of their mammalian counterparts, and it closely resembles mammalian receptor guanylyl cyclases. In trypanosomatids, the receptor adenylyl cyclases may replace the G-protein-coupled receptors of higher eukaryotes as the major sensory system.

Such novel structures in old pathways might result from the evolutionary adaptation of the trypanosomatids to their parasitic lifestyles, or from the evolutionary distance between them and their mammalian and insect hosts. The three groups of organisms are evolutionarily far apart; the trypanosomatids are among the oldest of the extant eukaryotes, whereas insects and mammals represent much more recent developments. They also might simply reflect a degree of evolutionary diversity that has remained largely undiscovered and unexplored due to an overly narrow focusing of contemporary cell biological studies to a very small number of model organisms such as man, mice, or *Drosophila*.

Studying the parasite-specific wrinkles of cyclic nucleotide signaling pathways not only is a fascinating challenge for the adventurous scientific mind, but it also holds great promise from a practical standpoint. For many of the major parasitic diseases, the current state of the art of chemotherapy is deplorable. The identification of new signaling molecules that differ significantly between the cyclic nucleotide signaling pathways of hosts and parasites might open up a treasure trove of new potential drug targets, not only for trypanosomatid-caused diseases, but just as well for many other protozoal diseases such as malaria, toxoplasmosis, or amebiasis.

However, the pharmacology of the PDEs, a class of enzymes that are similar between mammals and trypanosomatids, has been highly developed for the human enzymes. PDE inhibitors are currently on the market or in the pipelines as medication for an increasing number of clinical applications, and potency as well as isoenzyme and subtype specificity are ever increasing. This extensive know-how on developing inhibitors for mammalian PDEs could and should be applied to their homologs from trypanosomatids and other protozoal pathogens, possibly resulting in an assortment of effective and highly selective antiparasitic compounds.

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CHAPTER 211

Cyclic Nucleotide Specificity and Cross-Activation of Cyclic Nucleotide Receptors

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The homologous cAMP- and cGMP-dependent protein kinases (PKA and PKG) are generally believed to be activated relatively specifically by cAMP and cGMP. Certain physiological and pathophysiological conditions may exist in which elevation of one cyclic nucleotide may lead to cross-activation of the opposing kinase [1–3]. This chapter summarizes the current evidence supporting cAMP and cGMP cross-activation and the structural basis for cyclic nucleotide specificity of the cyclic nucleotide dependent protein kinases. The interaction of cyclic nucleotides with other eukaryotic cyclic nucleotide receptors is also discussed.

cAMP Cross-Activation of PKG

Though there is some evidence for cAMP cross-activation of PKG in gastric smooth muscle [4], the physiological paradigm for this type of cross-activation is vascular smooth muscle relaxation. In one line of experiments, doses of cyclic nucleotides sufficient to relax pig coronary arteries correlate with their potencies for PKG activation but not PKA activation [5]. Consistent with this, a mere twofold elevation of intracellular cAMP in this same tissue is sufficient to cause vasorelaxation and activation of both PKA and PKG [6]. A second and more frequently tried line of evidence correlates the pharmacological inhibition of PKG with the disruption of cAMP-induced functions such as vasorelaxation [7], phosphorylation of the IPs receptor in rat aorta [8], gating of the L-type Ca^{2+} channel in rabbit portal vein myocytes [9], and gating of the Ca^{2+}-activated potassium (BKca) channel in porcine coronary artery myocytes [10]. A third experimental approach demonstrates that cAMP reduces vasopressin-induced Ca^{2+} mobilization in primary cultures of rat aortic myocytes, but not in myocytes depleted of PKG [11]. This last experimental model suggests cAMP-induced vascular smooth muscle relaxation is mediated solely by PKG. Other models suggest, however, that cAMP can also relax vascular smooth muscle through PKA activation [7,9]. The latter is further supported by the observation that PKG I-deficient mice still undergo cAMP-induced smooth muscle relaxation even though these mice already suffer severe vascular and intestinal disregulation [12].

Although the affinity of PKG for cAMP is 50- to 100-fold lower than its affinity for cGMP [13], two factors may make PKG susceptible to cAMP activation in the cell. First, the concentration of cAMP is estimated to be fivefold higher than cGMP in some tissues [5]. Second, autophosphorylation of PKG improves its affinity for cAMP *in vitro* [13–15]. For example, autophosphorylation at Ser-79 of the bovine type IP isoform of PKG results in a three- to fourfold increase in basal kinase activity and a twofold decrease in its K_a for cyclic nucleotides [16]. Though *in vivo* autophosphorylation at this site has not been determined directly, replacement of Ser-79 with aspartic acid to mimic the effect of autophosphorylation results in a mutant kinase with constitutive activity when expressed in CV-1 cells [17].

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cGMP Cross-Activation of PKA

The best evidence for the cross-activation of PKA by cGMP is from studies with intestinal epithelial cell lines. The *Escherichia coli* heat-stable enterotoxin causes supraphysiological accumulation of cGMP and increased Cl^- permeability in the human intestinal T84 cell line, which expresses little or no PKG, but plenty of PKA [18]. Inhibitors of PKA activation also suppress increased Cl^- secretion in T84 cells upon stimulation with cGMP analogs or guanylin, an endogenous guanylyl cyclase activator peptide [19]. Similarly, induction of Cl^- conductance in the human colonic carcinoma cell line, Caco-2, by cAMP or cGMP analogs is suppressed by the PKA inhibitor PKI_{5-24} but not the PKG inhibitor KT5823 [20]. Other studies suggest that cGMP-dependent activation of PKA may also occur during porcine carotid artery relaxation [21] and during atrial natriuretic peptide-induced testosterone production in mouse Leydig cells [22].

The cross-activation of PKA may occur indirectly through cGMP inhibition of the cAMP-specific phosphodiesterase PDE3. Treatment of rat small intestine synaptosomal preparations with inhibitors of PKA, but not PKG, block the NO-induced release of bombesin-like immunoreactivity (BLI), considered to be important in enteric smooth muscle contractility [23]. Treatment with trequinsin, a blocker of PDE3, results in increased cAMP and mimics the NO-induced release of BLI. The effects of trequinsin and NO are not additive suggesting that the two agents act through a common mechanism.

Molecular Basis for cAMP/cGMP Selectivity of PKA and PKG

Central to the theme of cyclic nucleotide-dependent protein kinase cross-activation is the degree to which the cyclic nucleotide-binding domain (CNBD) can select for the appropriate cyclic nucleotide. The typical CNBD contains about 124 residues and is characterized by three α-helices, an eight-stranded anti-parallel (β-barrel), and a half-dozen invariant residues. Three invariant glycines are critical for proper folding of the β-barrel, an invariant glutamic acid forms a hydrogen bond with the ribose 2′-OH, and an invariant arginine interacts electrostatically with the cyclic phosphate of the cyclic nucleotide. The importance of these residues in cyclic nucleotide-binding has been demonstrated by site-directed mutagenesis of the type Iα regulatory subunit (RIα) of PKA, which contains two CNBDs termed the A and B domains (summarized in [24]). The contribution of the sixth invariant residue, an alanine, to high affinity cyclic nucleotide binding is not fully understood, though its mutagenesis destroys cAMP-binding activity [25]. Since all of these signature residues are present in cAMP- or cGMP-selective CNBDs, they are not involved in cyclic nucleotide selectivity.

The characteristic RAA and R(S/T)A motifs found in cAMP- and cGMP-selective CNBDs, respectively, have been identified as cAMP/cGMP selectivity determinants. The middle residues in each motif are bracketed by the invariant Arg and Ala residues described above. Mutagenesis of the first Ala in the RAA motif in the A and B domains of RIα increases its affinity 200-fold for cGMP with minor effects on cAMP affinity [26–28]. Likewise, mutation of Ser/Thr residues to Ala in the R(S/T)A motif in the A and B domains of PKG Iα [29], PKG Iβ [30], and PKG II [31] reduces their affinities for cGMP relative to cAMP. These site-directed mutagenesis studies along with cyclic nucleotide analog studies [32–34], molecular modeling [35,36], and determination of the structure of the RIα cAMP-binding domains [37] all support the interpretation that the selectivity of cGMP-binding domains is due to hydrogen bonding between the 2-amino position of cGMP and the Ser/Thr side chain.

Whereas cGMP selectivity can be explained in a large part by a single ligand-receptor interaction, identification of residues responsible for selective, high-affinity cAMP-binding has met with limited success. The RIα structure suggests that aromatic stacking between the A domain Trp-260 or the B domain Tyr-371 and the purine moiety of cAMP may contribute to cAMP/cGMP selectivity. Mutagenesis studies, however, demonstrate that Tyr-371, though important for general high-affinity cyclic nucleotide binding, does not affect cAMP/cGMP selectivity [38]. Other residues in the binding pocket close to the 6-position of the cyclic nucleotide have been implicated as potential selectivity determinants. A likely candidate is the peptide backbone carbonyl group of Asn-372, in the B domain of RIα. Mutagenesis of Asn-372 suggests that it contributes modestly to the cAMP selectivity in RIα (Comstock and Shabb, unpublished).

Another RIα B domain residue that may contribute to recognition of the 6-amino group is Ile-368. Conversion of Ile-368 to Tyr increases the affinity three- to fourfold for N^6-modified cAMP analogs [39]. The only fully converted cGMP-sensitive, cAMP-dependent protein kinase is a chimera where both of the cAMP-binding domains of RIα have been replaced by the cGMP-binding domains of PKG I [40].

Other Cyclic Nucleotide Receptors

Although cross-activation events are usually mediated by protein kinases, the potential involvement of other cyclic nucleotide receptors must be considered. For example, the homomeric expression of the olfactory cyclic nucleotide-gated (CNG) ion channel α subunit is more sensitive to cGMP than to cAMP [41,42]. However, heteromeric association with its β subunit tends to equalize the channel's affinity for cAMP and cGMP [43,44] suggesting that either cyclic nucleotide can act as its physiological ligand.

The CNBDs of the CNG ion channels are homologous to those found in the protein kinases. Like the kinases, mutagenesis of the invariant Arg in the ion channels results in reduced affinity for cGMP [45]. Substitution of the Thr in the R(S/T)A motif to an Ala in photoreceptor and olfactory CNG ion channels results in a reduction of cGMP-responsiveness

without affecting cAMP-induced channel activation [41]. Other mutagenesis [46,47], molecular modeling [48], and analog studies [49] suggest that the 2-amino group of cGMP may not always interact with the CNG ion channel R(S/T)A motif, but may, under certain conditions, bind cyclic nucleotides in a conformation distinct from that found with the protein kinases.

The recently identified cAMP-binding protein EPAC (exchange protein directly activated by cAMP) or cAMP-GEF (guanine-nucleotide exchange factor) [50,51] represents a new family of CNBD-containing proteins. Preliminary characterization of EPAC-1 cyclic nucleotide selectivity has been done [52]. Further studies of this and other cyclic nucleotide receptors should continue to shed light on the complex interrelationships of intracellular cyclic nucleotide signaling pathways.

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CHAPTER 212

Cyclic Nucleotide Analogs as Tools to Investigate Cyclic Nucleotide Signaling

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Introduction

Early after the discovery of the two naturally occurring cyclic nucleotide monophosphates (cNMPs) cAMP and cGMP, the lipophilic analog N^6, 2′-O-dibutyryl cAMP was synthesized [1] and used to elicit cAMP responses in intact cells. Several hundred cNMP analogs have since been synthesized. See [2] for a review.

The effects of cAMP in vertebrates appear to be mediated mainly through activation of cAMP-dependent protein kinase isozyme I (cA-PKI) and cA-PKII, but also through activation of small GTPase exchange factors [3,4] and direct binding to ion channels [5,6]. Specialized extracellular cAMP receptors exist on the surface of *Dictyostelium discoideum* [7], and bacteria have a cAMP receptor [8] acting as a catabolite gene activator protein (CAP). The known receptors of cGMP are the cG-kinases (types Iα, Iβ, and II) and ion channels [5], but cGMP can also bind directly to allosteric sites on cyclic nucleotide phosphodiesterases and thereby modulate their action [9]. In the first section of this chapter we will give guidelines and examples of the use of cNMP analogs. In the second section we will describe the chemistry and some properties of commonly used cNMP analogs.

Use of cNMP Analogs: Guidelines and Examples

Activating cNMP Analogs

In order to pinpoint the receptor responsible for a given cyclic nucleotide effect, a dominant positive form of the receptor (not readily available for most cNMP receptors) can be introduced by transfection or by microinjection into cells. The alternative is to use cNMP analogs activating the cNMP receptor. The analog approach does not depend on artificially overexpressed gene products and can be used when transfection or microinjection is not applicable, e.g. in blood platelets. An additional advantage of cNMP analogs is that they generally can be removed by washing the cells and act within minutes, rather than hours or days.

A major problem has been to discriminate between cAMP effects mediated through the EPAC (exchange protein directly activated by cAMP) family of cAMP-regulated GTP exchange factors, cA-PKI and cA-PKII, since all commercially available analogs that activate EPAC also activate cA-PKI and cA-PKII [10]. Recently, novel 2′-O-modified cAMP analogs have been synthesized that have about three orders of magnitude higher affinity (relative to cAMP) for EPAC than for the R subunits of cA-PKI or cA-PKII (Table I), and show EPAC

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Table I Relative Affinities of Some EPAC-1 Selective cAMP Analogs

cA analog	Rel. aff. EPAC	Rel. aff. RI: $\sqrt{(AI)(BI)}$	Rel. aff. RI: $\sqrt{(AII)(BII)}$	EPAC/RI	EPAC/RII
cA	1.0	1.0	1.0	1.0	1.0
8-CPT-cA	65	2.57	1.01	25.3	64.3
2′-O-Me-cA	0.12	0.0048	0.0052	24.8	23.3
8-CPT-2′-O-Me-cA	4.6	0.0089	0.0028	517	1626

The table lists the analog affinity for the single cNMP binding site of EPAC-1, and the average affinity for site AI and BI of the RI subunit of cA-PKI and for site AII and BII of cA-PKII. The two right-hand columns give the preference for binding to EPAC relative to RI and RII. The data are from [35].

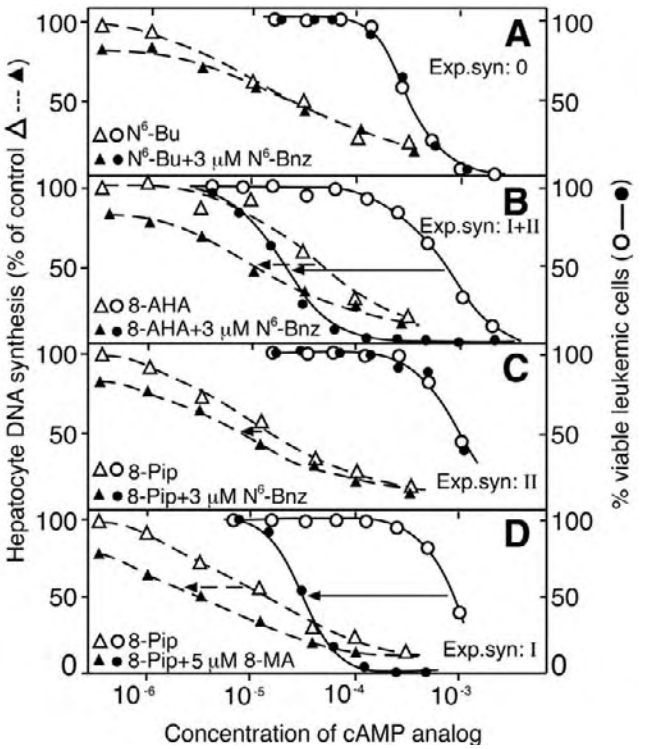


Figure 1 Effects of cAMP analog pairs selected for synergistic activation of cA-PKI and cA-PKII. Panel A shows that the two analogs N^6-benzoyl-cAMP and N^6-butyryl-cAMP (see Table III and Figure 2 for details of analog structure) fail to synergize in affecting hepatocyte DNA replication (Δ,▲) or leukemic cell apoptosis (○,●), as expected from lack of synergistic activation of either cA-PKI or cA-PKII (Table II). Panel B shows that N^6-benzoyl-cAMP synergized strongly with 8-aminohexyl-amino-cAMP, indicating that either cA-PKI or cA-PKII or both isozymes were able to induce the observed effects. Panel C shows that N^6-benzoyl-cAMP combined with 8-piperidino-cAMP, which only synergize for activation of cA-PKII (Table I), failed to synergize in induction of leukemic cell death but had a weak synergy in inhibiting hepatocyte DNA replication. Panel D shows that 8-piperidino-cAMP combined with 8-methylamino-cAMP, which selectively synergize for activation of cA-PKI (Table IIB), were strongly synergistic for leukemic cell death induction and moderately so for inhibition of hepatocyte DNA replication. The conclusion is that leukemic cell death can be induced by cA-PKI only, whereas hepatocyte DNA replication can be induced to a moderate extent by activation of cA-PKII, to a higher extent by cA-PKI, and in full by combined activation of cA-PKI and cA-PKII.

selectivity in intact cells [11]. N^6-modified cAMP analogs such as N^6-monobuturyl-cAMP and N^6-benzoyl-cAMP appear to activate cA-PKI and cA-PKII without activating EPAC [11a,35]. Such analogs do not bind to the retinal rod ion channel [12] and discriminate better than 8-Br-cAMP against cGMP-kinases [13].

No single cAMP analog is capable of selectively activating either cA-PKI or cA-PKII, but this can be achieved by analog pairs [9,14]. Examples are given in Fig. 1, where the end-point was either inhibition of hepatocyte DNA replication or induction of leukemic cell death by apoptosis [14]. The theoretical background for the approach is that the two cAMP-binding sites of cA-PKI (AI, BI) and cA-PKII (AII, BII) can be distinguished by cNMP analogs, and that both site A and B must be occupied in a given isozyme to achieve biologically relevant activation [15]. cNMP analogs preferring site AI and BI will therefore synergize in activation of cA-PKI, whereas AII- and BII-preferring analogs will activate cA-PKII synergistically (Table II). Synergistic activation is weaker for cG-PK [9,13], but N^2-substituted cGMP analogs can discriminate between the α and the β forms of cG-PKI [9]. The cyclic nucleotide-gated ion channels are less well characterized with respect to cNMP analog specificity; the photoreceptor channel was 30 times more sensitive to 8-Fl-cGMP (a fluorescein derived analog) than to cGMP itself [16]. Presumably, use of a battery of cGMP analogs, including 8-Fl-cGMP and analogs with cG-PK specificity, should allow a conclusion of whether an ion channel or cG-PK is responsible for a certain biological phenomenon.

Inhibitory cNMP Analogs

Considering the high latent energy of the cyclic phosphate bond it is no surprise that this part of the cNMP molecule is essential for biological action and that even minor modifications of the cyclic phosphate, as when either the axial (Sp) or equatorial (Rp) oxygen is replaced by sulphur, may interfere with activation. Sp-cNMPS analogs are generally agonistic, with the notable exception of the rod photoreceptor cGMP-gated cation channel, for which Sp-8-Br-PET-cGMPS

Table IIA Relative Affinities of Selected cN-analogs to cA-PK Binding Sites

cA analog	Rel. aff. AI	Rel. aff. BI	Rel. aff. AII	Rel. aff. BII
cA	1.0	1.0	1.0	1.0
N^6-Bu-cA	3.6	0.093	0.71	0.041
N^6-Bnz-cA	4.0	0.26	3.8	0.037
8-AHA-cA	0.056	4.1	0.010	0.39
8-Pip-cA	2.1	0.06	0.047	2.7
8-MA-cA	0.07	3.3	0.026	1.6
N^6-BC-cA	0.50	0.086	13	0.066
Sp-5,6diCl-cBIMPS	0.022	0.13	0.034	14

Table IIB Predicted Synergy between Pairs of cN-analogs

cA analog	Predicted synergy cA-PKI	Predicted synergy cA-PKII
x + y	$\frac{\sqrt{(AI^x+AI^y)(BI^x+BI^y)}}{\sqrt{(AI^x)(BI^x)}+\sqrt{(AI^y)(BI^y)}}$	$\frac{\sqrt{(AII^x+AII^y)(BII^x+BII^y)}}{\sqrt{(AII^x)(BII^x)}+\sqrt{(AII^y)(BII^y)}}$
N^6-Bu-cA + N^6-Bnz-cA	1.0	1.1
8-AHA-cA + N^6-Bnz-cA	2.8	2.9
8-Pip-cA + N^6-Bnz-cA	1.0	4.4
8-Pip-cA + 8-MA-cA	3.2	1.0
N^6-BC-cA + Sp-5,6diCl-cBIMPS	1.3	8.3

Panel A shows the affinity of selected cAMP analogs for the binding sites of cA-PKI (AI, BI) and cA-PKII (AII, BII). Data are from Øgreid *et al.* [36] or the authors'unpublished data. Panel B shows the formulas to predict synergy between analog pairs for activation of cA-PKI or cA-PKII. Numbers above 1.0 signify synergism. For details of cAMP analog structures, see Table III and Figure 2.

is an antagonist [17]. The Rp-cNMPS analogs are generally antagonistic or partially agonistic, with the exception of the cGMP-gated cation channel, where Rp-cGMPS analogs such as Rp-8-Br-cGMPS are agonists [18].

For practical purposes the comparative use of Rp-8-Br-cAMPS, Rp-cAMPS, and Rp-8-Br-cGMPS may be useful. Since these analogs must be used at high concentrations (0.1–1 mM in the extracellular medium) to be able to compete efficiently with cAMP or cGMP in the cell, it is important to test them against submaximal concentrations of the cNMP receptor activator. If Rp-8-Br-cAMPS is the more efficient inhibitor one can suspect that cA-PKI is the main mediator; whereas when Rp-cAMPS is the better inhibitor cA-PKII is more likely; and if Rp-8-Br-cGMPS is the most efficient inhibitor one can suspect cG-PK involvement. Further proof of cyclic nucleotide protein kinase involvement can be obtained by use of cell-permeable inhibitors directed against the kinase moiety of these enzymes, either broadly acting ATP antagonists [19] or more specific peptide inhibitors (Chapter 79 by Dostmann, this volume). If applicable, dominant negative forms of RI or RII (with deficient cAMP-binding sites) can be introduced by transfection or microinjection to further prove cA-PK involvement.

Chemistry and Properties of Cyclic Nucleotide Analogs

Cyclic AMP and cGMP are composed of four rings: the cyclic phosphate, the ribose, and a substituted aromatic purine double ring (pyrimidine and imidazole). The structure of most cNMP analogs can be described (Table III) as variously substituted derivatives of purine riboside-3′,5′-cyclic monophosphate (cPuMP), shown in Fig. 2A. The parent cAMP and cGMP molecules are virtually unable to penetrate cell membranes by diffusion and are readily broken down by cyclic nucleotide phosphodiesterases. Most cNMP analogs that are synthesized for use in cell biology have enhanced lipophilicity (Table III) and improved resistance toward cyclic nucleotide phosphodiesterase attack.

The imidazole ring of cPuMP has been subjected to a number of substitutions at the 8 position. Substituent charge, hydrogen bonding potential, hydrophobicity, and bulk determine the specificity toward cAMP-binding sites type A and B of cA-PKI and cA-PKII [20]. Bulky 8-substituents will force the cNMP molecule into a *syn* conformation about the torsion angle between C4-N9 of the purine ring and the O1′-C1′ of the ribose ring (Fig. 2A). This will deter cN binding to the

Table III Compounds Resulting from Substitution of cPUMP (fig. 2A)

Compound	R2′	R2	R6	R8	Relative lipophilicity	Sp/Rp isomers commercially available
cPUMP	–	–	–	–	–	–
cAMP	OH	–	NH_2	–	1.0	+
8-MA-cAMP	OH	–	NH_2	$NHCH_3$	1.0	–
N^6-Bu-cA	OH	–	NHC_4OH_7	–	3.5	–
N^6-Bnz-cA	OH	–	NH-benzoyl	–	6.5	–
N^6-BC-cA	OH	–	NH-t.-butyl-carbamoyl	–	32	–
8-AHA-cA	OH	–	NH_2	NH-$(CH_2)_6$-NH_3^+	–	–
8-Pip-cA	OH	–	NH_2	piperidino	12	+
8-CPT-cA	OH	–	NH_2	Chloro-phenyl-thio	36	+
8-Br-cA	OH	–	NH_2	Br	1.8	+
8-Azido-cA	OH	–	NH_2	N_3	–	+
2′-O-butyrate-cA	-O-C_4OH_7	–	NH_2	–	–	+
8-CPT-2′-O-Me-cA	-O-CH_3	–	NH_2	Chloro-phenyl-thio	–	–
cGMP	OH	NH_2	=O	–	0.48	+
8-Br-cG	OH	NH_2	=O	Br	1.2	+
8-CPT-cG	OH	NH_2	=O	Chloro-phenyl-thio	27	+
8-Fluo-cG	OH	NH_2	=O	Fluo	–	+

Compounds resulting from substitution of cPUMP (Fig. 2A). For numbering of substituents, see Fig. 2A. 8-Fluo-cGMP is 8-[[2-[(Fluoresceinylthioureido)amino]ethyl]thio]-cGMP. The lipophilicity data are adapted from [2] and www.biolog.de.

E. coli CAP protein that binds cAMP in the *anti*-conformation [21] and to the surface cAMP receptor of *Dictyostelium* [22], but not to cA-PK [23] and cG-PK, which bind cAMP in the *syn* conformation. 8-azido-cAMP can be used to photo-affinity label cA-PK in broken cell preparations [24]. This compound may not work in intact cells, since it is labile in a reducing environment, where it quickly decomposes to 8-NH_2-cAMP [25]. 8-Fluo-cAMP and 8-NBD-cAMP are both useful fluorescent cAMP analogs (for more information, see www.biolog.de). 8-Cl-cAMP is slowly metabolized intracellularly to toxic 8-Cl-AMP and 8-Cl-adenosine, and its antitumor properties [26,27] may be related to this fact [28,29]. The 7-deaza-cAMP molecule has intact ability to bind to cA-PK, but is metabolized to the highly toxic [30] compound 7-deaza-adenosine (tubercidine).

Most pyrimidine ring substituents are in the 6 or 2 position, in which cAMP and cGMP differ (Table III). The introduction of an electron-withdrawing group (-Cl, -CF3) in position 2 will enhance binding to site B of cA-PK, but decrease binding to cG-PK [13,31]. The introduction into cGMP of certain bulky NH-substituents in 2-position can greatly enhance the specificity of binding to cG-PKIα compared to cG-PKIβ [9]. The introduction of bulky hydrophobic NH-substituents in 6-position will produce molecules with preference for site A of cA-PK, low affinity for cG-PK, and resistance to degradation by cyclic nucleotide phosphodiesterases.

Modification of the purine ring structure itself has resulted in compounds with enhanced affinity for site B of cA-PKII (such as Sp-5,6-diCl-cBIMPS; Fig. 2B) or cG-PKI (such as 8-Br-PET-cGMP; Fig. 2C). Fluorescent analogs can also be produced by ring extension, as in 1,N^6-etheno-cAMP [32].

The ribose moiety of cAMP and cGMP is tolerant to modification regarding binding to the *Dictyostelium* surface receptor [22] and EPAC (Table I), but not regarding binding to cA-PK and cG-PK. The 2′-O-butyryl-substituents give at least 1000-fold decreased binding activity but are useful to enhance membrane penetration. Since the butyrate is removed quickly by ubiquitous esterases in the cell, the parent compound will be formed intracellularly (trapping effect).

In the cyclic phosphate moiety the equatorial (Rp) or axial (Sp) oxygen (O) can be substituted with a sulphur (S) (see also Fig. 2B and 2E). This modification greatly increases the stability toward phosphodiesterases. Sp-cAMPS and Sp-cGMPS and their derivatives are used as hydrolysis-resistant agonists of cA-PK and cG-PK. It is interesting that Sp-8-Br-PET-cGMPS is an antagonist for the retinal type ion channels and an activator of cG-PK [17]. Rp-cAMPS and Rp-cGMPS and their derivatives act as partial agonists or pure antagonists for cA-PK and cG-PK. Rp-8-Br-cAMPS is an excellent inhibitor of cA-PKI, whereas Rp-cAMPS preferentially inhibits cA-PKII. Rp-8-Br-cGMPS is an antagonist of cG-PK and an activator of the retinal cGMP-gated ion channel [18]. The ability of cNMP analogs to penetrate cell membranes is highly improved [33] by masking the cyclic phosphate charge by esterification (cNMP-acetoxymethyl ester, e.g. Sp-cAMPS-AM, Fig. 2E) or coupling to a coumarin derivative. The cNMP-AM analogs will be trapped intracellularly due to esterase cleavage of the AM ester. The coumarin

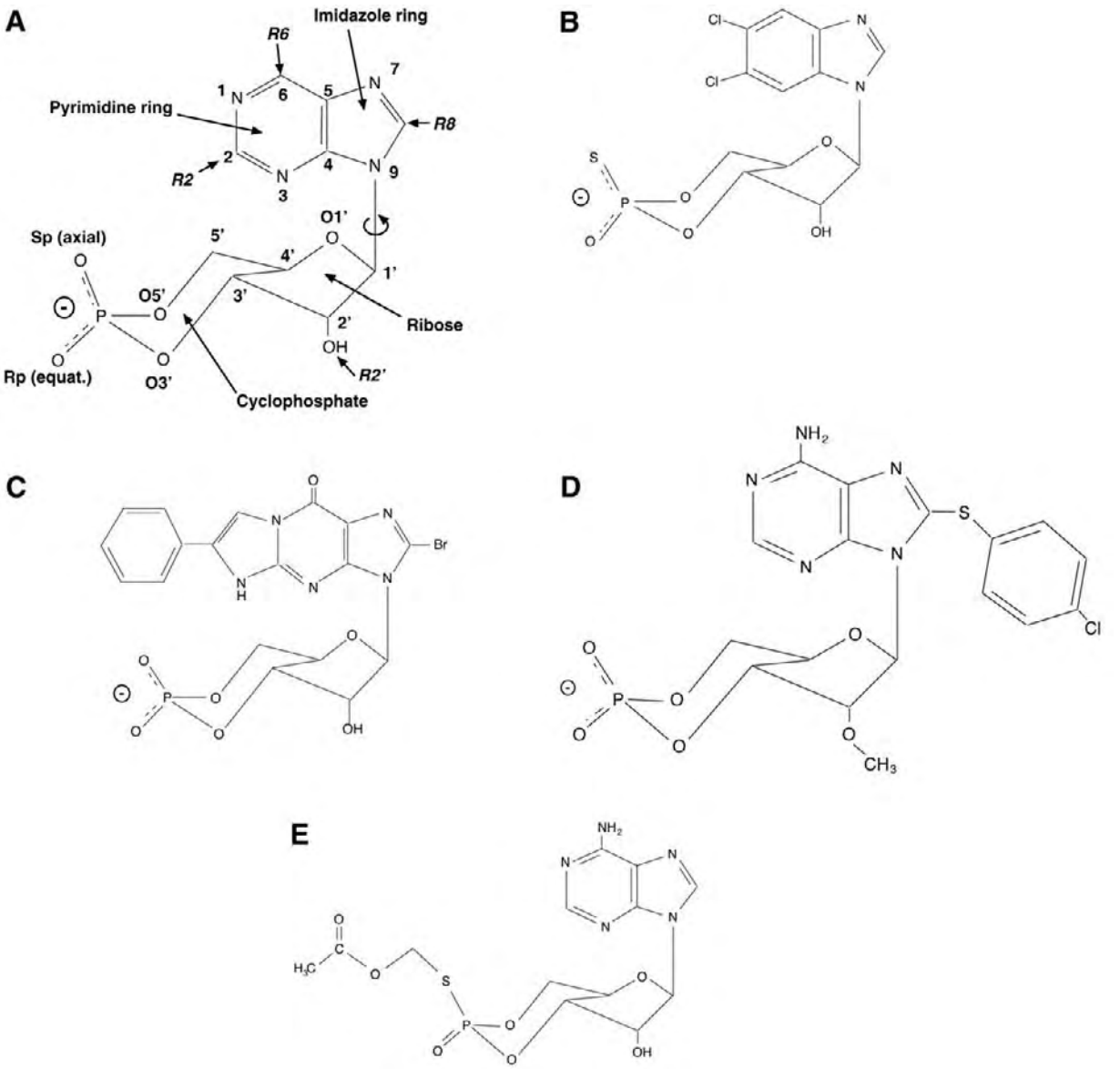


Figure 2 The structure of some cyclic nucleotide analogs. Panel A shows the structure of cyclic purine monophosphate (cPuMP), which can be considered the backbone of cAMP, cGMP, and the other analogs listed in Table III. The most common sites for substitution are indicated (R2′, R2, R6, and R8). Panel B shows Sp-cAMPS with modified pyrimidine ring (Sp-5, 6-di-chloro-cBIMPS), panel C shows 8-Br-cGMP with a phenyl-etheno extension of the pyrimidine ring, panel D shows 8-CPT-cAMP with a modified 2′-position (8-CPT-2′-O-Me-cAMP), and panel E shows the acetoxymethyl-modified Sp-cAMPS.

derivatives are caged, and in principle they are immediately converted to the active parent compound upon irradiation. Other modifications of the cyclic phosphate ring are replacements of the O3′ and O5′ by NH [22].

Future Developments

Currently available cNMP analogs have proven to be very useful tools in cell biology. There is nevertheless room for considerable progress regarding both the synthesis of more specific and potent analogs with improved pharmacokinetic properties and mapping of the cNMP receptors. An interesting novel approach is to synthesize polymer-linked cNMPs to simultaneously occupy two binding sites in the same receptor complex [34].

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SECTION G

G Proteins

Heidi Hamm, Editor

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CHAPTER 213

Signal Transduction by G Proteins—Basic Principles, Molecular Diversity, and Structural Basis of Their Actions

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Introduction

Cells do not live in isolation and respond to extracellular stimuli either with specific tasks such as neurotransmission, activation of substrate uptake, fatty acid release, or with adaptive changes that ensure homeostatic cohabitation with other cell types as is needed in multicellular organisms to ensure better survival (stress responses). Extracellular cues include nutrients, intoxicants, and of course, signaling molecules such as autacoids, growth factors, and hormones. Signaling molecules are either membrane-permeant (e.g., steroid hormones) or -impermeant (peptide hormones, biogenic amines) for which cells have evolved separate response mechanisms. For membrane-permeant signals, receptors are for the most part intracellular, cytosolic, or nuclear, and the changes they elicit are frequently modulations of gene expression with direct participation of the receptor in the regulatory complex that transcribes response genes. For membrane-impermeant signals, nature has evolved a repertoire of mechanisms by which binding of the ligand to its receptor on the cell surface leads to intracellular changes in one or more enzymatic activities and to activation or inhibition of regulatory signaling pathways. In many instances the action(s) of a receptor involve promotion or disruption of multimeric protein complexes. Some of the signaling pathways activated by receptors in response to membrane-impermeant ligands are wholly cytosolic or submembranous; others affect nuclear gene expression. The process by which the extracellular ligand-receptor interaction leads to changes inside the cell is commonly referred to as "signal transduction"—the ligand is the extracellular signal whose message is transduced into an intracellular signal of a different chemical nature.

Among the signals generated in this way are second messengers, such as ions entering through ligand-gated ion channels; signaling molecules derived from the receptor itself, such as the cytosolic domain of Notch; products of the activation of the receptors' intrinsic enzymatic activities, such as cGMP formed by the guanylyl cyclase activity of ANF and guanylin receptors; tyrosine phosphorylation, first of self and then of proteins recruited to the phosphorylated receptor, as happens when epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin, or nerve growth factor (NGF) bind to their respective homodimeric receptors; and sequential serine/threonine phosphorylation, also first of self (receptor II phosphorylating receptor I) and then of cytosolic transducing proteins such as R-SMADs by phosphorylated receptor I, as it happens in response to interaction of the transforming growth factor β (TGFβ) and

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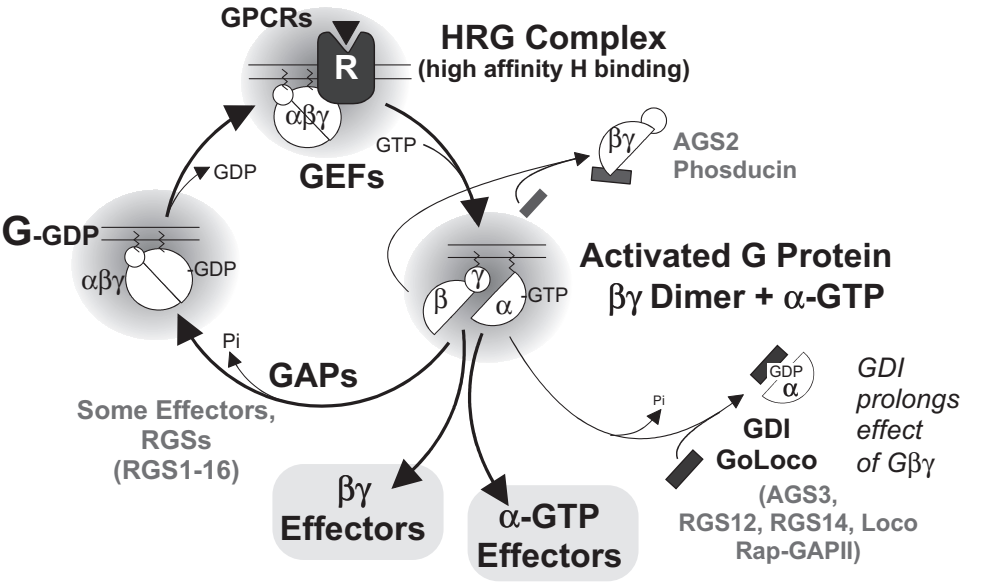


Figure 1 Hormone receptor complexes act as guanine nucleotide exchange factors (GEFs) to catalyze activation of the trimeric G protein with formation of α-GTP plus βγ, both of which modulate effector functions. Spontaneous decay of α-GTP to α-GDP can be modulated by GTPase activating proteins (GAPs) such as RGSs and some effectors, and by activators of G protein signaling (AGSs) that tilt the balance between effects through α-GTP versus those through βγ.

bone morphogenic protein (BMP) superfamily of signaling molecules with their heteromeric receptors. In the case of R-SMADs, their phosphorylation exposes a nuclear localization signal as well as a heterodimerization domain, leading ultimately to translocation into the nucleus and changes in gene expression. For general references see [1].

The use of heterotrimeric G proteins as signal transducers constitutes a completely different type of signal transduction process. In this case evolution has lead a structurally related superfamily of receptors that recognize peptide and protein hormones, neurotransmitters, autacoids, and sensory signals such as light, odor, and taste, to acquire the ability to recruit and regulate the intrinsic signaling capacity of a large family of structurally related heterotrimeric regulatory GTPases (Fig. 1). This chapter deals with the mechanism of activation/deactivation and the action of the heterotrimeric G proteins responsible for transducing receptor activation signals into cellular responses. Activation of a G protein by a receptor, first proposed by Rodbell and collaborators to be a two-step process in which a GTP-dependent transducer regulates an enzyme or amplifier without clear knowledge of the number of molecular components involved [2], is now known to be a multistep process that includes GDP/GTP exchange and subunit dissociation of the αβγ G protein, followed by spontaneous deactivation—hydrolysis of the activating GTP to GDP—and reassociation of the separated members in preparation for reactivation by a new round of GDP/GTP exchange promoted by the activating hormone receptor (HR) complex (bottom panel of Fig. 1). As shown in this figure, the basic cycle is affected by modulators of G-protein signaling, the RGSs, which increase the rate at which α-GTP deactivates and the AGS2- and AGS3-type modulators which sequester either α-GDP or Gβγ and thereby bias signaling through α-GTP versus signaling through Gβγ (see the following sections).

Ras, the Prototypic Regulatory GTPases

As the name indicates, regulatory GTPases are proteins that bind and hydrolyze GTP. Their regulatory power lies in the fact that their conformation differs when occupied by GTP or GDP. They are also referred to as molecular switches. Due to their intrinsic GTPase activity they carry a built-in inactivating timer that prevents the GTP state from being long-lived. The crystal structure of the 180 amino acid regulatory GTPase *ras*, in its GTP- and GDP-liganded forms, shows two principal regions that differ in the GTP state as compared to the GDP state, referred to as switch I and switch II [3]. Switch I, amino acids 32 through 40, is a large loop connecting α-helix 1 (α1) to β-strand 2 (β2). Mutations in this region interfere with activation of several of the downstream effectors of *ras*. Switch II, amino acids 60 through 75, changes conformation even more drastically than switch I, to the extent that upon binding of GTP, amino acids 66 through 74 rearrange into a well-ordered α-helix (α2). Switches I and II are required for activation of downstream effector(s) by *ras*-GTP, the activated form of *ras*. *Ras*-GDP appears to be neutral. Mutations of Gln-61, (at the start of switch II after β3), and of Gly-12 (at the base of α1), reduce the intrinsic GTPase activity of *ras* prolonging the life span of the activated GTP state. As is the case for most, if not all regulatory GTPases, the *ras* GTPase is under regulation of two types of proteins, a GEF or guanine nucleotide

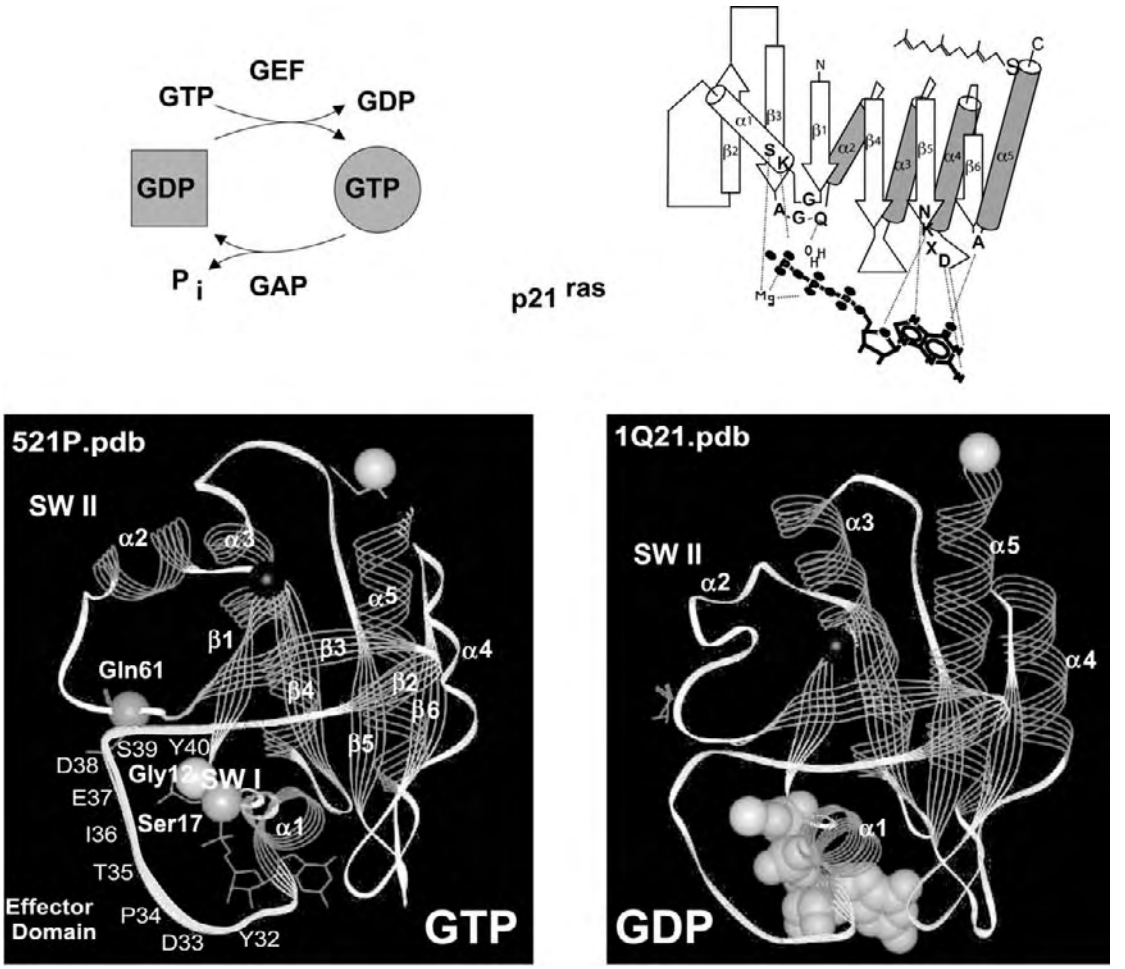


Figure 2 Model of the crystal structure of *ras* in its GTP and GDP states (PDB accession #521P and 1Q21) highlighting secondary structures (α-helices and β-strands as well as switch 1 (effector domain) and switch 2 regions. Bottom left illustrates the basic GTPase regulatory cycle and its two principal modulators: GEFs, responsible for activation, and GAPs, responsible for deactivation of the regulatory GTPases. The bottom right diagram is a two-dimensional representation of the three-dimensional features of the basic GTPase fold found in all regulatory GTPases.

exchange factor, responsible for promoting the transition of *ras*-GDP to *Ras*-GTP, and several GAPs or GTPase activating proteins, which, as their name indicates, shorten the life span of *ras*-GTP by increasing the catalytic efficacy of its intrinsic GTPase (see [4,5], and references therein). SOS (son of sevenless) and *ras*-GAP1, are the prototypes of *ras* GEF and *ras* GAP, respectively. Mutation of Ser-17 to Asn-17, roughly in the middle of α2, interferes with the nucleotide exchange reaction and locks *ras*-GDP in an inactive conformation in which it may still bind to proteins regulated by *ras*-GTP, but without affecting their activity. By reason of occupying the site to which *ras*-GTP should bind to activate effectors, Asn-17 *ras* interferes with signaling by wild-type *ras*-GTP and is referred to as a dominant negative form of *ras* [6]. Figure 2 depicts *ras* in its GTP and GDP conformations and highlights the locations of Gly-12 (G12), Ser-17 (S17), and Gln-61 (Q61), as well as switch I (in blue) and switch II. The upper left panel of Fig. 2 presents this basic GTPase cycle in schematic form with GEF and GAP regulating the life-times of the GTP and GDP sates. The upper right panel is a schematic two-dimensional representation of the main three-dimensional features of this regulatory GTPase (adapted from reference [3])

Heterotrimeric G Proteins

Trimeric G proteins are responsible for transducing the effects of the seven-transmembrane superfamily of receptors, and are regulatory GTPases which, while more complex than *ras*, nevertheless preserve the basic features of the *ras*-type regulatory GTPases. G proteins activated by seven-transmembrane receptors are α, β, γ trimers, of which the α subunit is the GTPase-bearing subunit. The β and γ subunits form a dimer that exists either free or in association with α-GDP. Gβ and Gγ have never been found isolated as individual proteins.

Subunit Structure

α-Subunits were crystallized in the laboratories of PaulSiegler at Yale University and of Steven Sprang at

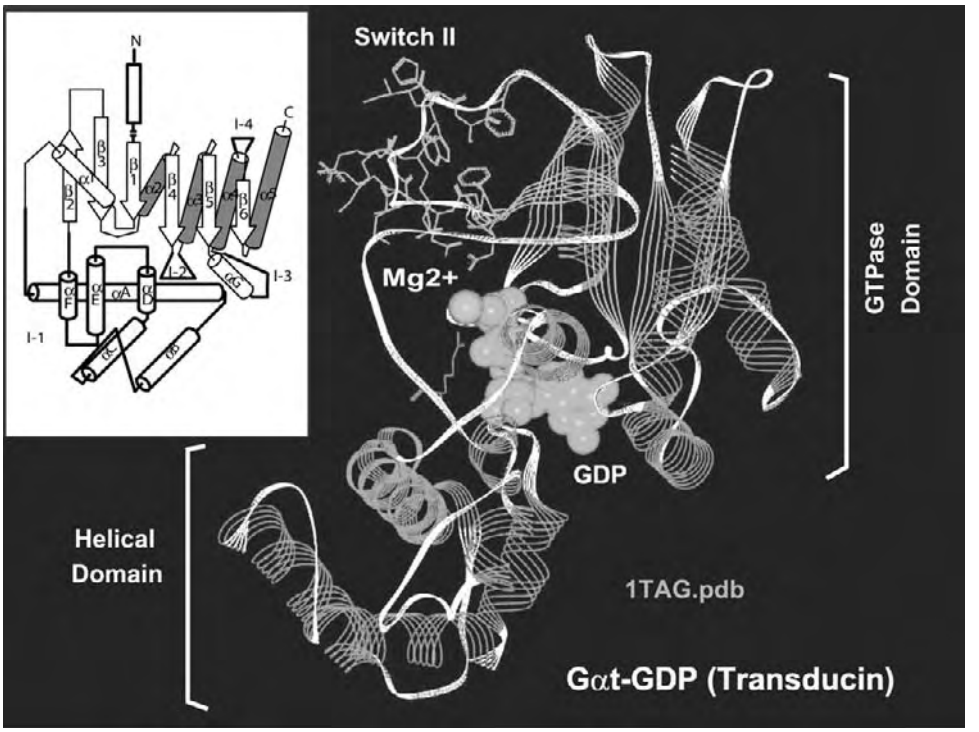


Figure 3 Crystal structure of an α subunit of a heterotrimeric G protein in its GDP state (PDB #1TAG) Its GTPase domain is oriented in the same way as *ras* in Fig. 2. Atoms of amino acid side chains of switch II two are displayed as sticks; space-filling CPK atoms were used to represent GDP. Mg is a cyan ball. Upper right inset: Two-dimensional diagram of three-dimensional features of heterotrimeric G-protein α subunit. Additions to the *ras* structure are in heavy lines. GTPase and helical domains are highlighted.

the University of Texas at Dallas, in collaboration with Heidi Hamm and Alfred Gilman, respectively [7,8]. From a structural viewpoint, α subunits of heterotrimeric G proteins are made up of a *ras*-like GTPase domain and a helical domain with 6 α-helices (αA through αF) inserted in the center of what would be switch I of the *ras* (Figs. 3 and 5). In its GDP-liganded form, Gαs have a disorganized switch II region and exhibit high affinity for Gβγ. Gα-GDPs are therefore found associated with Gβγs as heterotrimers. Gβγ locks GDP into its binding site on Gα, causing its dissociation rate to drop by a factor of 10 [9]. At the same time, Gβγ also shields the switch II region from interacting with possible effectors (Fig. 5). Consequently, activation of a heterotrimeric G protein requires two events: (1) exchange of GTP for GDP, and (2) dissociation of Gβγ from Gα. Subunit dissociation has not been measured in intact cells in an unequivocal way, but it is readily seen *in vitro*. Dissociation exposes the regulating surface of Gα-GTP and allows for effector regulation with direct involvement of switch II ([10]; PDB accession #1CS4). The converse also applies. That is, Gβγ is a signaling molecule able to regulate effector functions and its association with Gα-GDP resulting in occlusion of the signaling surface of its Gβγ.

The structure of Gβγ deserves separate comment ([11,12]; Figs. 4 and 5). Gβ is a seven-bladed propeller of which each blade is made up of four anti-parallel β-strands running from the center to the periphery. The innermost β-strand runs parallel to the axis of rotation of the propeller. The next two change pitch to approach the orientation of the outermost, which runs along the periphery of the propeller and is coplanar with the circle described by the rotating propeller. Blade seven is made up of three β-strands contributed by the very C terminus of Gβ and a fourth (outermost) "zipping" β-strand recruited from the sequence immediately preceding those that create blade I. Preceding the zipping β-strand is an extended N terminus with a long α-helix that interacts with the Gγ by forming a coiled-coil. Gβγ dimers have so far been crystallized only in association with Gα subunits or with a retinal regulatory protein, phosducin, but not in isolation. Conformational changes in Gβ that occur upon dissociating from Gα, if they occur, have not been observed as yet.

LIPID MODIFICATIONS

Gα and Gβγ subunits engaged in signal transduction are membrane bound by virtue of lipid modifications. Gβγ dimers are anchored to membranes through a C15 or C20 polyisoprene attached to the Cys of a C-terminal CAAX motif. Posttranslational processing not only attaches the polyisoprene, but also removes the last three amino acids and methylates the new C terminus. Prenylation is not necessary for association of Gγ with Gβ, but is required for association of Gβγ to Gα-GDP and for regulation of effector, e.g., adenylyl cyclase. Further, prenylation contributes to the association of the Gγ dimer to membranes.

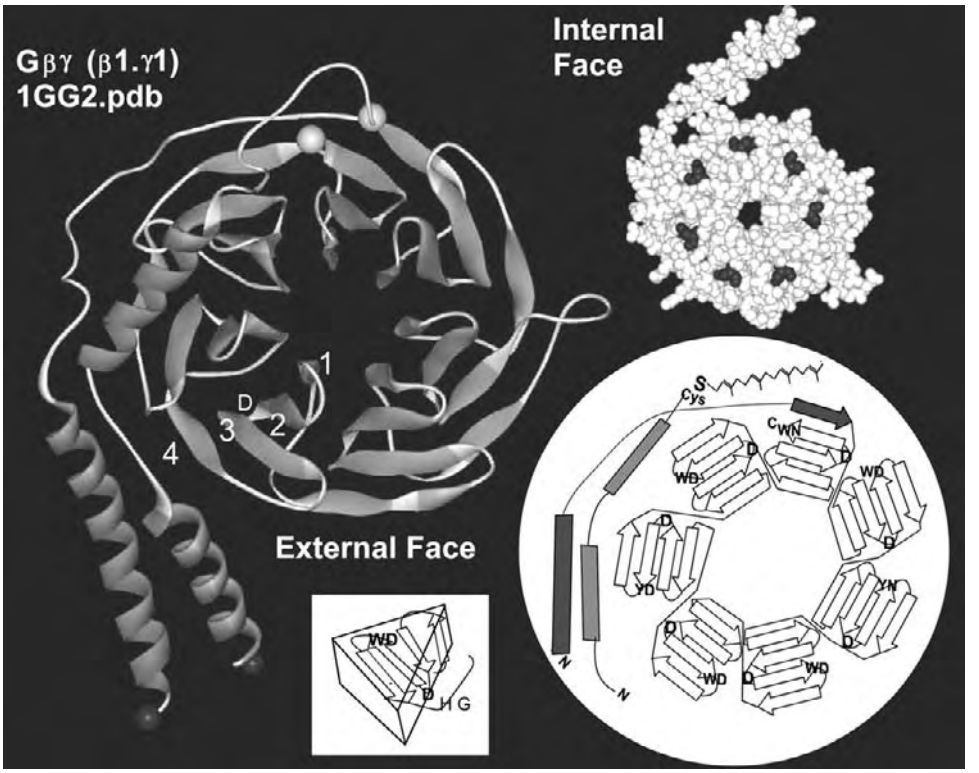


Figure 4 View of a seven-bladed Gβ propeller from the "external" side determined by its orientation when complexed with α-GDP. Upper right inset: CPK representation of the same propeller viewed from the side facing α-GDP in its trimeric form. Blue: Conserved aspartic acids (D) present in each propeller blade. Bottom right: Two-dimensional diagram of the three-dimensional features of a Gβ propeller highlighting the "zipping," outermost β-strand of blade seven, and location of conserved D residues shown in blue in the space-filling representation of the model. Note the γ coiled-coil interaction of the γ subunit with the N terminus of the Gβ subunit.

Most Gα subunits engaged in signal transduction are palmitoylated near their N terminus. Palmitoylation facilitates their anchoring to the plasma membrane. Gα subunits of the Gi/Go family are also myristolated at N-terminal glycines (Gly-2 of the primary transcript). Gα myristylation increases affinity for Gβγ dimers. Removal of myristic acid by Gly-2 to Ala-2 mutation renders Giα subunits inactive as inhibitors of adenylyl cyclase. Some but not all nonpalmitoylated Gα subunits fail to localize to membranes and are found in the cytosol. Lipid modification of Gα and Gγ subunits are therefore essential for their normal biological activity (Reviewed in reference[13]).

Molecular Diversity of G Proteins

Each of the subunits that make up a heterotrimeric G protein is encoded by a family of structurally homologous genes. There are 16 Gα (one with two splice variants), 5 Gβ, and 11 Gγ genes, raising the theoretical possibility of close to 1000 distinct heterotrimers. Gα subunits are the longest of the three subunits, ranging from 350 to 390. Their sequence similarities vary from almost identical when Gi1α is compared to Gi3α (86% identical) to up to 60% different when Gsα is compared to G16α. Amino acid sequence alignments show Gβs to be structurally a very closely related family with β1-4 being 350 amino acids long and differing by no more than 17% in their amino acid sequence. Gβ5 with 395 amino acids, exhibits the same degree of similarity, differing primarily by a 45amino acid N-terminal extension. Gγs are the shortest (68–75 amino acids) and are the most diverse, differing in amino acid sequence between 40 and 65%. GGL (Gγ-like) domains of RGS 6, 7, 9, and 11 (for RGS see below) constitute an additional group of Gγ subunits that interact with the atypical N-terminal extension of Gβ5′.

The actual number of G-protein isoforms in any given cell is much lower than 1000 for two reasons: (1) there is no cell known that expresses all G-protein subunit genes, and (2) there are structural limitations that do not allow all βγ dimers to form, e.g., while β1 interacts with γ1, γ2, and γ3, β3 does not interact with γ1 or γ2, but partners with γ4. Vice versa, γ1 partners with β1 but not with either β2 or β3; and γ2 partners with β1 and β2, failing to do so with β3. The complete spectrum of permissible interactions among the 5β subunits and 11 γ subunits still needs to be worked out. One Gβ, Gβ5, interacts preferentially with the GGL domain of 6, 7, 9, and 11. It is quite possible that even for biochemically permissible interactions there may be βγ dimers that never form because they are not co-expressed in the same cell.

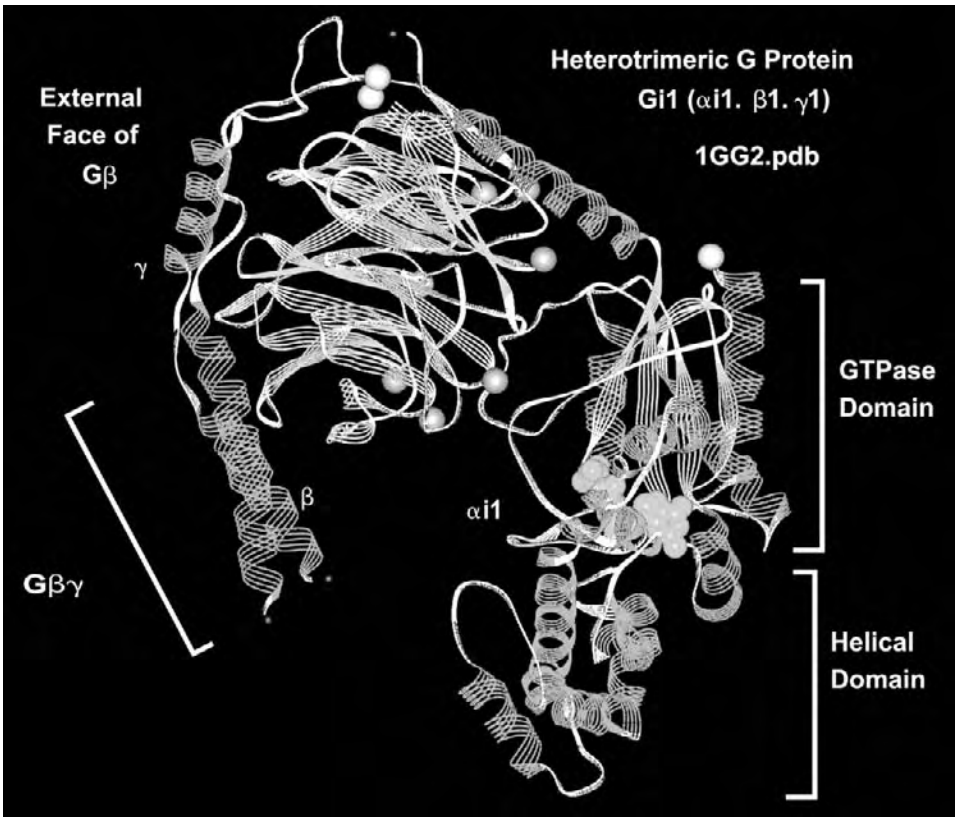


Figure 5 Model of Gi1 heterotrimer with bound GDP deduced from crystal x-ray diffraction studies (PDB accession #1GG2). Note that the internal face of Gβ shields the switch II of Gα subunit from being accessible to effectors. Blue balls, N-terminal α carbons; brown balls, C-terminal α carbons; gray balls on Gβ, α carbons of conserved aspartic acids.

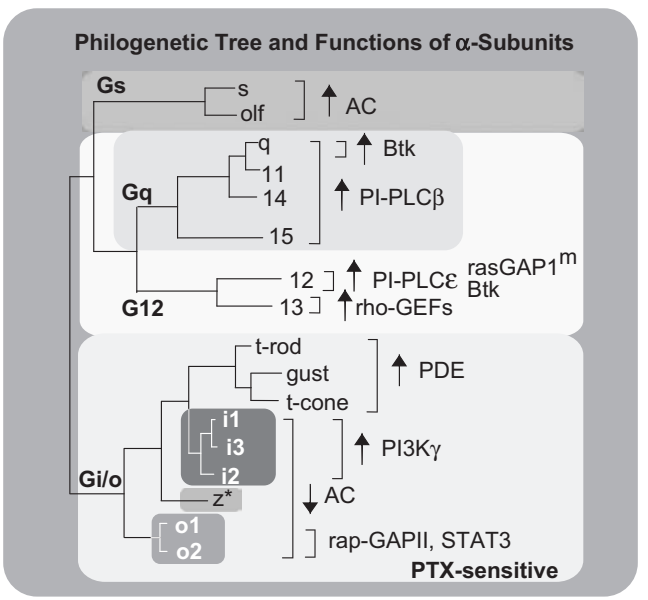


Figure 6 Phylogenetic analysis of G-protein α subunits reveals structural subdivisions that have functional correlates.

G proteins are named after their α subunit. This has its origin in the fact that for the first two G proteins discovered, Gs and transducin (Gt), it was established that the major (then sole) signaling function resided in their α subunits. Gαs, activates adenylyl cyclases (ACs), and Gαt subunits activate visual phosphodiesterase (PDE, a tetramer of one α, one β, and two inhibitory γs). Activation of visual PDE results from the association of αt-GTPs with the PDEγs, thereby suppressing their inhibitory effects on PDEαβ. A phylogenetic tree of G-protein α subunits (Fig. 6), clusters their sequences into four subfamilies: Gs, Gq, G12/13, and the pertussis toxin (PTX)-sensitive Gα subunit subfamily. The latter includes three α subunits that play roles in light and taste perception, plus the α subunits of the Gi/Go family. PTX-sensitive α subunits not only show higher sequence similarity to each other than to the remaining α subunits, but as their name indicates, they are substrates for the ADP-ribosyl-transferase activity of the S1 subunit of pertussis toxin. The ADP-ribosylated amino acid is a Cys at position –4 from their C termini. PTX uncouples this group of G proteins from activation by receptors by virtue of creating of a steric hindrance to the G-protein::receptor interaction. Included in this structural group is the α subunit of Gz (αz), which lacks a Cys at -4 from the C terminus and is PTX-insensitive. It is functionally a Gi, its closest structural homologue. As illustrated in Fig 6, and in more detail in Fig. 7, α subunits modulate the activity of a large and diverse group of enzymes, including ACs, phospholipase Cβs (PLCβs), phosphatidylinositol 3-OH kinases (PI3Ks), as well as type 6 visual and type 3 gustatory PDEs and a Rho-GEF for regulation of cytoskeletal remodeling. An analysis of the evolution of the

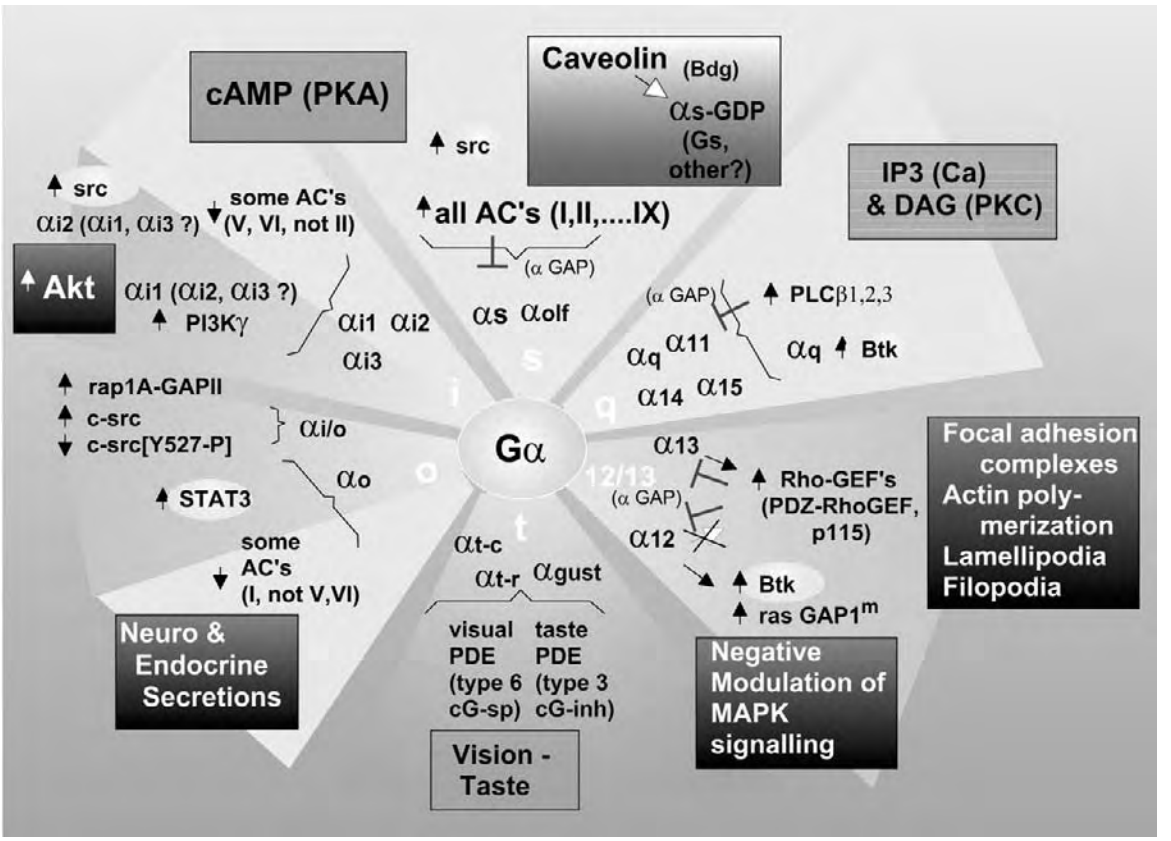


Figure 7 Effectors of activated α subunits. This figure shows the different signaling systems affected by α subunits at the periphery, with ever increasing detail toward the center. —|, inhibition.

intron-exon structure of G protein α subunits (Fig. 8) clusters the genes into the same groups: Gs, Gq/11, G12/13, and Gi/o and G-taste/vision. This type of analysis places Gz in the Gi/Go group solely on the basis of open reading frame sequence similarity, but shows no similarity in its intron-exon organization to those of any G protein as it has only three exons (two coding) instead of the eight to nine exons of the Gi/o family of α subunits. The loss of introns is highly suggestive of reactivation of a pseudogene with attendant chance mutation of the Cys at –4 from the C terminus.

Both Gα subunits and Gβγ dimers modulate, positively or negatively, a diverse set of cellular functions. In some instances, the effects of Gβγ dimers are in concert with those of α subunits, in others regulation of effector by Gβγ is unrelated to regulation by a Gα. It is not clear at this time whether effectors distinguish Gβγ isoforms. As illustrated on Fig. 9 the gamut of Gβγ effectors is as complex and as diverse as that of the α subunits. Among the regulated functions worth mentioning are inhibition of type 1 AC, but co-stimulation with Gsα of type 2 and type 4 ACs; stimulation of type 3 PLCβ independently of co-existing stimulation by the Gq/11 group of α subunits; co-stimulation with Gαi2 (possibly also αi1 and αi3) of PI3Kγ; and co-stimulation of PI3Kβ with tyrosine-phosphorylated p85, the regulatory subunit of PI3K. *In vitro* reconstitution experiments in which PI3Kβ was incubated with Gβγ and a tyrosine-phosphorylated peptide corresponding to the tyrosine-phosphorylated sequence of p85, showed that stimulation by each was three to fivefold, but became 100-fold when the tyrosine-phosphorylated peptide and the Gβγ were added together. This type of cross-dependence on dual inputs is highlighted in Figs. 7 and 9. Gβγ also modulates, sometimes positively and sometimes negatively, a variety of ion channels and thereby provides a nexus between regulation of second messenger formation by enzymes and regulation of cell excitability by voltage, which it can augment (activation of potassium channels) or dampen (inhibition of presynaptic Ca^{2+} channels) (Fig. 9).

In conclusion, signal transduction by G proteins is the result of structurally similar receptors activating structurally very similar G proteins, which then regulate positively or negatively the activity of a diverse gamut of structurally unrelated cellular functions that affect intracellular levels of cAMP, inositol tris-phosphate (IP3), Ca^{2+}, diacylglycerol (DAG), and phosphatidyl inositol 3 phosphates (PIP3s) as well as ion channel activity and formation of lamellipodia and filopodia and the attendant cytoskeletal changes. Moreover, because second messengers such as cAMP, DAG, and Ca^{2+} affect protein kinases, it is unlikely that there is a cellular function that in one way or another is not under the controlling or modulatory influence that emanates from activation of heterotrimeric G proteins. Indeed, as indicated in Figs. 7 and 9, cellular responses included are the PI3K-PDK-Akt-NF.kB anti-apoptotic response of cells to extracellular

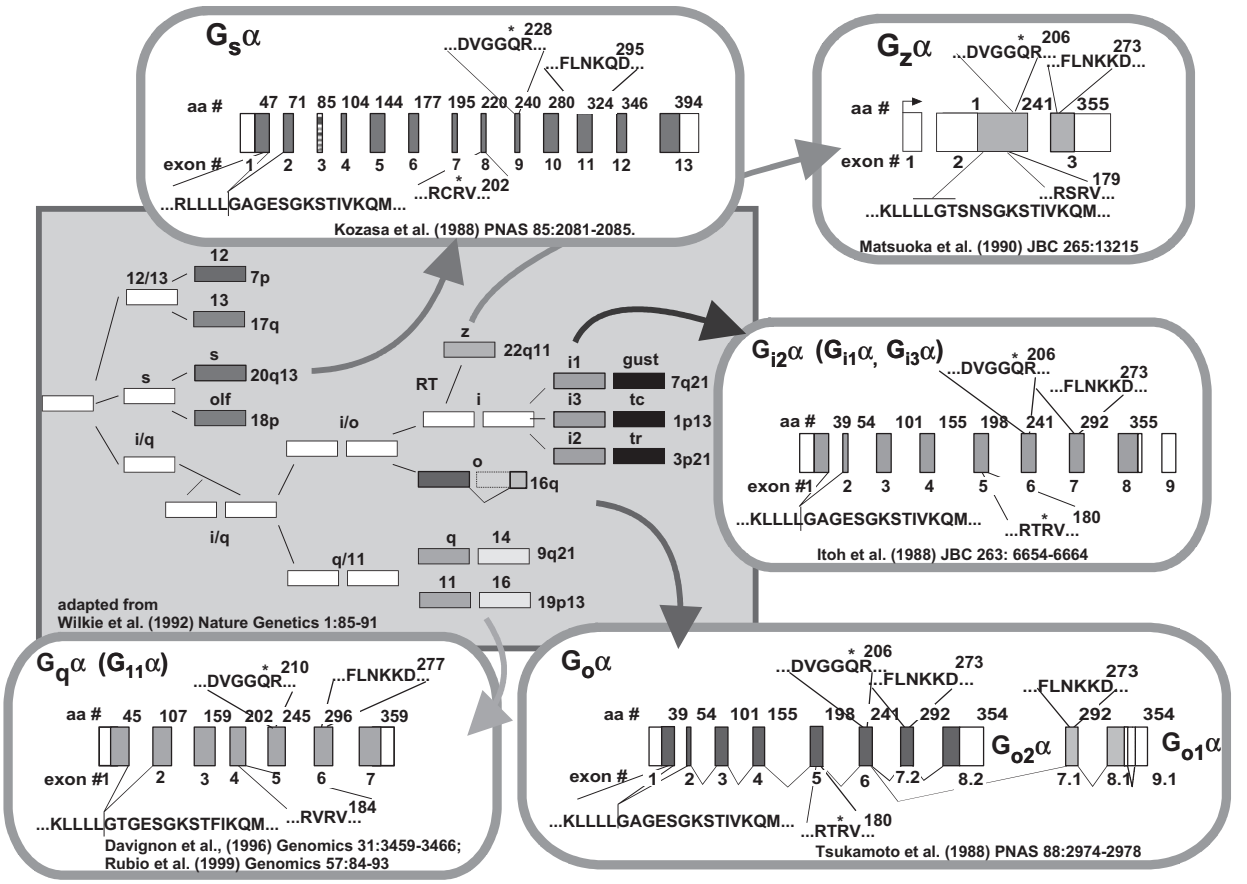


Figure 8 Analysis of the intron-exon structure and sequence similarities among proteins encoded in the exons, allow for development of model for the evolution of α subunit genes in which a series of gene duplications, loss of one intergenic region including most of the exons of the downstream gene followed by rescue of a pseudogene lead to the present day G-protein α subunits. The general layout of this figure was adapted from a similar layout in reference [26]. (Adapted from Wilkie *et al*., *Nat. Genet*., **1**, 85–91, 1992. With permission). Other references on the figure refer to the publications in which the intron-exon structures shown were described.

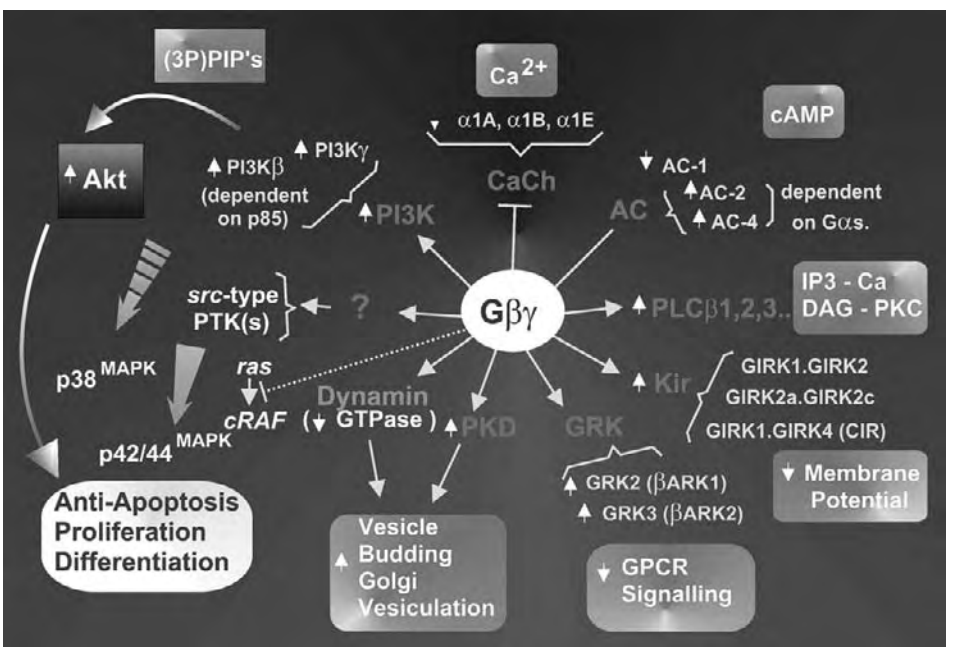


Figure 9 Effectors of βγ dimers. This figure shows the different intracellular signaling systems affected by Gβg dimers at the periphery, with ever increasing detail toward the center. →, stimulation; —|, inhibition; ——, stimulation or inhibition.

signals and the effects of Gβγ on dynamin, Golgi vesiculation, and vesicle budding.

Mechanism of G-Protein Activation by Receptors and Modulation of Activity

Receptors acting through heterotrimeric G proteins are referred to as GPCRs or G-protein-coupled receptors. They play the role of GEFs in the regulatory GTPase cycle. Ligand binding to GPCRs, all of which belong to the rhodopsin superfamily of seven-transmembrane receptors, has as its final effect the GDP-GTP exchange with attendant subunit dissociation into Gα-GTP plus Gβγ (Fig. 1). The final effect of a hormone acting through a GPCR on any given cell depends on the type of G protein activated by the receptor, and the repertoire of effectors, i.e., regulatable enzymes, ion channels, and other affected molecules in the target cell.

Mechanism of Activation of a G Protein by a Receptor

At the molecular level, activation of a G protein by a rhodopsin-like receptor is still poorly understood. This is because of lack of knowledge of which amino acids of the receptor make contact with which amino acids of the G protein. In contrast, the regions of each molecule important for productive interaction are well known, as are some of the kinetic and molecular state changes that occur when a receptor under the influence of an activating ligand, i.e., agonist, interacts with and activates a G protein. Thus, binding of an agonist to a GPCR in the absence of guanine nucleotide (GTP or GDP), as can be done *in vitro* with purified membranes, has two consequences: (1) a shift of the equilibrium between two states of the receptor, from being mostly in state I (inactive) characterized by having low affinity for agonist as well as for the G protein(s), to mostly in state II (active) having higher affinity for the activating ligand, and (2) the stable association of the agonist-receptor complex to the G protein. The latter causes the G protein to reduce its affinity for GDP. Bound GDP, or prebound [3H]GDP, will thus dissociate under these conditions. Mg ion has to be present if the receptor is to cause GDP dissociation. Addition at this point of GTP or a GTP analog such as GTPγS or GMP-P(NH)P, leads to its binding in place of GDP and to the activation of the G protein as seen by stimulation of the activity of an effector such as adenylyl cyclase or visual phosphodiesterase. For most of the cases where this has been studied, a high concentration of Mg ion, about 50 mM, mimics the action of the agonist-activated receptor. With a purified G protein, incubation with Mg ion and GTPγs or GMP-P(NH)P leads not only to accumulation of Gα-bound guanine nucleotide, but also to subunit dissociation, i.e., formation of Gα-GTPγS plus free Gβγ, Dissociation is evident in several ways, the easiest being by a shift in sedimentation velocity from that corresponding to an approximate M_r of 100,000 protein (Gαβγ) to that of two co-sedimenting proteins of an approximate M_r of 50,000 (Gα-GTPγS + Gβγ). M_rs of α subunits are in the 40,000–50,000 range and those of Gβγ complexes are also approximately 50,000.

In intact membranes, where activation of a G protein of the αβγ type by agonist occupancy of a receptor can be measured in terms of stimulation of the activity of an effector, e.g., adenylyl cyclase, phospholipase C, visual phosphodiesterase, or an inwardly rectifying potassium channel, the net effect of receptor activity is thus facilitation of the activation of the G proteins by Mg ion. This comes about as a consequence of a receptor-induced shift in the apparent K_m for Mg ion from high millimolar to low micromolar. In other words, a receptor appears to act by reducing the concentration of Mg required for activation of the G protein by GTP from being above physiologic to being below physiologic. Free cytosolic Mg is in the order of 0.5 mM. The effect of glucagon (receptor) shifting the concentration of Mg ion required for activation of liver Gs, (the stimulatory regulatory component of adenylyl cyclase) by GTPγS, is illustrated in Fig. 10.

Structural Determinants

At the molecular level, mutational analysis has shown that amino acids in the third intracellular loop of GPCRs are involved in the ability of a GPCR to activate a G protein. Swapping intracellular loops between receptors of different G-protein preference, such as between M1 and M2 muscarinic receptors, or β and αl adrenergic receptors, also points to the third intracellular loop as responsible for defining G-protein specificity. Further, most of the receptor mutations that are of the gain-of-function type are in the distal (C-terminal) end of the third intracellular loop. It is not known, however,

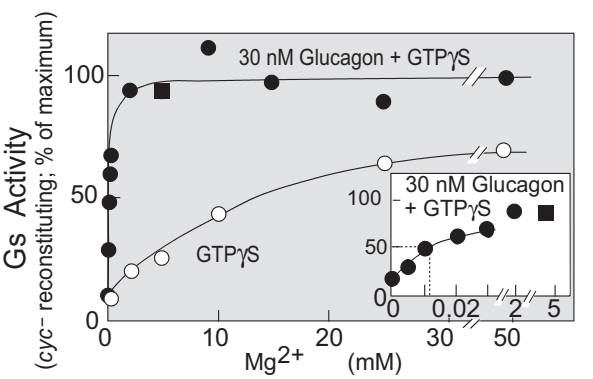


Figure 10 GPCRs reduce the concentration of Mg ion required for G-protein activation by GTP. The results of a three-step reaction are shown. In the first step (not shown), the adenylyl cyclase enzyme of liver membranes was inactivated by treatment with *N*-ethyl maleimide, leaving an intact receptor-G-protein system in its natural membrane environment. The second step tested for the effect of varying Mg ion on GDP/GTPγS exchange in the absence and presence of the hormone glucagon. In the third step, the Gsα-GTPγS complexes formed were extracted and quantified in a standard reconstitution assay. This figure shows the effect of the glucagon-activated receptor on the Mg required for G-protein activation. Note that (1) hormone was not necessary for Gs activation as long as high enough (supraphysiologic) Mg was present during incubation with GTPγS, and (2) that in the presence of the hormone, the Mg required to activate Gs was approximately 1000-fold lower than in its absence. Inset: Same as main panel but with an expanded Mg concentration scale. (Adapted from Iyengar, R. and Birnbaumer, L., *Proc. Natl. Acad. Sci. USA*, **79**, 5179–5183, 1982. With permission).

why some mutations are activating and others inactivating. Moreover, it is also not known whether these amino acids actually contact the G protein, and if so, which G-protein subunit (for further discussion see references [14–17,141]).

Mutational analysis and sequence swapping experiments with G protein α subunits indicate that receptors interact with the very C terminus of the G protein α subunit. Indeed, swapping as few as three of the last ten amino acids between two α subunits can lead to a switch in the type of receptor that activates the G protein. The C terminus is not the only region of interaction of an α subunit with a receptor. Multiple sites have been identified by mutational analysis, including the α3β5 and α4β6 loops of the GTPase domain (Figs. 2 and 12; references [16] and [18]). The α subunit C terminus and the α3β5 and α4β6 loops are part of the same face of the molecule presumed to be immediately juxtamembranous. Receptors not only interact with the α subunit of a trimeric G protein. Free Gα subunits are not recognized by receptors; they are only recognized in the context of the heterotrimer. In agreement with this conclusion, injection of subunit specific antisense oligonucleotides, or subunit specific antibodies, leads to loss of receptor-mediated effector regulation, not only upon suppressing Gα but also either Gβ or Gγ subunits, all in a gene-specific manner. It has been shown that in pituitary cells the M4 muscarinic receptor activates a Go G protein of subunit composition αo1β3γ4, while the somatostatin receptor activates a Go of subunit composition αo2β1γ3. It follows that receptors "proofread" the subunit subtypes that make up the particular trimer with which they enter in contact. Figure 5 shows that only one of the two faces of the Gβ propeller is exposed to the milieu, while the other one faces the switch II region of the α subunits. The exposed face and the sides of the propeller are therefore available for interaction with a receptor. In turn, because the receptor interacts with Gβ and Gγ, it can reasonably be expected that receptors may affect the Gβγ interaction with Gα. One model, based on these considerations, as well as the results shown in Fig. 10, is that while the interaction of the receptor with the C terminus of Gα is essential for selection of the type of Gαβγ that will be activated through the GDP-GTP exchange reaction, the "activating" effect of the receptor may in fact be mediated by the Gβγ. In this sense the Gβγ dimers would have a receptor dependent GEF activity. Thus, while Gβγ alone prevents GDP dissociation by acting as guanine nucleotide dissociation inhibitor (GDI), the Gβγ receptor complex has GEF activity facilitating binding of GTP to its site on α. Artificial "bending" of the N terminus, mimicking what Gβγ might do if it were acting as GEF, does indeed lead to an apparently constitutively active G protein [19]. It has been speculated that Gβγ may be the site of action of Mg ion [20]. If so, the role of receptor would be simply to promote binding of Mg ion to Gβγ. Mg ion would then be responsible for changing the activity of Gβγ from GDI to GEF.

Regardless of the final outcome regarding the events responsible for G-protein activation by a receptor at the submolecular level, the overall reaction for a receptor activating a G protein is facilitation of the action of Mg ion to promote GDP-GTP exchange, followed by dissociation of the trimer into Gα-GTP plus Gβγ.

RGSs or Regulators of G-Protein Signaling

As there are receptors that by virtue of their GEF activity promote activation of the heterotrimeric G proteins, there are also GAPs that accelerate the GTPase activity of activated, GTP-liganded Gα subunits. Two types of Gα GAPs have been identified. One type are the RGSs. RGSs accelerate GTPase activity by 100-fold or more and exhibit Gα subunit selectivity. Sixteen RGSs are known and many of them are multidomain, multifunction proteins, and thus not only affect the Gα subunits but also aid in the organization of multicomponent "signaling complexes" [21]. The second type of GAPs are some of the effectors regulated by the α subunits. GAP activities of effectors increase k_{cat} of the Gα-GTP complexes by 10- to 20-fold only. In both instances, increased GTP hydrolysis ensures not only prompt turn-off of the signaling protein, but also a faster approach to equilibrium, therefore increasing the rate at which responses to an extracellular stimulus can be obtained. Indeed for RGS proteins, faster on/off rates of the regulated function may be a primary raison d'être. In contrast, when effectors act as GAPs, the primary purpose may be to ensure that they are indeed affected by the activated α-GTP complex. The intrinsic GTPase activity of Gα subunits is very low, in the order of 4–8 per s, giving them a rather long half-life, ensuring that they "find" their effector(s) while still in their GTP state. Once the effector has been found and the receptor message is delivered, the recipient of the message "kills" the messenger through activation of its deactivating mechanism. Thus, continued stimulation of effector, if this is desirable, requires continued presence of receptor agonist and constant reactivation of the G protein. Figure 11, illustrates the effect of GAPs on accelerating the rate at which equilibrium is established in a simple on/off reaction, such as binding of a hormone H to its receptor R. The three panels show: (1) the basic rate at which a bimolecular reaction reaches equilibrium; (2) the effect of increasing the k_{off} rate on the rate at which equilibrium is reached, expressed as HR formed in absolute concentrations; and (3) the fact that while the number of complexes at equilibrium decreases with increasing values of k_{off}, the rate at which equilibrium is reached increases with increases in k_{off}. It follows that, given the low intrinsic GTPase activity of Gα subunits, the need for a rapid response can only be satisfied by both existence of a GAP and a very high concentration of reactants so that the amplitude of the read-out signal (regulated effector) is large enough upon activation of only a small fraction of the GTPase. This is, in fact, the case with the activation of transducin (heterotrimeric G protein) by rhodopsin. The GTPase of transducin is stimulated by RGS9, a GGL RGS. This ensures that the physiological rapid turn-off occurs within a tenth of a second as opposed to having a half-life 10 s ($t_{1/2} = \ln 2/k_{off} = 10$ s, where k_{off} corresponds to published intrinsic GTPase activity of Gα subunits of 4 per min) [22]. However, to ensure that sufficient

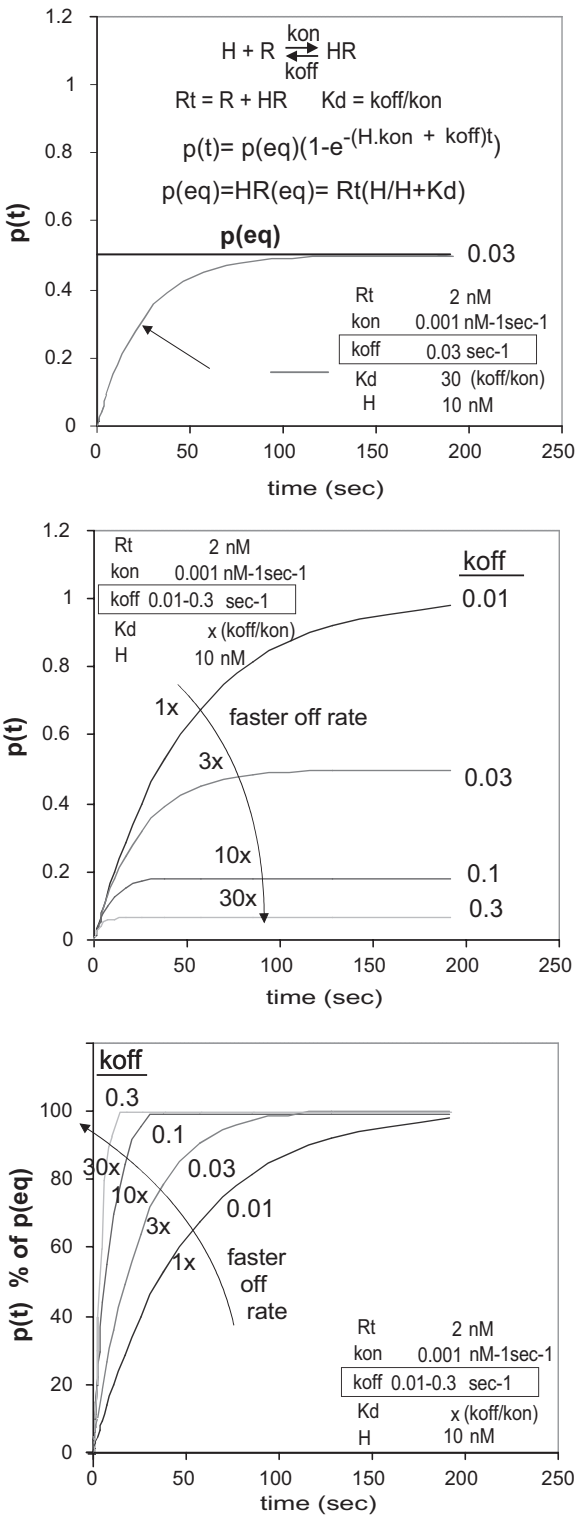


Figure 11 Approach to equilibrium of a reversible bimolecular reaction,

$$H + R \underset{koff}{\overset{kon}{\rightleftarrows}} HR$$

HR = 0 at t_o. These panels illustrate the effect of increasing the koff rate, equivalent to the effect of introducing a GTPase-activating RGS into a regulatory GTPase cycle.

active transducin will be formed, Mother Nature endowed the visual system with the highest known concentrations of receptor (rhodopsin; 40% of disk membrane protein) and regulated G protein (transducin; 10% of disc protein).

Other Forms of G-Protein Modulation: ASGs or Activators of G-Protein Signaling—the GoLoco domain

As described above, the free Gα-GTP and the Gβγ released upon G-protein activation, are both active regulators of effector systems. RGSs and effectors acting as GAPs shorten the lifetime of the active Gα with formation of Gα-GDP. Due to the high affinity of Gα-GDP for Gβγ, a GAP activity not only accelerates deactivation of Gα-regulated effectors, but also that of the Gβγ-regulated effectors from which Gβγ is sequestered by Gγ-GDP. AGSs, or activators of G-protein signaling, were uncovered in a search for molecules that potentiate the effect of α factor in baker's yeast [23]. α Factor acts by activating an αβγ heterotrimeric G protein and initiating a Gβγ-mediated cascade of reactions that leads to growth arrest and preparation for mating to the opposite mating type. One of the AGSs, AGS3, was found to potentiate Gβγ signaling by binding preferentially to Gα-GDP and to do so via a domain found in unrelated proteins and referred to as GoLoco. In mammalian systems, GoLoco domains act as they do in yeast. They bind preferentially to GDP-liganded Gα subunit of the Gi/Go type, thus prolonging Gβγ signaling. In addition to AGS3, proteins with a GoLoco domain include several RGSs and Rap-GAPII. This hints at a role that transcends its direct function of binding the GDP forms of Gi/Go and involves participation in integration of multicomponent signaling pathways (see reference [24] for recent review).

Even though they were identified in the same type of bioassay, AGS1, AGS2, and AGS3 differ in their mode of action. AGS1 (also Rasdex1) is a ras-related protein that appears to act as a GEF, whereas AGS2 interacts with Gβγ and AGS3 binds to α-GDP. Why a Gβγ-interacting protein (AGS2) would enhance the effectiveness of either a Gβγ or a Gα-GTP remains to be determined. Another Gβγ-interacting protein, phosducin, serves to attenuate the action of transducin in the retina. The existence of Gβγ- and Gα-interacting proteins (RGSs, GoLoco proteins, phosducin, and non-GoLoco AGSs) points to the fact that fine-tuning of the basic regulatory G-protein cycle is required for proper cell homeostasis. These fine-tuning mechanisms are therefore responsible for the ultimate ability of a cell to live a productive life that is in concert with the needs of the whole organism.

In Memoriam

This chapter is written 32 years after the first data on the GTP requirement in hormonal stimulation of liver adenylyl cyclase (then adenyl cyclase) were obtained in Martin Rodbell's laboratory where I was a postdoctoral fellow. It was then that we made the proposal that receptors may be acting on adenylyl cyclase through a signal transducer

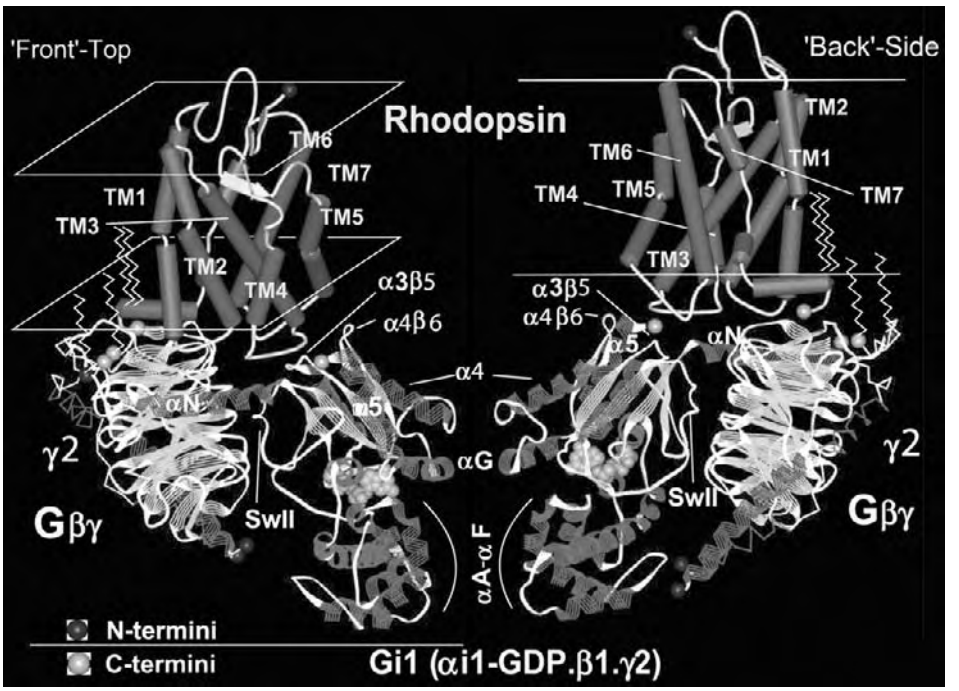


Figure 12 Model of orientation of a Gα subunit with respect to both the inner plane of the plasma membrane and a membrane-embedded GPCR. This is based on the crystal structures of bovine rhodopsin (PDB accession #1F88) and the GDP-occupied Gi1 trimer (PDB accession #1GG2). The molecules are shown in a near-docking situation with receptor in its "inactive" conformation and the G protein with GDP bound to it. Two views are shown and the main structural features are annotated. Vertical zigzag lines denote the α subunit N-terminal myristylation and/or palmitoylation and the γ subunit C-terminal polyisoprenylation. Positions of lipids are approximate because the modified N- and C-terminal amino acids were not resolved in the crystal structure of Gi1. Double zigzag approximately 13 amino acids after the TM7 of rhodopsin denotes double palmitoylation of the C terminus at that position. Single and double palmitoylation of GPCR C termini is a common but not universal feature of GPCRs.

driven by GTP—in some unknown way (Fig. 12). Two great thinking minds of that era are no longer with us. Martin Rodbell passed away in 1998 [25], as did Michael (Mickey) Schramm on June 8, 2002. Mickey visited often in the late 1960s, and fed us ideas that were incorporated into our thinking without us even realizing it. Seldom, if ever, have we properly given credit to Mickey's influence on our thinking. Better late than never, goes the saying, but I wish I would have done it earlier. While life is destined to end, our task as researchers dedicated to extracting nature's secrets never ends. Even though 32 years have passed, the mystery of signal transduction through G proteins has not been fully resolved, as the recent discoveries of RGS and AGS proteins demonstrates. While looking forward to new discoveries, we should also remember those that contributed in major ways to the way we think today. This chapter was written with the idea of introducing signaling through G proteins to the next generation of investigators. I hope not to have failed too badly.

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CHAPTER 214

Genetic Analysis of Heterotrimeric G-Protein Function

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Introduction

For a surprisingly long time, the yeast *Saccharomyces cerevisiae* has been the only organism in which signaling by heterotrimeric G proteins has been analyzed genetically. More recently, mutants affecting G-protein function have also been analyzed in *C. elegans* [1–3], *Dictyostelium* [4,5], and *Drosophila* [6] and have yielded some quite unexpected results. In this chapter, I want to first summarize the results obtained in yeast and then focus on more recent experiments that have revealed a function for heterotrimeric G-proteins in cell polarity in *Drosophila*. The beautiful experiments done in *C. elegans* and *Dictyostelium* are summarized in other chapters (see the contributions by Julie Ahringer and Peter Devreotes) and will not be reviewed here.

Signaling by Heterotrimeric G Proteins in Yeast

In response to the pheromone α-factor, yeast cells polarize their actin cytskeleton and extend a process called "shmoo." α-factor binds to a seven-transmembrane receptor in the plasma membrane and activates the only heterotrimeric G protein present in the yeast genome, Gpa1 [7]. G-protein activation leads to polarization of the actin cytoskeleton, arrest of the cell cycle, and a transcriptional response in the nucleus. In mutants affecting the β-subunit (Ste4) or the γ-subunit (Ste18) of the heterotrimeric G-protein complex, no response to α-factor can be detected, whereas in mutants affecting the α-subunit the signaling pathway is constitutively activated. Thus, signal transduction occurs via the free Gβγ subunit, but the GTP-bound Gα subunit does not seem to play a role [7]. It is interesting that the defects observed in Gβ mutants can be rescued by a Gα-Gβ-fusion protein in which Gα and Gβγ are covalently linked but can still undergo conformational changes [8], indicating that the βγ-subunit does not have to physically separate from the α-subunit for signaling to occur.

The best studied signal transduction cascade that operates downstream of the heterotrimeric G protein involves the protein kinases Ste11, Ste7, and Fus3 (Fig. 1A). Upon G-protein activation, Ste11 phosphorylates Ste7, which in turn phosphorylates Fus3 and induces its translocation into the nucleus. Fus3 belongs to the MAP-kinase family of protein kinases and therefore this cascade of protein kinases is the prototype of a so-called MAP-kinase cascade. Activation of the MAP-kinase cascade occurs in two ways: First, Gβγ binds and activates the protein kinase Ste20 when released from the α-subunit and Ste20 initiates MAPK-signaling by phosphorylating its most upstream member, Ste11. Second, all kinases of the cascade bind to the scaffold protein Ste5. Ste5 can bind to free Gβγ and therefore G-protein activation recruits the whole kinase cascade to the site of receptor activation. Thus, G-protein signaling in yeast involves the relocalization of downstream signaling components to the site of G-protein activation by direct binding to free Gβγ.

Although the Map-kinase cascade transduces a signal into the nucleus, the most important downstream target for polarizing the actin cytoskeleton is the guanine exchange factor Cdc24 (Fig. 1A). Cdc24 binds free Gβγ through the

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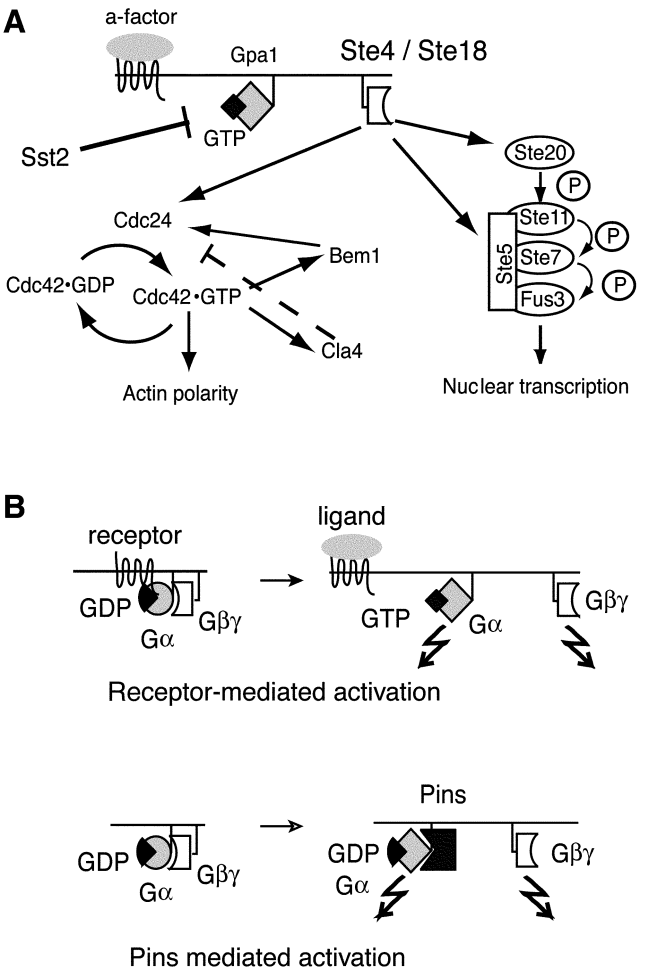


Figure 1 Outline of G-protein signaling in yeast (A) and Drosophila (B).

adaptor protein Far1 [9,10]. In the absence of α-factor, Far1 shuttles between the cytoplasm and the nucleus and sequesters Cdc24 in the nucleus [11,12]. Upon α-factor exposure, however, Far1 binds to free Gβγ and the trimeric complex is assembled, leading to an accumulation of Cdc24 at the site of receptor activation. Once it is localized, Cdc24 binds the adaptor protein Bem1 and this association stabilizes the cortical localization of the protein. Cdc24 is an exchange factor that catalyzes GDP/GTP exchange on the small G-protein Cdc42. Cdc42, in turn, is a well-characterized modulator of the actin cytoskeleton, and it is the local activation of Cdc42 by Cdc24 that is thought to polarize the actin cytoskeleton toward the site of a-factor exposure.

Two feedback loops have been shown to operate downstream of the G proteins. In its GTP bound form, Cdc42 can directly bind to Bem1 and recruit this adaptor protein to the cell cortex [13]. Bem1 in turn recruits Cdc24, which generates more Cdc42 and therefore amplifies the signal. Activated Cdc42 also recruits the protein kinase Cla4, which will phosphorylate Cdc24 at a site that is needed for it to bind to Bem1 [14]. This phosphorylation event will eventually terminate the signal and allow the signal transduction cascade to return to its inactive state. Thus, positive and negative feedback loops that operate downstream of the G-proteins both amplify and terminate the signal that is initially generated by receptor activation.

Regulation of signaling also occurs at the level of the G protein itself. The protein Sst2 is required for yeast cells to recover and reenter the cell cycle after exposure to α-factor. Sst2 directly binds to the G-protein α-subunit. It contains a so-called RGS (regulator of G-protein signaling) domain that enhances the rate of GTP hydrolysis and therefore increases the rate of G-protein inactivation. RGS proteins were also found in higher eukaryotes and represent an evolutionarily conserved protein family that acts as GAPs for heterotrimeric G-proteins [15].

Heterotrimeric G-Protein Function in *Drosophila*

A genetic analysis of heterotrimeric G-protein function in *Drosophila* has recently revealed another important protein family that regulates G-protein activity by direct interaction. *Drosophila* neural precursor cells called neuroblasts divide asymmetrically along the dorso-ventral axis. Before division, a protein called Inscuteable localizes to their apical cell

cortex and establishes an axis of polarity that is used during mitosis for correct apical-basal spindle orientation and for asymmetric localization of protein determinants to the basal cell cortex [16]. Upon cytokinesis, these determinants are inherited by only one of the two daughter cells and ensure its correct cell fate. Inscuteable binds to a protein called Pins and recruits it to the apical cell cortex. Pins in turn recruits the heterotrimeric G protein Gαi through its C-terminal GoLoco domains, and both proteins are required for establishing an axis of polarity [17]. Thus, Inscuteable and Pins are adaptors that function by polarizing the distribution of heterotrimeric G-proteins.

Besides localizing the G proteins, Pins and its vertebrate homologs AGS-3 [18] and LGN [19] also seem to regulate their activity. Binding of Pins or just its GoLoco domains to Gαi induces the release of the βγ-subunit *in vitro* without the need for receptor activation [6]. βγ-release seems to occur in the GDP-bound form, since Pins does not induce nucleotide exchange and only binds the GDP-form of the G protein. In fact, GoLoco domains inhibit nucleotide exchange, suggesting they function as GDIs (GDP dissociation inhibitors) for heterotrimeric G-proteins [20]. The structural basis for these activities was recently revealed by X-ray crystallography [21]. GoLoco domains bind a region on Gα that overlaps the Gβγ-binding domain and induce a conformational change that precludes coincident Gβγ interaction. In addition, they interact with the α- and β-phosphates of Gα-bound GDP and inhibit its dissociation from the G protein. Thus, GoLoco domains represent a novel class of G-protein regulators that activate G proteins via a nonclassical mechanism that involves neither a seven transmembrane receptor nor GDP/GTP exchange on the α-subunit.

The function of heterotrimeric G-proteins in *Drosophila* has been addressed genetically. The *Drosophila* genome contains one of each of the major classes of Gα-subunits, Gαi, Gαo, and Gαs. Gαs mutants die for unknown reasons but do not show defects that can be associated with a lack of PKA activity or defects in any of the major developmental signaling pathways [22]. Mutants in Gαo have defects in heart development because epithelial polarity in cardiac precursors is not correctly established [23]. Mutants in Gαi have not been generated, but the function of this G protein was addressed by mutating the associated β-subunit [6]. Based on sequence similarity, Gβ13F is the common *Drosophila* homolog of vertebrate Gβ1-4. Gβ13F binds to Gαi, and in its absence Gαi can no longer be detected. Presumably, a homeostasis mechanism degrades Gαi in the absence of its associated β-subunit, ensures that cells contain equal amounts of Gα and Gβ, and prevents the accumulation of free Gαi in Gβ13F mutants. Besides the defects in asymmetric cell division mentioned above, these mutants have defects in gastrulation. This phenotype resembles the one described for *concertina*, the *Drosophila* homolog of Gα13. Whether Gα13 is also degraded in Gβ13F mutants cannot be addressed due to the lack of a good antibody, but this results suggests that both Gαi and Gα13 use Gβ13F for signal transduction in *Drosophila*.

Conclusions

Together with genetic results obtained in *C. elegans* and *Dictyostelium* (reviewed in the chapters by Devreotes and Ahringer in this volume), these experiments tell us that heterotrimeric G proteins—besides their well-studied function in hormone and neurotransmitter signal transduction—have an important role in cell polarity. To exert this role, G proteins may not always be activated by classical mechanisms involving seven-transmembrane receptors. Unconventional G-protein activators have been identified, and their further analysis may reveal more widespread functions that might change some of the classical textbook views on the role of this important protein family.

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CHAPTER 215

Heterotrimeric G Protein Signaling at Atomic Resolution

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Introduction

Heterotrimeric G proteins (subunits $G_{\alpha\beta\gamma}$) mediate a variety of physiological responses, including sensory perception, hormone action, polarization, chemotaxis, and growth control [1–3]. In the conventional paradigm for G-protein signaling, ligand-bound (or light-activated) heptahelical receptors catalyze release of GDP from the G_α subunit, resulting in a complex between the receptor and the nucleotide free $G_{\alpha\beta\gamma}$ heterotrimer. Association of GTP with G_α triggers release from the receptor and dissociation of G_αGTP and $G_{\beta\gamma}$. Depending on the particular signaling pathway, either G_αGTP or the released $G_{\beta\gamma}$ subunits interact with downstream effectors until the G_α subunit is deactivated by GTP hydrolysis. Although the G_α subunit possesses an intrinsic GTP hydrolytic activity, regulator of G protein signaling (RGS) domains, present in a variety of modular proteins, accelerate the rate of GTP hydrolysis [4].

Crystallographic studies of two different G-protein signaling pathways, involving the visual G protein transducin (G_t) as well as the hormone activated G proteins that stimulate (G_S) and inhibit (G_i) adenylyl cyclase (AC), reveal a highly conserved structural basis for heterotrimer assembly, activation by nucleotide exchange, and deactivation by GTP hydrolysis. The various structures explain a wealth of biochemical and cell biological data accumulated over the years and provide a springboard for mutational analyses aimed at dissecting structure-function relationships for the myriad diverse biological responses mediated by G proteins. The most salient observations are highlighted below. Interested readers are encouraged to consult the cited references for in-depth discussion of particular structures.

Architecture and Switching Mechanism of the G_α Subunits

As illustrated in Fig. 1A, the G_α subunits share a conserved architecture with a Ras-like domain, consisting of a core six stranded β sheet (β1–β6) surrounded by five helices (α1–α5), and a helical domain comprising a long helix (αA) enveloped on three side by five shorter helices (αB–αF) [5]. Compared with monomeric GTPases, the helical domain of the G_α subunits represents an insertion within the α1/β2 loop (known as the "effector binding loop" in Ras). Consequently, the Ras-like and helical domains are connected by two extended strands, one joining α1 of the Ras-like domain to αA of the helical domain and the other traversing from αF of the helical domain to β2 of the Ras-like domain. In all of the G_αGDP and G_αGTP structures determined to date, the αD/αE loop of the helical domain engages the Ras-like domain, thereby capping the nucleotide binding site [5–10]. In contrast, the ribose and phosphate moieties of the nucleotide are partially exposed in monomeric GTPases, particularly in the GDP-bound conformation in which the α1/β2 loop is highly flexible. Consistent with the structural observations,

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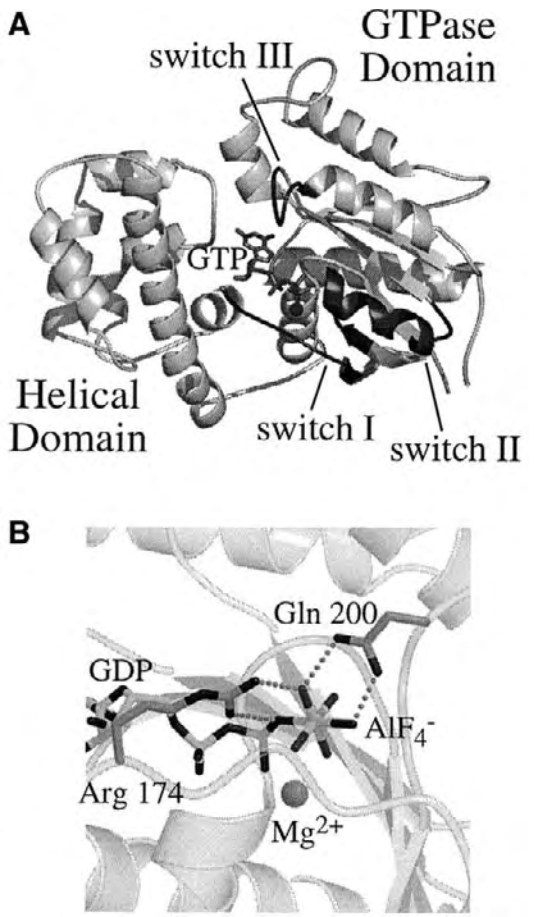


Figure 1 Active and transition states of a G_α subunit. (A) Structure of the active form of $G_{t\alpha}$ bound to GTPγS, a poorly hydrolyzeable GTP analog. The conformational switch regions, deduced by comparison with the inactive (GDP-bound) form, are highlighted in dark gray. The Mg^{2+} ion is represented as a dark gray sphere. (B) Structure of AlF_4^- activated $G_{t\alpha}$GDP.

the G_α subunits bind GDP with high affinity in the absence of Mg^{2+} whereas monomeric GTPases require Mg^{2+} for high-affinity binding, exhibiting rapid rates of nucleotide release in the presence of EDTA [11]. As an obvious consequence of the conserved domain architecture in the G_α subunits, intrinsic as well as receptor-catalyzed GDP release necessarily involves a transition to an "open" intermediate in which the helical domain has rotated away from the Ras-like domain.

Structural changes between the GDP-bound (inactive) and GTP-bound (active) conformations are localized to three nonconsecutive conformational switch regions corresponding to the first linker strand (switch I), a region extending from the C-terminus of β3 to the C-terminus of the α2/β4 loop (switch II), and the β4/α3 loop (switch III) [6,9]. In the GDP-bound structures, the switch regions are either disordered [9] or adopt a relaxed conformation stabilized by crystal contacts [6]. In the GTP-bound form, the γ phosphate is detected by direct hydrogen bonding interactions with the side chain hydroxyl of the invariant threonine residue in the RxxxT motif of switch I and the backbone NH group of the invariant glycine residue in the DxxGQ motif of switch II. The conformation of switch III is indirectly coupled to the nucleotide state by a hydrogen-bonding interaction between the side chain carboxylate of an invariant glutamate residue in switch III and a main chain NH group in switch II. The active conformation is further stabilized through Mg^{2+} coordination by the side chain hydroxyl of the invariant threonine residue in switch I and through an extensive hydrophobic/ionic interface between the α2 helix of switch II and the α3 helix. Finally, the zippering of the β2 and β3 strands, resulting in two additional hydrogen bonds in the GTP-bound form, suggests cooperativity in the switching mechanism.

Insight into the GTP Hydrolytic Mechanism from an Unexpected Transition State Mimic

Aluminum fluoride (AlF_4^-) binds to G_αGDP near the binding site for the γ phosphate of GTP, inducing a conformational change that results in artificial activation of G_αGDP and dissociation from $G^{\beta\gamma}$ Crystal structures of the GDP•AlF_4^--bound forms of $G_{i\alpha}$ [7] and $G_{t\alpha}$ [8] revealed an unexpected finding. Although the conformation of all three switch regions closely resembles that of the GTP-bound form, AlF_4^- does not mimic the tetrahedral geometry of the γ phosphate but rather adopts an octahedral geometry with four fluoride ligands arranged in an equatorial plane and two axial ligands consisting of an oxygen from the β phosphate of GDP and a water molecule (Fig. 1B). The bound aluminum fluoride interacts with and orders the side chains of two critical residues previously implicated in GTP hydrolysis, namely the arginine of the switch I RxxxT motif and the glutamine of the switch II DxxGQ motif. These observations led to the hypothesis that AlF_4^- activates G_αGDP by approximating the expected stereochemistry of the pentavalent intermediate for GTP hydrolysis. This notion is strongly supported by the remarkable observation that AlF_4^- also binds to and stabilizes complexes of both Ras and Rho GTPases bound to their respective GAPs [12,13].

$G_{\beta\gamma}$ with and without G_α

Parallel crystallographic studies revealed the stunningly beautiful structure of the $G_{\beta\gamma}$ heterodimer alone [14] and in complex with G_αGDP [15,16]. The seven distinctive WD repeats of G_β fold into a seven bladed β propeller in which each blade consists of a four stranded antiparallel β sheet (Fig. 2A). It is interesting that the WD repeats do not coincide precisely with the individual blades of the propeller, but rather each repeat begins with the outer strand of one blade and extends through the inner three strands of the next blade. G_γ possesses an N-terminal helix, which forms a parallel coiled coil with the N-terminal helix of G_β, followed by an internal helix and a region of coil that extends across the bottom of the G_β propeller. Although G_γ has well-defined secondary structure, it is devoid of intramolecular interactions characteristic of tertiary structure and thus could not

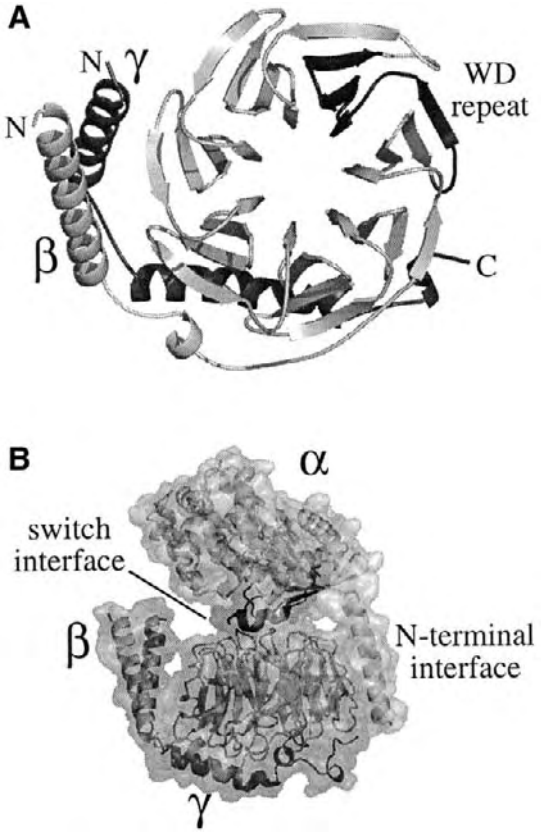


Figure 2 Structure of a heterotrimeric G protein. (A) Ribbon rendering of $G_{t\beta\gamma}$ with the γ subunit and one of the WD repeats in the β subunit shown in dark gray. (B) Heterotrimeric complex of a $G_{t\alpha}/G_{i\alpha}$ chimera and the unprenylated form of $G_{t\beta\gamma}$.

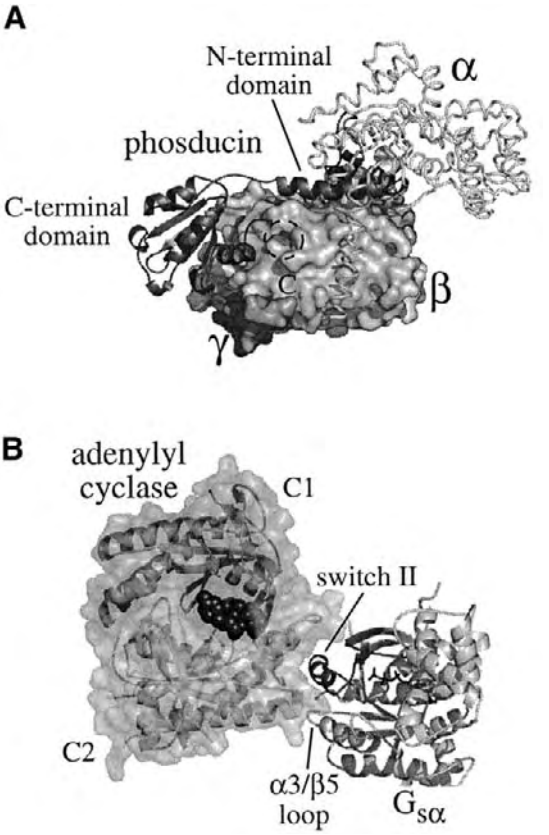


Figure 3 Regulatory and effector complexes with phosducin and adenylyl cyclase. (A) Structure of the unprenylated $G_{t\beta\gamma}$ (light surface) in complex with retinal phosducin (dark ribbon). For comparison, the $G_{t\alpha}/G_{i\alpha}$ chimera from Fig. 2B is overlaid as a light coil. A dashed circle denotes the location of the proposed binding cavity for the farnesyl moiety. (B) Structure of an active adenylyl cyclase C1-C2 heterodimer (ribbons and semitransparent surface) bound to forskolin (dark spheres) and $GS_\alpha GTP_\gamma S$ (ribbons). The active site is located to the left of the forskolin-binding site. Note that the interaction epitope of GS_α comprises the switch II region and the α3/β5 loop.

adopt a properly folded structure in the absence of G_β. Indeed, roughly half of the residues in G_γ are buried in an extensive hydrophobic interface with G_β, a finding that explains the unusually high stability of the $G_{\beta\gamma}$ heterodimer.

The interaction between G_αGDP and $G_{\beta\gamma}$ occurs at two distinct interfaces (Fig. 2B). The most extensive interface involves the switch I and II regions of G_α, which contact residues from the loops and turns at the top of the G_β propeller. The second interface forms between the N-terminal helix of G_α and the side of G_β. In the complex with $G_{\beta\gamma}$, the switch regions of G_α adopt a well-ordered conformation that is incompatible with the active conformation of G_αGTP. In contrast, it appears that the interaction with the N-terminal helix of G_α would not be directly influenced by the state of the bound nucleotide, consistent with a residual low-affinity interaction between G_αGTP and the released $G_{\beta\gamma}$ subunits.

Phosducin and $G_{\beta\gamma}$

The first insight into how phosducin engages $G_{\beta\gamma}$ and promotes membrane dissociation came from the crystal structure of a phosducin complex with an unprenylated form of retinal $G_{\beta\gamma}$ [17]. Phosducin contains two domains, a small N-terminal helical domain composed primarily of hydrophilic residues and a C-terminal domain with a thioredoxin-like fold (Fig. 3A). The N-terminal domain interacts with the top of the G_β propeller, overlapping extensively with the epitope for interaction with the switch regions of G_α, whereas the thioredoxin domain contacts the side of G_β at a site distinct from the N-terminal epitope for G_α. These observations explain why the interaction of $G_{\beta\gamma}$ with phosducin and G_α is mutually exclusive. Furthermore, electrostatic calculations indicate that the presence of phosducin's thioredoxin-like domain introduces a substantial negative electrostatic potential near the prenylation site at the C-terminus of G_γ, thereby destabilizing the association with acidic membranes. Finally, the interaction with phosducin's N-terminal domain perturbs the conformation of three loops at the top of the G_β propeller. A subsequent structure of phosducin bound to farnesylated $G_{\beta\gamma}$ suggested that the conformational changes in G_β open a pocket of appropriate dimensions to accommodate the hydrophobic farnesyl group of G_γ [18].

$G_{S\alpha}$ and Adenylyl Cyclase

Adenylyl cyclase (AC) consists of two hexahelical transmembrane domains, each followed by a similar cytoplasmic domain referred to as C1 and C2. Expressed independently, the isolated C1 or C2 domains form soluble but inactive homodimers. When mixed, the C1 and C2 homodimers spontaneously equilibrate to form catalytically active heterodimers that retain the ability to be stimulated by $G_{S\alpha}GTP$ and inhibited by $G_{i\alpha}GTP$ [19]. The structure of a C1-C2 heterodimer in complex with the GTP-bound form $G_{S\alpha}$ and forskolin, a plant terpenoid that activates AC, provided the first glimpse of how a GTP-bound G_α subunit recognizes and activates a downstream effector [20]. The inactive C2 homodimer binds two molecules of forskolin at symmetrical sites located at the dimer interface [21]. In contrast, the pseudo-symmetrical C1-C2 heterodimer binds a single forskolin molecule at an analogous site (Fig. 3B). The ATP-binding site is located at a pseudo-symmetrical site analogous to the second forskolin site in the C2 homodimer. The switch II region and $\alpha3/\beta5$ loop of $G_{S\alpha}$ contact the C1-C2 domains at a location remote from the active site, thereby inducing a domain rotation that brings key catalytic and ATP-binding residues into register.

Filling in the GAP

RGS domains present in a variety of modular proteins accelerate GTP hydrolysis for Gα subunits [4]. The underlying structural basis was established by the crystal structure of the helical RGS domain of RGS4 in complex with $G_{i\alpha}GDP$ and AlF_4^- [22]. In contrast to GAPs (GTPase-activating proteins) for Ras and Rho GTPases, which supply an "arginine finger" analogous to the catalytic arginine in switch I of G_α, RGS proteins promote GTP hydrolysis by engaging the switch I and II regions so as to reorient the catalytic arginine and glutamine residues of $G_{i\alpha}$ to stabilize the pentavalent intermediate (Fig. 4A).

Visual Fidelity

Structures of the GTPγS-bound and AlF_4^- activated forms of $G_{t\alpha}$ in complex with RGS9 and/or the inhibitory subunit of the retinal phosphodiesterase (PDEγ) provided further insight into the cooperative mechanism of effector recognition and RGS stimulation of GTP hydrolysis in the visual system [23]. PDEγ forms a predominately hydrophobic interface with residues in the switch II/α3 cleft of $G_{t\alpha}$, consistent with mutational data. This interaction sequesters C-terminal residues of PDEγ implicated in PDEαβ inhibition. RGS9 engages the switch I and II regions of $G_{t\alpha}$ in a manner analogous to that observed for RGS4 and $G_{i\alpha}$. As shown in Fig. 4B, a small interface between PDEγ and a unique loop of RGS9, near the critical aspargine residue involved in positioning the catalytic glutamine of $G_{t\alpha}$, couples the maximal GAP activity of RGS9 to the interaction of $G_{t\alpha}$ with PDEγ, thereby enhancing the fidelity of visual signal transduction.

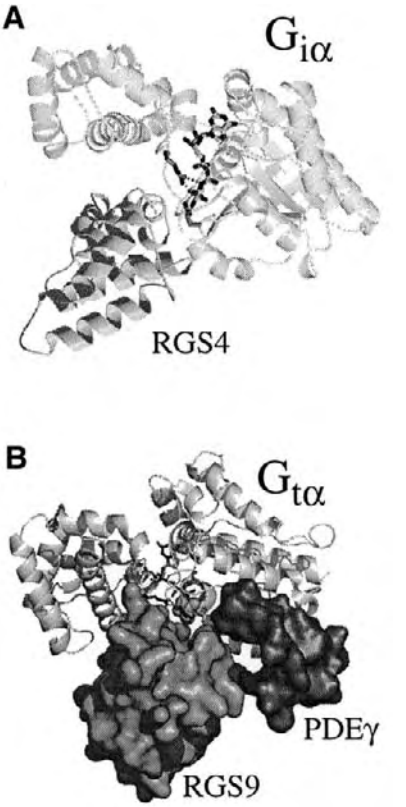


Figure 4 RGS and effector complexes with G_α subunits. (A) Structure of RGS4 (dark ribbon) bound to AlF_4^- activated $G_{i\alpha}GDP$ (light ribbon). Also shown are the conserved arginine and glutatmine residues in the switch regions as well as GDP-AlF_4^-. (B) Structure of AlF_4^- activated $G_{t\alpha}GDP$ (ribbons) bound to PDEγ (dark surface) and RGS9 (light surface). AlF_4^- is depicted as light spheres whereas GDP as well as the conserved arginine and threonine residues are shown as bonded cylinders.

What Structures May Follow

Clearly the most important unresolved structural question is how an activated receptor engages a heterotrimeric G protein so as to catalyze nucleotide exchange on the G_α subunit. The resolution of this question requires the crystal structure of a complex between a ligand-bound or light-activated receptor and the nucleotide free form of a G-protein heterotrimer. Only then can we claim to have glimpsed the conversion of extracellular signals into intracellular second messengers at atomic resolution.

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CHAPTER 216

In Vivo Functions of Heterotrimeric G Proteins

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Introduction

The transmembrane signaling system, which uses heterotrimeric G proteins to couple heptahelical receptors to various effectors, operates in all cells of the mammalian organism and is involved in many physiological and pathological processes. The main properties of individual G proteins are determined by the identity of their α-subunits. To elucidate the role of G-protein-mediated signaling processes in the intact mammalian organism, almost all known genes encoding G-protein α-subunits have been inactivated by gene targeting in mice (Table I). So far, no mouse line was reported carrying targeted mutations of Gβ- or Gγ-genes. This short review summarizes the main phenotypical changes observed in mice lacking G protein α-subunits.

Development

Various Gα-deficient mouse models have pointed to the involvement of G-protein-mediated signaling pathways in certain developmental processes. For example, lack of $G\alpha_{13}$ results in embryonic lethality at about midgestation due to a defect in angiogenesis [1]. Mice deficient in both $G\alpha_q$ and $G\alpha_{11}$ suffer from a defect in heart development and die *in utero* (see below). In addition, signaling through G_q class members has also been implicated in the proliferation and/or migration of craniofacial neural crest cells [2]. The complete loss of $G\alpha_s$ in mice homozygous for an inactivating $G\alpha_s$ mutation leads to embryonic lethality before embryonic day 10 [3]. It is interesting that heterozygotes show varying phenotypes depending on the paternal origin of the intact allele; these are probably caused by genetic haploinsufficiency and/or tissue-specific imprinting of the maternal $G\alpha_s$ allele [4].

Central Nervous System

In the central nervous system (CNS), many mediators and neurotransmitters function through G-protein-coupled receptors to modulate neuronal activity or morphology. Neurotransmitters that induce an inhibitory modulation typically act on receptors that are coupled to members of the $G_{i/o}$ family, whereas G_q- and G_s-family members are primarily involved in excitatory responses.

The G-protein G_o is highly abundant in the mammalian nervous system and has been shown to mediate inhibition of neuronal (N-, P/Q-, R-type) voltage-dependent Ca^{2+} channels via its βγ-complex, thereby reducing the excitability of the cell. $G\alpha_o$-deficient mice suffer from tremors and have occasional seizures [5,6]; severely abnormal motor behavior can be observed in these animals (http://www.anes.ucla.edu/~lutzb/realmice.htm) as well. In addition, $G\alpha_o$-deficient mice appear to be hyperalgesic when tested in the hot plate assay [6]. The latter finding is consistent with the observation that opioid receptor-mediated inhibition of Ca^{2+} currents in dorsal root ganglia (DRG) from $G\alpha_o$-deficient animals was reduced by about 30 percent compared to those in wild type DRGs [6].

G_z, a member of the $G_{i/o}$-family of G proteins, shares with G_{i1}, G_{i2}, and G_{i3} the ability to inhibit adenylyl cyclases but has a rather limited pattern of expression, being found in brain, adrenal medulla, and platelets. $G\alpha_z$-deficient mice exhibit altered responses to a variety of psychoactive drugs. Cocaine-induced increases in locomotor activity were more pronounced, and short-term antinociceptive effects of

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Table I Phenotypical Changes in Mice Lacking α-Subunits of Heterotrimeric G-Proteins

Family/Type		Gene	Expression	Effectors	Phenotype	Reference
$G\alpha_s$	$G\alpha_s$[a]	*Gnas*	ubiquitous	AC (all types)↑	embryonic lethal[d]	[3]
	$G\alpha_{olf}$	*Gnal*	olf. epithelium, brain	AC ↑	anosmia, hyperactivity	[9]
$G\alpha_{i/o}$	$G\alpha_{i1}$	*Gnai1*	widely distributed	AC ↓[e]	no obvious phenotype seen so far	[i]
	$G\alpha_{i2}$	*Gnai2*	ubiquitous	”	inflammatory bowel disease	[14]
	$G\alpha_{i3}$	*Gnai3*	widely distributed	”	no obvious phenotype seen so far	[i]
	$G\alpha_o$[b]	*Gnao*	neuronal, neuroendocr.	Ca^{2+}-ch. ↓[f]	various CNS defects	[5,6,20]
	$G\alpha_z$	*Gnaz*	neuronal, platelets	AC ↓; ?	viable, increased bleeding time	[7,8]
	$G\alpha_{gust}$	*Gnag*	taste cells, brush cells	?	impaired bitter and sweet sensation	[21]
	$G\alpha_{t\text{-}r}$	*Gnat1*	retinal rods, taste cells	cGMP PDE ↑	mild retinal degeneration	[19]
	$G\alpha_{t\text{-}c}$	*Gnat2*	retinal cones	cGMP PDE ↑	no mouse mutant available	–
	$G\alpha_{i1} + G\alpha_{i3}$				no obvious phenotype seen so far	[i]
	$G\alpha_{i2} + G\alpha_{i3}$				lethal	[i]
$G\alpha_q$	$G\alpha_q$	*Gnaq*	Ubiquitous	PLC-β ↑[g]	ataxia, defective platelet activation	[12,23]
	$G\alpha_{11}$	*Gna11*	almost ubiquitous	”	no obvious phenotype seen so far	[2]
	$G\alpha_{14}$	*Gna14*	kidney, lung, spleen	”	no obvious phenotype seen so far	[j]
	$G\alpha_{15}$[c]	*Gna15*	hematopoietic cells	”	no obvious phenotype seen so far	[26]
	$G\alpha_q + G\alpha_{11}$				myocardial hypoplasia (lethal e11)	[2]
					cardiomyocyte-restricted:	
					pressure overload induced hypertrophy ↓	[16]
	$G\alpha_q + G\alpha_{15}$				like $G\alpha_q$ (–/–)	[26]
$G\alpha_{12}$	$G\alpha_{12}$	*Gna12*	ubiquitous	?	no obvious phenotype seen so far	[k]
	$G\alpha_{13}$	*Gna13*	ubiquitous	?[h]	defective angiogenesis (lethal e9.5)	[1]
	$G\alpha_{12} + G\alpha_{13}$				embryonic lethal (e8.5)	[k]

[a]several splice variants
[b]2 splice variants
[c]mouse form ($G\alpha_{16}$, human counterpart)
[d]parent of origin specific defects in heterozygotes
[e]adenylyl cyclase types I,V,VI
[f]N-,P/Q-type; effector is regulated through βγ-subunits
[g]β4;β3 ≥ β1 >> β2
[h]RhoGEF-proteins (p115RhoGEF)
[i]L. Birnbaumer, M. Jiang, G. Boulay, K. Spicher (personal communication);
[j]H. Jiang and M.I. Simon (personal communication)
[k]S. Müller, S.O., M.I. Simon (unpublished data). AC, adenylyl cyclase; Ca^{2+}-ch., Ca^{2+}-channel; cGMP PDE, cGMP-phosphodiesterase, PLC-β, β-isoforms of phospholipase C.

morphine were altered [7,8]. In addition, behavioral effects of catecholamine reuptake inhibitors were abolished in $G\alpha_z$-deficient mice [7], indicating that G_z is involved in signaling processes regulated by various neurotransmitters.

$G\alpha_{olf}$ is expressed in various regions of the CNS, including olfactory sensory neurons and basal ganglia. $G\alpha_{olf}$-deficient mice exhibit clear motoric abnormalities such as hypermotoric behavior [9]. Recent data indicate that G_{olf} is critically involved in dopamin(D_1)- and adenosine(A_{2A})-receptor-mediated effects in the striatum [10,11].

The two main members of the G_q family, G_q and G_{11}, are widely expressed in the central nervous system. Mice lacking $G\alpha_q$ develop an ataxia with clear signs of motor coordination deficits, and functional defects could be observed in the cerebellar cortex of $G\alpha_q$-deficient mice [12]. In addition, lack of $G\alpha_q$ resulted in defective cerebellar and hippocampal long-term depression (M. Kano *et al.*, unpublished; [13]).

Immune System

Mice lacking $G\alpha_{i2}$ develop a lethal, diffuse inflammatory bowel disease that resembles in many aspects ulcerative colitis in humans [14]. In subsequent studies, dramatic changes in the phenotype and function of intestinal lymphocytes and epithelial cells have been described that are likely to be due to defective lymphocyte homing in enteric epithelia [15]. On a cellular level, G_{i2} may be involved in the regulation of T-cell function and trafficking. These processes are regulated through chemoattractant and chemokine

receptors that show a predominant coupling to G_i-type G-proteins. In addition to the colitis, many $G\alpha_{i2}$-deficient mice develop colonic adenocarcinomas, which are probably secondary to colonic inflammation [14].

Heart

The $G\alpha_q/G\alpha_{11}$-mediated signaling pathway appears to play a pivotal role in the regulation of physiological myocardial growth during embryogenesis. This is demonstrated by the phenotype of $G\alpha_q/G\alpha_{11}$-double deficient mice that die at embryonic day 11 due to a severe thinning of the myocardial layer of the heart [2]. Adult cardiomyocytes are terminally differentiated post-mitotic cells that respond to stimulatory signals with cell growth rather than proliferation. Myocardial hypertrophy in the adult heart following mechanical stress depends on $G\alpha_q/G\alpha_{11}$-mediated signaling as demonstrated by the absence of a hypertrophic response in adult mice with cardiomyocyte-specific $G\alpha_q/G\alpha_{11}$ deficiency [16].

Inhibition of L-type Ca^{2+} channels in the heart through muscarinic M_2 receptors was found to be abrogated in hearts lacking $G\alpha_o$ as well as $G\alpha_{i2}$ [5,17]. This unexpected finding suggests that both G proteins may regulate this downstream signaling event in a complex fashion.

Sensory Systems

Odors, light, and many tastants act directly on G-protein-coupled receptors. The G protein G_{olf} is centrally involved in the transduction of odorant stimuli in olfactory cilia, and $G\alpha_{olf}$-deficient mice exhibit dramatically reduced electrophysiological responses to all odors tested [9]. Since nursing and mothering behavior in rodents is mediated a great deal by the olfactory system, most $G\alpha_{olf}$-deficient pups die a few days after birth due to insufficient feeding, and rare surviving mothers exhibit inadequate maternal behavior. In contrast to the olfactory epithelium, the vomeronasal organ, which detects pheromones, expresses receptors that are coupled to $G_{i/o}$. Absence of $G\alpha_o$ results in apoptotic death of receptor cells that usually express $G\alpha_o$ [18].

Rod-transducin ($G_{t\text{-}r}$) and cone-transducin ($G_{t\text{-}c}$) play well-established roles in the phototransduction cascade in the outer segments of retinal rods and cones, where they couple light receptors to cGMP-phosphodiesterase. In mice lacking $G\alpha_{t\text{-}r}$, the majority of retinal rods does not respond to light anymore, and these animals develop mild retinal degeneration with age [19]. The light response is transferred from the receptor cell to bipolar cells of the retina. In mice lacking $G\alpha_o$, modulation of ON bipolar cells in response to light is abrogated, indicating that G_o is critically involved in the tonic inhibition of these cells mediated by metabotropic glutamate (mGluR6) receptors [20].

Among the four taste qualities—sweet, bitter, sour, and salty—bitter and sweet tastes appear to signal through heterotrimeric G-proteins. Gustducin is a G protein mainly expressed in taste cells, and $G\alpha_{gust}$-deficient mice show impaired electrophysiological and behavioral responses to bitter and sweet agents [21]. The residual bitter and sweet taste responsiveness of $G\alpha_{gust}$-deficient mice could be further diminished by a dominant-negative mutant of gustducin-α, suggesting the involvement of other G proteins related to $G\alpha_{gust}$ [22].

Hemostasis

Hemostasis is a complex process involving platelet adhesion and aggregation as well as formation of fibrin through the coagulation cascade. Platelet activation results in a rapid shape-change reaction immediately followed by secretion of granule contents, as well as inside-out activation of the fibrinogen receptor, integrin $\alpha_{IIb}\beta_3$, leading to platelet aggregation. Most physiological platelet activators act through G-protein-coupled receptors, which in turn activate $G_{i2/3}$, G_q, G_{12}, and G_{13}. In platelets from $G\alpha_q$-deficient mice, the effect of various platelet stimuli on aggregation and degranulation was abrogated, demonstrating that $G\alpha_q$-mediated phospholipase C activation represents an essential event in platelet activation [23]. However, platelet shape change can still be induced in the absence of $G\alpha_q$, indicating that it is mediated by G proteins other than G_q, most likely G_{12}/G_{13} [24]. The defective activation of $G\alpha_q$-deficient platelets results in a primary hemostasis defect, and $G\alpha_q$ (–/–) mice are protected against platelet-dependent thromboembolism.

The role of G proteins of the $G_{i/o}$ family in platelet activation has recently been elucidated. Platelets contain at least three members of this class, G_{i2}, G_{i3}, and G_z. ADP, which is released from activated platelets and functions as a positive feedback mediator during platelet activation, induces platelet activation through the G_q-coupled $P2Y_1$ receptor as well as through the G_i-coupled $P2Y_{12}$ purinergic receptor. The general importance of the G_i-mediated pathway is indicated by the fact that responses to ADP but also to thrombin were markedly reduced in platelets lacking $G\alpha_{i2}$ [25]. In contrast to ADP or thrombin, epinephrine is not a full platelet activator *per se* in murine platelets. However, it is able to potentiate the effect of other platelet stimuli. In platelets from $G\alpha_z$-deficient mice, epinephrine's potentiating effects were clearly impaired, while the effects of other platelet activators appeared to be unaffected by the lack of $G\alpha_z$ [7]. Thus, members of the G-protein families G_q, G_{12}, and $G_{i/o}$ are involved in processes leading to platelet activation.

Conclusions

Mouse models lacking almost all known genes encoding G-protein α-subunits have been generated, and they provide a first insight into the biological roles of G-protein-mediated signaling pathways. To overcome embryonic lethality or complex phenotypes of some Gα null mutations and to understand the degree of functional redundancy of closely

related G proteins researchers have begun to cross individual mutants and to generate mouse lines that allow for the conditional inactivation of genes in a time- and tissue-specific manner. These approaches will soon provide more detailed views on the functions of G-protein-mediated signaling pathways in the developing and adult mammalian organism.

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CHAPTER 217

Regulation of G Proteins by Covalent Modification

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Introduction

G proteins are subject to a number of covalent modifications that affect their subcellular localization, protein-protein interactions, and regulation. The first modifications known were those catalyzed by bacterial toxins. The discovery of ADP-ribosylation of G-protein α subunits by cholera and pertussis toxins provided significant insights into G-protein function and is discussed in detail in another chapter in this volume (Chapter 100 by Di Girolamo and Corda). Regulation of G-protein activity by phosphorylation is also covered in a separate chapter (Chapter 99 by Luttrell and Luttrell). Other posttranslational modifications of G-protein subunits that have been reported include deamidation of $G_{o\alpha}$ [1,2] and ADP-ribosylation of G_{β} by a cellular ADP-ribosyltransferase [3]. Here we focus on the covalent modification of G proteins by lipids. All G_{α} subunits are fatty acylated by amide-linked myristate and/or thioester-linked palmitate. All G_{γ} subunits are modified at the C-terminus by a farnesyl (C15) or geranylgeranyl (C20) isoprenoid. Two themes have emerged concerning the role of lipid modifications in G-protein signaling. First, by conferring membrane affinity, they have a role in properly positioning G proteins at the plasma membrane, where they must reside in order to interact with receptors and effectors. Once the G protein is in place, lipids further affect G-protein signaling by modulating protein-protein interactions. This chapter will focus on the latter role, as subcellular localization is discussed elsewhere (Linder, Volume 1, Chapter 60).

Separating the roles of lipid modification in membrane affinity from protein affinity is challenging. *In vivo*, mutation of lipid modified residues often results in mislocalized G protein. Defects in signaling can be attributed to the mislocalization, making it difficult to assess additional roles of the lipid modification. Scientists have turned to *in vitro* methods to study effects of lipid modification on affinity between proteins. However, due to the hydrophobic nature of the proteins involved, detergents must often be present in the assays. Detergents at concentrations above the critical micelle concentration (CMC) form micelles. Lipid modifications mediate association with the micelles, concentrating proteins at the micelle surface. Thus, an apparent change in affinity could reflect the ability of lipid-modified proteins to cluster at the micelle surface. The use of soluble effector domains, lipidated peptides, and x-ray crystal structures have allowed more definitive analysis of lipid effects on protein interactions.

N-Terminal Acylation of G_{α}

As presented in Table I, fatty acids are found singly or in combination on G_{α} subunits. N-myristoylation is a cotranslational modification of G-protein subunits of the $G_{i\alpha}$ subfamily [4]. Myristic acid is added through an amide linkage to a glycine residue exposed after removal of the initiator methionine. The enzyme that catalyzes this modification, N-myristoyltransferase, is associated with ribosomes in mammalian cells [5].

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All G-protein α subunits with the exception of $G_{t\alpha}$ and gustducin are substrates for palmitoylation [4]. Palmitate is linked through a labile thioester bond to one or more cysteine residues near the N-terminus of G_α. Palmitoylation of G-protein α subunits is believed to occur at the plasma membrane. In contrast to N-myristoylation, palmitoylation is a reversible and regulated modification. This topic is explored in a separate chapter (Chapter 106 by Wedegartner).

Acylation and Subunit Interactions

In the inactive state, G_α forms a high-affinity complex with $G_{\beta\gamma}$. The crystal structure of this complex reveals that the lipid-modified termini of G_α and G_γ are proximal to each other [6,7]. Although the crystallized proteins lacked lipid moieties, it is predicted that the lipids extend away from the protein complex and into nearby membrane. *In vitro*, myristoylated $G_{o\alpha}$ has higher affinity for $G_{\beta\gamma}$ than the nonmyristoylated form [8]. Studies with $G_{t\alpha}$ suggest that this apparent increase in affinity is due to interactions of the myristoylated subunit with detergent micelles, rather than direct interaction of the fatty acid with $G_{\beta\gamma}$ [9].

The N-terminal modifications found on $G_{s\alpha}$ are also important for subunit interactions. $G_{s\alpha}$ is palmitoylated on Cys3 and contains an additional unidentified hydrophobic moiety at the N-terminus [10]. $G_{s\alpha}$, which has both modifications (purified from liver or Sf9 insect cells), has higher affinity for $G_{\beta\gamma}$ than $G_{s\alpha}$, which lacks the modifications (Sf9 $G_{s\alpha}$ treated with palmitoyl esterase or purified from *E. coli*) [10,11]. The relative contributions of the modifications cannot be determined from these experiments. Detergent at concentrations above the CMC is present in the assays, and the effect of lipidation may be indirect.

Interaction of G_α with Effectors

Activated $G_{s\alpha}$ and $G_{i\alpha}$ work to stimulate and inhibit membrane-bound adenylyl cyclase (AC), respectively. The lipid modifications found on these subunits are important for this activity. The unidentified modification on $G_{s\alpha}$ supports high-affinity binding to AC, independent of the presence of palmitate [10,12]. It is not clear whether this modification directly binds AC or increases affinity by concentrating the G protein at membranes. It is clear, however, that the myristoyl group on $G_{i\alpha}$ directly affects affinity for AC independently of membrane localization [13]. Myristoylation was required for $G_{i\alpha}$ to interact with a soluble domain of AC in an assay free of micelles or membranes. It is unknown whether the myristoyl group has a binding site on AC or affects the conformation of $G_{i\alpha}$ to mediate its interaction with the effector [13]. In contrast to N-myristoylation, palmitoylation does not appear to have an important role in promoting G_α-effector interactions [10,14].

Palmitoylation and Signal Downregulation

Activation of receptors increases palmitate turnover on $G_{s\alpha}$ and $G_{i\alpha}$ [4]. This may serve to regulate the amount of G_α at the plasma membrane [15], although this has been controversial [16], and also to modify interactions with $G_{\beta\gamma}$ as mentioned previously in the section on acylation and subunit interactions. In addition, palmitoylation may be involved in signal down-regulation through effects on interactions with regulators of G-protein signaling (RGS), which stimulate the intrinsic GTPase activity of G_α subunits. Palmitoylated $G_{z\alpha}$ has reduced affinity for its RGS protein G_z GAP [17]. This same phenomenon was observed with other G_α-RGS pairs and is not due simply to the hydrophobicity of the palmitate moiety, since myristoylation of $G_{z\alpha}$ has the opposite effect and promotes interaction with G_z GAP. Thus, depalmitoylation after G-protein activation may be important for returning the G protein to the inactive state [17].

C-Terminal Modification of G_γ

All known G_γ subunits contain C-terminal CaaX motifs, where C is Cys, "a" is an aliphatic residue, and X is the C-terminal amino acid (Table I). These motifs direct prenylation of the cysteine residue, proteolysis of the three C-terminal residues (-aaX), and carboxylmethylation of the C-terminal prenylated cysteine [18]. G_γ is modified with farnesyl or geranylgeranyl depending on the identity of the C-terminal amino acid. Most G_γ subunits are geranylgeranylated; $G_{\gamma 1}$, $G_{\gamma c}$, and $G_{\gamma 11}$ are farnesylated. Prenylation occurs in the cytoplasm, and subsequent processing steps are likely to take place at the endoplasmic reticulum [19,20].

Prenylation and Subunit Interactions

Prenylation of G_γ is not required for the assembly of $G_{\beta\gamma}$ dimers [21]. However, like G_α fatty acylation, prenylation promotes interaction of $G_{\beta\gamma}$ with G_α [22]. The type of prenyl group attached affects the affinity of the interaction for many G_α subunits. Generally, G_γ modified with the more hydrophobic geranylgeranyl group displays higher apparent affinity for G_α. This is likely to be an indirect effect due to enhanced association with the membrane surface. For example, changing the prenyl group from farnesyl to geranylgeranyl on $G_{\gamma 1}$ increases affinity of $G_{\beta 1\gamma 1}$ for both membranes and G_α [23].

Prenylation and Receptor Coupling

Prenylation is essential for receptor-G-protein coupling. Farnesylated peptides corresponding to the C-terminus of G_γ inhibit coupling to rhodopsin, suggesting that the prenyl group is directly involved in binding to receptor [24]. The type of prenyl moiety with which the peptides are modified influences the ability of the peptide to stabilize activated rhodopsin, a measure of affinity. Farnesylated peptides have greater affinity for rhodopsin than either geranylated (C10) or geranylgeranylated peptides [25]. Since the observed affinity does not correlate with hydrophobicity, this is suggestive of a specific binding site on rhodopsin that

Table I Lipid Modifications of Heterotrimetric G Proteins

Subunit	Lipid modifications	Modified sequence[a]
αi, αo, αz	N-Myr, S-palm	H_2N-M**GC**–
αt	N-Myr	H_2N-M**G**A–
αs	Unknown hydrophobic, S-Palm	H_2N-MG**C**–
αq	S-Palm	H_2N-MTLESIMA**CC**–
α12	S-Palm	H_2N-MSGVVRTLSR**C**–
α13	S-Palm	H_2N-MAD–^{14}**C**FPG**C**^{18}–
γ1, γc, γ11	Farnesyl	–**C**aaS-COOH[b]
γ2, γ3, γ4, γ5, γ7, γ8, γ10, γ12	Geranylgeranyl	–**C**aaL-COOH[b]

[a]modified residues are in boldface

[b]"a" is an aliphatic residue, additional processing includes proteolysis of the three C-terminal residues and carboxymethylation of the prenylated cysteine

preferentially recognizes farnesylated G_γ. However, in assays of rhodopsin-stimulated GTPγS binding, geranylgeranylated $G_{\beta\gamma}$ dimers are more effective than their farnesylated counterparts, arguing against a specific farnesyl binding site on rhodopsin [23,26]. How the prenyl moiety functions in receptor recognition requires further investigation.

Prenylation and Effector Interactions

Prenylation and primary sequence of G_γ are both important determinants of $G_{\beta\gamma}$ effector activation. Similar to receptor coupling, the same $G_{\beta\gamma}$ complex is more effective in activating phospholipase C β (PLCβ) or AC when G_γ is geranylgeranylated than when farnesylated [27]. In the case of PLCβ activation, there is evidence that the prenyl group is not simply promoting effector activation indirectly through membrane interactions, but is directly mediating protein-protein interactions. Peptides corresponding to the C-terminus of $G_{\gamma 2}$ inhibit activation of PLCβ by $G_{\beta\gamma}$ in a prenylation-dependent manner. A fluorescence-based binding assay demonstrated a direct interaction of the prenylated peptide with $PLC\beta_2$ [28]. Precedence for prenyl-binding sites in proteins is provided by the structure of prenylated Cdc42, a Rho family GTPase, bound to Rho-GDI, a guanine nucleotide dissociation inhibitor, which regulates interaction of Rho family members with membranes. In this structure, the C-terminal geranylgeranyl group on Cdc42 inserts into a hydrophobic pocket formed by Rho-GDI [29].

A second mechanism for prenylation-dependent effector interactions is suggested by the structure of the $G_{\beta\gamma}$-phosducin complex. Phosducin binds tightly to free $G_{\beta\gamma}$, extracting it from membranes and preventing its reassociation with G_α. Phosducin induces several local conformational changes in G_β that are not seen in the structures of free $G_{\beta\gamma}$ or the heterotrimer, including the opening of a pocket between blades 6 and 7 [30,31]. Based on their structure of farnesylated $G_{\beta\gamma}$, with phosducin, Loew *et al*. proposed that the farnesyl group is sequestered in the crevice [30]. $G_{\beta\gamma}$ may undergo a similar conformational change when bound to effectors. Consistent with this model, mutations in G_β that perturb the putative prenyl binding pocket exhibit reduced potency in effector activation assays [32].

Carboxymethylation of G_γ

Methylation of the C-terminal prenylated cysteine residue is of interest because it is the only step in the post-translational processing of G_γ that is reversible and thus has the potential to be regulated. Methylation affects protein properties by neutralizing the negative charge on the C-terminal carboxylate ion. The effects of methylation on $G_{\beta\gamma}$ have only been studied for the farnesylated $T_{\beta\gamma}$ [33,34]. The most significant effect observed was that on effector interactions [34]. $T_{\beta\gamma}$ activation of phophoinositide-3-kinase and PLCβ, assayed in phospholipid/detergent micelles, was strongly dependent on methylation. Demethylated $T_{\beta\gamma}$ binds to phospholipid/cholate micelles, albeit less well than its methylated counterpart [9,34]. This suggests that the effect of methylation on effector interactions is not simply due to membrane affinity, but may involve direct protein contacts.

Conclusions

Lipid modifications found on G_α and $G_{\beta\gamma}$ modulate interactions with membranes and other proteins, playing an essential role in signal transduction. There is evidence for prenyl-binding sites on receptors and the effector PLCβ, and a direct role for myristate in binding of $G_{i\alpha}$ to AC. How these modifications mediate these protein interactions awaits additional structural studies with lipidated proteins. Novel roles for lipid modifications in protein interactions will surely be revealed in the future.

Note Added in Proof

The unknown hydrophobic modification on αs (Table I) has been identified as amide-linked palmitate at Gly2.

Kleuss, C. and Krause, E. (2003). Gαs is palmitoylated at the N-terminal glycine. *EMBO J*. **22**, 826–832.

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CHAPTER 218

G-Protein-Coupled Receptors, Cell Transformation, and Signal Fidelity

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G protein-coupled receptors (GPCRs) represent the largest class of cell surface receptors and govern a multiplicity of intracellular signaling mechanisms, fulfilling a wide range of critical physiological and cell-specific actions. This vast signaling potential of GPCRs, however, leaves cell-specific transduction pathways vulnerable to profound alterations when G-protein coupled receptors are aberrantly expressed out of their cellular context or persistently activated by either mutatagenesis or excess availability of their ligands. This review covers some of the mechanisms that GPCRs modulate in order to stimulate normal and pathological cell proliferation, including the communication between heterotrimeric G proteins and guanine-nucleotide exchange factors for the Ras and Rho families of small GTPases, signaling cassettes regulating the activity of members of the MAP kinase superfamily, and more recent findings that identify a number of G-protein-independent effectors. We also explore the emerging role of scaffolding molecules in physically organizing the components of each transducing pathway downstream of GPCRs. These organizing molecules are likely to provide cell specificity to GPCR-driven signaling by preventing inappropriate cross-talk in physiological contexts, and may be one of the missing components that allow aberrantly expressed GPCRs or ectopic activation of their targets to cause cell transformation.

Introduction

The family of G-protein-coupled receptors (GPCRs) encompasses the largest group of transmembrane proteins implicated in signal transmission. Also known as heptahelical receptors, these molecules are sensitive to large variety of ligands, including peptide and nonpeptide neurotransmitters, hormones, growth factors, odorant molecules, and light. Such a wide spectrum of sensitivity is reflected in the number of genes that encode GPCRs in animal genomes, including *Drosophila* (1 percent of total genes), *Caenorhabditus elegans* (more than 5 percent of all genes), and even humans, where more than 2 percent of human genes are responsible for over 1,000 proteins with heptahelical structure [1,2]. Numerous GPCRs have been implicated in physiology and in the progression of hereditary diseases [3] and this link has transformed the investigation of new therapeutic drugs: GPCRs and the signaling pathways that they control have become a major focus among pharmaceutical firms [1,2,4].

Heptahelical receptors are called G-protein-coupled receptors because of their well-studied signaling relationship to heterotrimeric G proteins (α, β, and γ subunits). GPCRs become stabilized in an active conformation upon ligand-binding, allowing them to catalyze the exchange of GDP for GTP bound to the G-protein α subunit. Gα and G$\beta\gamma$ subunits subsequently activate effector proteins such as

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adenylyl and guanylyl cyclases, phosphodiesterases, phospholipase A_2 (PLA_2) and C (PLC), and phosphatidylinositol-3 kinases (PI3Ks). These downstream molecules modulate the synthesis of various second messengers such as cAMP, cGMP, diacylglycerol, IP_3, PIP_3, and arachidonic and phosphatidic acid and can also lead to increases in cytosolic [Ca^{2+}] through the opening or closing of a variety of ion channels. Although much work has been focused on the signaling relationship between GPCRs and trimeric G proteins, recent findings suggest that heptahelical receptor activation can direct biochemical responses that are independent of heterotrimeric G proteins (reviewed in [5]).

In this review we will attempt to give a sense of the multiplicity of classical and novel signaling systems that transmit heptahelical receptor-induced changes in cell behavior. We emphasize that the complexity of known GPCR-initiated signals is likely not to exist in one single cell type; that the signaling pathways available to GPCRs in a particular tissue are much more limited. However, because of the vast signaling potential of GPCRs, these critical cell-specific transduction pathways are in danger of being overidden by disregulated mechanisms that are sensitive to heptahelical receptors. Thus, we will also focus on mechanisms that enforce GPCR signaling fidelity to physiological pathways as a barrier against GPCR-induced pathological outcomes such as cell transformation.

Heptahelical Receptors and Tumorigenesis

GPCRs can promote tumor formation in two different ways: through the stimulation of unregulated growth of cancer cells and by the recruitment of nascent blood vessels to tumor sites. Both tumor-promoting activities have received a great deal of attention since they suggest that drug-induced blockade of specific receptors might allow for the selective inhibition of cell growth or blood vessel formation in particular tumor types, and that such approaches would provide a mechanistic handle that could be exploited in the treatment of neoplastic diseases.

Growth factors such as thrombin, lysophosphatidic acid (LPA), bombesin, vasopressin, bradykinin, substance K, acetylcholine receptor agonists, angiotensin II, and many others induce cell division by binding to their cognate GPCRs in many different cell types (reviewed in [6–8]. The heptahelical protein encoded by the *mas* oncogene was the first clue of a connection between cancer and GPCRs. Unlike other oncogenes, the *mas* gene product does not contain any activating mutations when compared to other heptahelical receptors and requires ligand-binding for its transforming ability. This observation taken together with other findings showing that ectopic expression of serotonin 1C and muscarinic m1, m3, and m5 receptors transforms mouse cells in an agonist-dependent fashion [9,10] suggested that endogenous GPCRs can be tumorigenic in the presence of excess ligand and do not require to be mutated to be transforming.

The role of cellular GPCRs in tumorigenesis is an area of active research, and many heptahelical receptors, and their ligands, are coopted by tumors to support cell proliferation (Table I). For example, GPCR ligands such as bombesin, gastrin-releasing peptide (GRP), neuromedin B (NMB), bradykinin, cholecystokinin (CCK), galanin, neurotensin (NT), and vasopressin are secreted by small cell lung cancer cells (SCLC). These tumors also express the GPCRs sensitive to these agonists and thus use heptahelical receptors to stimulate their own proliferation in an autocrine or paracrine fashion (see [11] for an extensive review). A variety of neuropeptide receptors and their ligands play a role in the progression of colon adenomas and carcinomas, gastric hyperplasia and cancer, prostate cancer, and pancreatic hyperplasia and carcinoma (reviewed in [8,11]). Thus, ectopic expression of GPCRs allows tumor cells to override the heptahelical receptor-driven physiological pathways that are intrinsic to their original cell type and use the unchecked potential of GPCR-controlled signaling mechanisms to drive cell proliferation.

Ligand-dependence, however, is by no means an absolute requirement. Mutagenesis can cause GPCRs to become transforming even in an agonist-independent fashion, as is the case for α_{1b} adrenergic receptors [12]. Moreover, constitutively activating mutations do occur in nature. For example, 30 percent of hyperfunctioning human thyroid adenomas and a minority of differentiated thyroid carcinomas contained constitutively active TSH receptors [13], again linking GPCRs to human cancer. Hypermorphic mutations have also been detected in other GPCR such as LH receptors, which can cause hyperplastic growth of Leydig cells in a form of familial male precocious puberty [14], and Ca^{2+}-sensing G protein linked receptors, which can cause autosomal dominant hypercalcemia [15] and may be involved in certain cancers [16].

Genes that encode heptahelical receptors are also present in the genome of several DNA viruses, including the human cytomegalovirus (HCMV) [17], herpes virus saimiri (HVS) [18], and the Kaposi's sarcoma associated herpesvirus (KSHV) [19]. These receptors, such as the HCMV-encoded GPCRs, have a high degree of homology to chemokine receptors [20] and may help virally infected cells escape detection by the immune system. Moreover, viral GPCRs can also function in paracrine or autocrine fashion, encouraging inappropriate cell behaviors such as pathological cell proliferation. For example, the HVS-GPCR contributes to fatal lymphoproliferative diseases caused by HVS infection, such as leukemias and lymphomas, in several nonhuman primates [18].

Another way in which cellular and viral GPCRs can promote tumorgenesis is by promoting the development of blood vessels that support tumor growth. A variety of G-protein-coupled receptors, including those binding sphingosine-1 phosphate, LPA, PAF, thrombin, IL-8, GROα-γ, MCP-1, and SDF-1, have been implicated in tumor-induced angiogenesis and vasculogensis [21]. The sphingosine-1-phosphate (S1P) receptor $S1P_1$/EDG-1 is a particularly interesting example

Table I G Proteins and G Protein-Coupled Receptors in Tumorigenesis

Activating mutations	
a) G proteins	
$G\alpha_s$	Thyroid toxic adenomas, thyroid carcinomas, growth hormone-secreting pituitary adenomas, McCune-Albright syndrome
$G\alpha_{i2}$	Ovarian sex cord tumors, adrenal cortical tumors
b) G protein-coupled receptors	
TSH receptor	Thyroid adenoma, thyroid carcinoma
FSH receptor	Ovarian sex cord tumors, ovarian small cell carcinoma
LH receptor	Leydig cell hyperplasia, male precocious puberty
CCK-B receptor	Colorectal cancer
Ca^{2+}-sensing receptor	Autosomal-dominant hypocalcemia, neoplasms
Autocrine and paracrine activation	
Neuromedin B receptor	Small cell lung carcinoma
Neurotensin receptor	Prostate cancer Small cell lung carcinoma
Gastrin receptor	Gastric cancer Small cell lung carcinoma
Cholecystokinin receptors	Pancreatic hyperplasia, pancreatic carcinoma, Gastrointestinal cancer Small cell lung carcinoma
Vasopressin receptors	Small cell lung carcinoma
Virally encoded G protein-coupled receptors	
Kaposi's sarcoma associated herpesvirus (KSHV)	Kaposi's Sarcoma
Herpes virus saimiri (HVS)	Leukemias and lymphomas in non-human primates
Jaagsiekte sheep retrovirus (JSRV)	Ovine pulmonary carcinoma

because of its interactions with receptor tyrosine kinases [22]. This GPCR was originally cloned from endothelial cells and supports G_i-dependent cell migration and Rac activation in human embryonic kidney (HEK) cells [22,23] and mouse embryonic fibroblasts (MEF) [24]. It is interesting that MEF derived from EDG −/− animals not only exhibit deficits in S1P-directed Rac activation and cell migration but also in that elicited by other mitogens such as PDGF [25,26]. Previous data showing that the enzyme that makes S1P, sphingosine kinase, is stimulated by growth factors such as PDGF [27] led to a transactivation model in which S1P generated by PDGF receptor stimulation activated the EDG-1 receptor in a paracrine or autocrine way. Since cell migration is essential for blood vessel formation, this signaling relationship between the $S1P_1$/EDG-1 and the PDGF receptors might be a critical step in angiogenesis, including that promoted by tumors.

Viral GPCRs have also been implicated in pathological blood vessel formation. The KSHV-GPCR is a constitutively active G_q-coupled receptor and has been shown to be transforming when overexpressed in murine fibroblasts [19]. Recent work has shown that the KSHV-encoded GPCR can appropriate signaling pathways that are normally active in cell proliferation and use them to stimulate the inappropriate expression of VEGF [28,29] and promote cell survival [30], thus participating in the hyper-angiogenic response that characterizes Kaposi's sarcoma lesions.

G-Protein Signaling in Cancer

A minimum of ten of the seventeen Gα subunits have been described to have transforming potential (reviewed in [31]), including members of the four trimeric G-protein classes: $G_{12\text{-}13}$, G_q, G_i, and G_s. In many cases, these proteins are similar to heptahelical receptors in that they stimulate carcinogenesis in their intact form when they are overexpressed outside their normal cellular context. However, mutations that inhibit the basal GTPase activity of two of these Gα subunits, $G\alpha_s$ and $G\alpha_{i2}$, have been described in several tumors types.

Oncogenic mutations of $G\alpha_q$ family members have not been found in human cancers, and research carried out with laboratory-generated active forms of these proteins have yielded contradictory data. It seems that, depending on cell type, activated $G\alpha_q$ is transforming when expressed at low levels [32] but can lead to apoptosis when present at high levels [31]. It is interesting that highly transforming receptors, such as serotonin-1C, muscarinic m1 and α_1-adrenergic receptors, are coupled to G_q, and that the KSHV-GPCR, a constitutively active Gq-coupled receptor, has been implicated in Kaposi sarcoma progression [19], suggesting that parallel pathways emanating from Gq receptors may be necessary in addition to the activation of the $G\alpha_q$ subunit itself.

Examples of transforming G-proteins include the *gep*, *gip2*, and *gsp* oncogenes. The *gep* gene was simultaneously

identified as an oncogenic sequence present in Ewing's sarcoma and as a transcript that induces strong transformation of NIH 3T3 cells [33,34]. *gep* turned out to be a wild-type $G\alpha_{12}$ subunit, belonging to the $G\alpha_{12/13}$ family. This result has been consistent with subsequent findings: increased expression of $G_{12\text{-}13}$ subunits has been detected in many human cancers, but it is interesting that no mutations have been found. For example, breast, colon, and prostate adenocarcinoma-derived cell lines express elevated levels of wild-type $G\alpha_{12/13}$ (reviewed in [35]). The *gip2* oncogene is a constitutively active mutant of $G\alpha_{i2}$. This mutation has been found in human ovarian sex cord stromal tumors and adrenal cortical tumors [36], although how often $G\alpha_{i2}$ mutations occur in these cancer types remains a point of controversy. Fibroblast transformation resulting from transfection of *gip2* has been suggested to result from the derepression of the Ras-ERK1/2 pathway after cAMP/PKA inhibition [37]. However $G\alpha_{i2}$ can also stimulate the Ras-ERK1/2 cascade via Rap1 inhibition [38] or stimulation [39], depending on the cellular context, and by stimulating the release of $\beta\gamma$ G-protein subunits [40]. The *gsp* oncogene codes for a GTPase-deficient mutant of $G\alpha_s$ and is found in thyroid toxic adenomas (30 percent), thyroid carcinomas (10 percent), growth hormone-secreting pituitary adenomas, and McCune-Albright syndrome. It is interesting that responses to *gsp* expression are cell-type specific. Increases in cAMP and activated PKA resulting from the presence of this oncogene can inhibit Raf1 and prevent transformation in some cells [41]. By contrast, the presence of *gsp* and the same downstream second messengers can inhibit cell growth in other cell types such as PC12 and thyroid cells [41–43].

gsp illustrates the importance of cellular context in regard to the transformation potential of GPCRs and G-proteins. Cell types that express the small GTPase Rap1 and the downstream kinase B-Raf, such as PC12 and thyroid cells, are predisposed to ERK activation in response to cAMP increases [42,43]. Thus the availability of the transforming ERK pathway to *gsp* depends on the organization of the signaling pathways present in a particular cell type. The cell type dependence of *gsp* transformation is only one specific case of a more general phenomenon. For example, the ERK pathway belongs to the MAP kinase group of related signaling cascades that are downstream of GPCRs, and many of these transduction pathways can be regulated as "signaling cassettes" to provide cell-type specific responses after heptahelical receptor stimulation.

A Matrix of MAPK Cassettes Links GPCRs to Biological Outcomes

Many intracellular cascades have been found to mediate GPCR cell growth effects. A major contributor to this function is a group of highly related proline-targeted serine-threonine kinases, generally known as MAP kinases (MAPKs). Although new members of this group have been recently discovered, we will focus on the three best-known classes of MAPKs: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNK), and the p38 kinases. These molecules are phosphorylated by a family of proteins known as the MAP kinase kinases (MAPKKs), which are, in turn, themselves phosphorylated by the MAP kinase kinase kinase (MAPKKK) class of proteins. Under physiological conditions, there is great specificity among the MAP kinases in a particular cascade, with only a very limited number of proteins at each step. For example, in the ERK cascade the MAPKKK Raf-1 will not phosphorylate a MAPKK other than MEK1 or MEK2, two isotypes that perform the same function in that specific MAP Kinase pathway. Thus, MAP kinase molecules such as ERK are downstream of phosphorylation cascades, forming separate and parallel signaling cassettes.

G-protein coupled receptors can signal to a variety of MAPKKKs that are linked to MAP kinases, including those of the ERK, JNK, and p38 pathways. After activation, members of the MAPKs translocate to the nucleus, where they regulate the expression of genes that play a key role in physiological and pathological cell growth. These signaling molecules affect gene transcription by phosphorylating transcription factors that control the synthesis of these critical mRNAs [44]. Each MAP kinase signaling cassette has a different range of intracellular targets and is therefore able to induce different cellular responses such as cell proliferation, apoptosis, and migration. Much work has been done on the essential molecular events that GPCRs use to regulate the function of the MAPKs, and this research has provided insights into the underlying biochemistry that GPCRs use to govern a wide range of biological events.

ERK Cassette

Many GPCR agonists stimulate p42 and p44 MAPK (MAPK/ERK1/2) in multiple cell contexts (Fig. 1) (reviewed in [45]). Research focusing on the molecular mechanisms regulating this signaling cassette has revealed an assortment of cell-specific signaling cascades. These include the recognition of tyrosine kinases, PI-3 kinases, and PKC as possible downstream targets for GPCRs.

Tyrosine kinase inhibition reduces both the activation of ERK1/2 by GPCRs [46] and the rapid tyrosine phosphorylation of the adapter molecule Shc, a posttranslational modification that induces Shc-GRB2 complex formation [47]. These findings provided the first clue of a tyrosine kinase link between GPCRs and the Ras-ERK cassette. A subset of nonreceptor tyrosine kinases (NRTKs) and receptor tyrosine kinases (RTKs) have been suggested as mediators of this response. Src or Src-like kinases can phosphorylate Shc upon stimulation of β-adrenergic receptors, or $\beta\gamma$ subunits [48]. NRTKs such as Csk, Lyn, Btk, Pyk2, and Fak have been implicated in signaling to mediate the ERK cassette by $G\alpha_i$ and $G\alpha_q$-coupled receptors in many cell contexts (reviewed in [49,50]). The variety of tyrosine kinases mediating the activation of the ERK cascade is a reflection of the multiplicity of cellular contexts in which this transduction

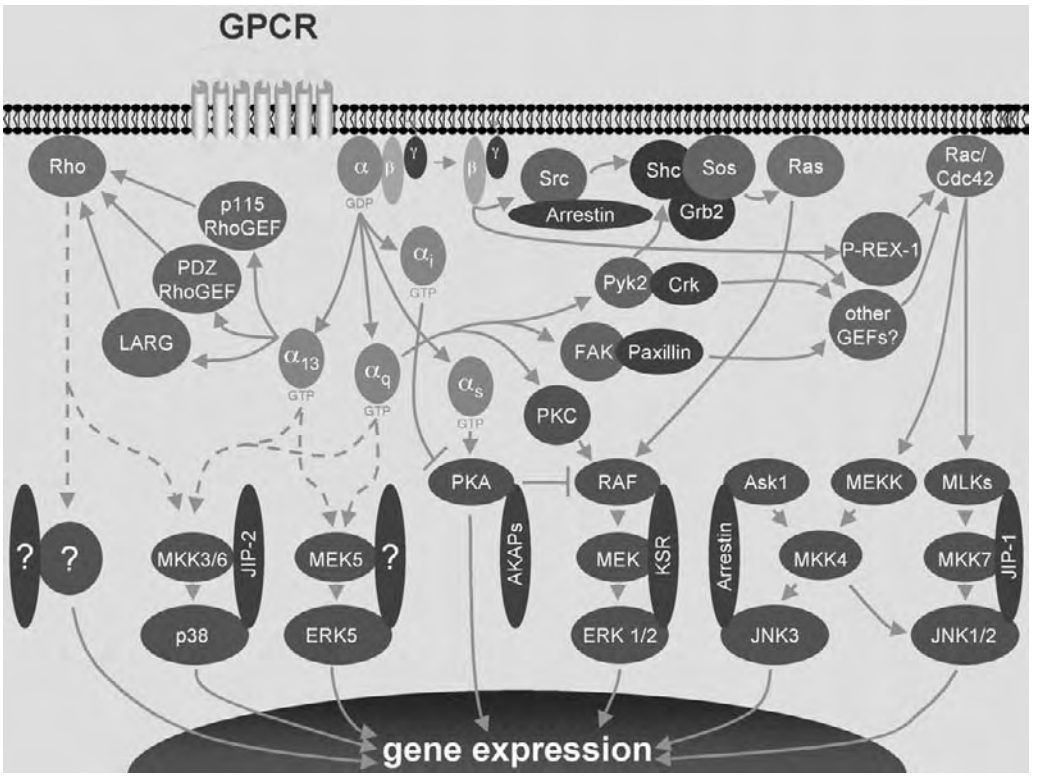


Figure 1 Signaling pathways connecting G-protein-coupled receptors to gene expression are embedded in scaffolding complexes. A multiplicity of signal transduction cascades can link G-protein-coupled receptor stimulation to nuclear events. Thus, aberrant expression of heptahelical receptors, or their cognate ligands, can lead to the activation of a large variety of biochemical routes that can promote neoplastic transformation. Under physiological conditions, scaffolding molecules ensure signal fidelity by physically linking multiple members of signaling cascades to proper subcellular locales and preventing inappropriate crosstalk between related pathways. Arrows represent positive stimulation; dashed lines, functional interactions where precise mechanisms are yet to be elucidated (see text for details).

pathway is involved and its modular nature. Thus, multiple tyrosine kinases in separate pathways may converge in the same signaling cascade to perform different functions.

The protein and lipid kinases of the PI3K family are also essential for GPCR signaling to ERKs. PI3Kγ activity is stimulated upon binding to Gβγ subunits and upon stimulation PI3Kγ activates by signaling to tyrosine kinases that phosphorylate Shc, leading to increases in ERK activity [51]. The PI3Kβ isoform can also mediate GPCR-directed signaling to the ERK cassette [52]. In this mechanism, it is possible that PI3K activates Rac and PAK in combination with Ras to stimulate Raf kinase activity [53]. The variety of pathways stimulated by PI3Kγ and β is an example of the complex mechanisms by which GPCRs can activate the ERK cascade. It is interesting that these pathways all converge on the ERK cassette, impinging on the MAPKKK Raf-1 and suggesting that although the GPCR-driven signals mediated by PI3 kinase and tyrosine kinases are complex, they are fundamentally ordered, as they all "plug-in" to a canonical mechanism leading to ERK activation.

$G\alpha_q$-coupled receptors can also use alternative mechanisms to activate the ERK pathway. These variations include signaling cascades that are mediated by protein kinase C, signals that are transmitted by Ras, and transduction pathways that require the function of both Ras and PKC. Which mechanism functions in a given case depends strongly on the cell type and the extracellular stimulus given. Examples of this specificity include the second messengers that are synthesized in response to G_q, such as diacylglycerol and elevated levels of intracellular Ca^{2+}, that can stimulate Ras through the guanine nucleotide exchange factors (GEFs) RasGRF and RasGRP (also called CalDGEF). These two GEFs are expressed only in certain tissues and stimulate only Ras and Ras-related GTPases (reviewed in [49]). By contrast, the mechanism by which PKC stimulates ERK activity is not yet completely elucidated, since direct phosphorylation of Raf does not seem to be sufficient to fully activate MEK and MAPK [54]. PKC may also modulate other molecules that regulate the interaction between Ras and Raf.

The consistent theme in GPCR signaling to ERK1/2 is that there is a complex matrix of transduction pathways that are ultimately integrated by a canonical set of three kinases (Raf, MEK, and ERK). This multiplicity of known signaling links that impinge on one cascade is unlikely to coexist in one single cell type but represents the summation of the different mechanisms by which GPCRs can connect to ERK in many cell types. In order to prevent pathological responses,

it is critical for GPCRs to activate ERK in a controlled fashion, restricting its duration and intensity by cell-specific pathways that can ensure the fidelity of each signaling step. There is always potential, however, for GPCRs to inappropriately stimulate the ERK cassette via signal transduction pathways that are extraneous to cellular context. For example, overexpression of gastrin receptors in cancer cells leads to an activation of c-Src and, in turn, the ERK pathway. Thus, inappropriate c-Src activation may contribute to the transforming effects of gastrin receptors. When mechanisms of ERK activation are placed out of their normal cellular context, they are likely to be disregulated and, given the transforming potential of the ERK cascade, likely to contribute to tumor progression. Thus ensuring cell-specific signal transduction with respect to the ERK cassette is a critical aspect of proper GPCR physiology.

JNK Cassette

The detailed mechanisms by which GPCRs stimulate the MAP kinase cassette that terminates in the c-Jun NH2-terminal kinase (JNK) remain to be fully elucidated. This molecule, also known as stress-activated protein kinase (SAPK), has sequence similarity to ERK1/2, but is activated by GPCRs through distinct pathways. The most important difference in how these two MAP kinase cassettes are regulated is that whereas ERK1/2 stimulation often depends on Ras, the JNK cassette is downstream of the small G proteins Rac and Cdc42. Constitutively active mutants of Rac and Cdc42, for example, can stimulate JNK activity [55], and these two small G-proteins also mediate the activation of JNK by free Gβγ dimers and by $G\alpha_{12}$, $G\alpha_{13}$, Gq, and G_i [49,50,56]. Little is known about how GPCRs activate JNK beyond these general constraints, but recent work greatly advanced the field by identifying the first GEF known to be responsive to Gβγ and PI-3-kinase, P-REX [57]. This GEF, however, was purified from neutrophils and it remains to be seen whether P-REX or similar GEFs stimulate Rac in response to Gβγ in other cell types.

Another area that remains unclear is how $G\alpha_{12}/G\alpha_{13}$ stimulates Rac1/Cdc42 and the downstream JNK cassette. Current candidates that might mediate this effect include two Rac/Cdc42 GEFs, Tiam1 and Dbl, and two Ras GEFs, which may also catalyze Rac GTP exchange, Ras-GRF1 and Ras-GRF2. Further, the nonreceptor tyrosine kinases PYK2 and FAK that are stimulated by stress-fiber and focal complex formation can also stimulate the JNK cassette by interacting with the adaptor proteins Crk [58] or paxillin [59]. Crk and paxillin can, in turn, stimulate GEFs for Rac and Cdc42 (reviewed in [49]).

Still, many of the open questions concerning the activation of the JNK kinase pathway are in the end questions about how Rac and Cdc42 respond to GPCRs. The question of which Rac effectors stimulate the JNK pathway is also an important, outstanding issue. Here again, the presence of a variety of mechanisms impinging on the JNK cassette reflects the general theme of GPCR-signaling complexity and the exquisite cell specificity that is possible from such a wide range of alternative biochemical routes.

p38 Cassette

The p38 MAP kinases, like the JNK family, are stimulated by cellular stress and membrane-bound receptors [60]. There are presently four p38 MAPKs known: p38α(CSBP-1), p38β, p38γ (ERK6/SAPK3), and p38δ (SAPK4) [61].

Although the GPCRs and agonists that elicit increased activity from the p38 family of MAP kinases have been the focus of much investigation, there is no clear picture of the downstream mechanisms directly controlling p38 kinases. There have been some reports showing that $G\alpha_q$ and βγ dimers stimulate p38α [62] and that two NRTKs, BTK [63] and Src [64], are involved in this mechanism. Receptors that couple to $G\alpha_q$ can also stimulate the p38α, p38γ, and p38δ isoforms [65]. Recent work indicates that $G\alpha_q$ mediates p38 activation through the MKK3 and MKK6 MAPKKs [66]. Further, research using electrophysiological techniques suggest that p38 MAP kinases are downstream from G_{13} [67], and it has been proposed that this heterotrimeric G protein can initiate the activity of the p38 pathway by stimulating Ask, a MAPKKK for this cascade [68]. It is expected that the use of novel techniques, such as RNA interference or the generation of knockout animals for molecules acting upstream of p38, will enable the molecular dissection of the mechanisms by which GPCRs and other cell surface receptors activate each member of the p38 family of MAPKs.

G-Protein-Independent Signaling

It has now become evident that the extent of heptahelical receptor signaling reaches beyond signal transduction pathways that are downstream of Gα and Gβγ subunits. Recent work suggests that GPCRs interact with a wide range of signaling molecules besides heterotrimeric G proteins. Molecules containing protein-protein interaction domains such as the PDZ, SH2, and SH3 motifs, as well as polyproline-containing regions, have been reported to directly interact with GPCRs (reviewed in [69]).

These specialized domains could serve GPCRs as cell-specific bypasses from trimeric G-proteins to activate intracellular signaling in some cellular contexts. Several signaling proteins containing these protein-protein interaction motifs have been shown to bind heptahelical receptors. For example, the PDZ domain interacts with proteins containing a C-terminal S/TxV(L/I) sequence common in GPCRs. SH2-containing molecules such as the adaptor Grb2 and the SH2-containing tyrosine phosphatase SHP (reviewed in [69]) have been reported to bind to the β_2-adrenergic and AT_{1A} receptors, respectively. Other domains that have been reported to bind GPCRs are the polyproline-binding domains, such as SH3, WW, and EVH domains [5,70]. For example, metabotropic glutamate receptors (mGluRs) interact with a class of molecules that harbor Enabled/VASP

homology (EVH)-like domains, such as Homer (1a-c, 2, and 3), which binds mGluRs through a C-terminal polyproline sequence (PPXXFP) (reviewed in [69]).

This expanded view of GPCR signaling, combined with emerging results showing that the *frizzled, smoothened,* and *Dictyostellium* cAMP receptors elicit biological responses that are independent of heterotrimeric G proteins, suggests a reevaluation of the "G protein-coupled receptor/heterotrimeric G protein associated effector" concept of heptahelical receptor function. Thus, some workers prefer terms other than "GPCR" in order to avoid using a designation that suggests a more limited range than the vast array of signaling cascades that these receptors actually control. Alternative terms include serpentine, seven-transmembrane, or heptahelical receptors [71]. Such a wide range of signaling possibilities requires strict organization among the signaling molecules that are downstream of GPCRs. One emerging example of how these downstream cascades can be physically organized intracellularly is a variety of scaffolding molecules that tether multiple components of signal transduction pathways in specific configurations.

GPCR Effectors Are Organized by Scaffolding Molecules

Heptahelical receptors stimulate physiological cell growth *via* a variety of tightly regulated signaling modules. In the past decade, scaffolding proteins have emerged as general regulatory mechanisms ensuring the fidelity of intracellular pathways. These proteins bind components of signaling pathways, physically organizing them to enable physiological responses. The prototypical signaling scaffold is the yeast protein Ste5p, which binds the components of the yeast MAP kinase cascade leading to the mating response after activation of the pheromone GPCR [72–75] and has been suggested to mantain the signaling fidelity of this cascade [76]. Ste5p is particularly important to the mating pathway of yeast because unlike multicellular organisms, MAP kinase cascades share most of their components such as the PAK-like kinase Ste20p and the MEKK-like protein Ste11p [77–80]. Like scaffolds in multicellular organisms, however, Ste5p is expressed in a specific cell type: the haploid yeast cell. Thus Ste5p allows a generalized MAP kinase module to elicit a specialized physiological response in a particular cellular context.

Several scaffolds binding a variety of signaling pathways have now been described in multicellular organisms. These include the various MAP kinase pathways organized by scaffolding proteins such as kinase suppressor of RAS (KSR) and c-Jun terminal kinase interacting protein family (JIPs), the cAMP dependent cascades that rely on the A-kinase anchoring proteins (AKAPs) for tethering, and the multiple cell signals downstream of proteins that bind molecules of the β-arrestin class. In many cases, overexpression of these scaffolding proteins will inhibit cell transformation, suggesting that these molecules play an important role in restricting the propagation of signaling events and preventing signal transduction cascades from causing pathological cell proliferation. Although there are very few published reports directly connecting G-protein-coupled receptors to these organizing molecules, we can expect that the relevance of scaffolding proteins to GPCR-induced transformation will become increasingly apparent in the foreseeable future because of their fundamental role in maintaining the integrity of biochemical routes connecting cell surface receptors to the nucleus.

KSR

KSR was first cloned in RAS suppressor screens by using *Caenorhabditis elegans* and *Drosophila* [81–83]. However, recent work has shown that KSR function is required for proper signaling between RAF-1 and ERK in both *Drosophila* [84,85] and *C. elegans* [86]. In these invertebrate systems, ablation of KSR by either RNAi [85] or mutation [86] prevented the efficient activation of MEK and ERK by constitutively active Ras. An intriguing finding is that overexpression of KSR has similar negative effects on preventing Ras-V12-induced tumorigenesis in *Drosophila* imaginal discs [84]. Roy *et al.* [85] have observed that wild-type KSR overexpression can only stimulate the ERK pathway if all other components binding KSR are concomitantly transfected; they suggest that the mechanism by which the overexpressed scaffolding protein prevents efficient signaling to ERK is through a stoichiometric excess of KSR isolating signaling components from each other.

The murine form of KSR was cloned concurrently with its invertebrate homologs [83,87]. Experiments in mammalian systems have paralleled results obtained in *Drosophila* and *C. elegans*. Transfection studies in NIH3T3 cells showed that KSR overexpression blocks RAS-induced transformation [88]. Moreover, KSR knockout mice are resistant to polyomavirus middle T-induced tumor formation [89], a finding that parallels results obtained with *Drosophila.* Mammalian KSR has been reported to bind βγ subunits, preventing ERK activation [90]. This result is particularly interesting because it is the first known link between the KSR scaffold and G-protein-coupled receptors. Further work remains to elucidate the physiological role of βγ subunit/KSR interactions and its effects on GPCR signaling to the ERK pathway.

JIP/IB Family of Scaffolding Molecules

The JIP/IB family of scaffolding proteins consists of three mammalian homologs JIP/IB-1, JIP/IB-1b, and JIP/IB-2 [91–93]. These molecules bind members of the mixed-lineage group of MAPKKK's (MLK) that have been reported to activate both JNK and p38 [94,95]. In addition, both IB1/JIP1 and IB2/JIP-2 have been found to bind the JNK MAPKK, MKK7 [93,96], and IB2/JIP-2 has also been reported to bind the p38 MAPKK MLK3 [97] and p38γ [98].

JIP-1 was originally described as an inhibitor of the JNK pathway that could block JNK-dependent cell growth and transformation [91]. Soon thereafter IB1, an alternatively transcribed isoform of JIP-1, was found in insulin-secreting cells [92]. Like KSR, the overexpression of JIP proteins inhibits the transmission of signals through the JNK pathway. However, the physiological function of this set of protein scaffolds seems to be to temper the activity of the JNK pathway, since cells derived from JIP-1 +/– heterozygous mice or cells depleted of endogenous JIP-1 with antisense RNA have augmented JNK activity [99–101]. These large increases lead to enhanced apoptosis in IL-1β/TNFα/IFN-treated pancreatic β cells. In fact, a mutation in the human *IB1/JIP-1*gene has recently been linked to adult onset diabetes [100]. An emerging property of these scaffolding proteins is their ability to bind certain GDP exchange factors that activate the small G-proteins Rho and Rac. The Rho exchange factor p190RhoGEF binds JIP-1through its phosphotyrosine binding domain (PTB) in neuronal and PC12 cells [102]. Recent work describes the binding of IB2/JIP-2 to the RAC exchange factor TIAM and the RAC/RAS exchange factor RAS-GRF1, thereby physically linking known activators of RAC to components of the p38 pathway, which have previously been reported to be downstream of this small GTPase [97]. TIAM is phosphorylated in response to the G-protein-coupled receptor ligands LPA, endothelin-1, bombesin, and bradykinin [103], and this protein modification is required for the proper localization of TIAM after LPA stimulation [104]. Thus, the JIP scaffolding proteins might link G-protein-coupled receptors to the JNK and p38 pathways by physically interacting with the small GTPase exchange factors activated by heptahelical receptors.

AKAPs

cAMP signaling was the first second messenger described [105,106]. Production of this nucleotide is controlled by adenylyl cyclases, a class of enzymes that are mostly controlled by heptahelical receptors coupling to G proteins of the G_S class [107]. The best described target of cAMP is protein kinase A (PKA), a tetrameric enzyme that becomes activated upon binding to this nucleotide [108]. Activation of PKA does not occur randomly; instead a class of scaffolding proteins, the A kinase anchoring proteins (AKAPs), localizes PKA to specific sites of action [109]. These scaffolding proteins cannot be grouped together by homology, and are defined as AKAPs solely in terms of their capacity to bind PKA.

A common theme among AKAPs, besides their ability to bind PKA, is their multifunctional nature. A growing number of large, multivalent proteins that bind the PKA holoenzyme have been described. These proteins often serve as scaffolds for other signal transduction pathways or exhibit other functions in addition to binding PKA. For example, AKAPs now include proteins such as gravin, a protein that was first identified in patients with the autoimmune disease myasthemia gravis, and also binds protein kinase C (PKC) [110] and three orthologous members of the WASP family of proteins that interact with the ARP2/3 actin polymerization unit WAVE1-3 [111] and respond to RAC signals [112,113]. Moreover, recent evidence suggests that WAVE3 and gravin function as tumor suppressors [114–116], and it is probable that more of these PKA-binding proteins will be shown to function as tumor suppressors.

One reason AKAPs might serve as tumor suppressors is that the G_s/cAMP pathway often serves as an inhibitor of cell proliferation. For example, PKA phosphorylates RAF-1 at serines 43, 259, and 621 [117]. Phosphorylation at serine 259 is critical for PKA-dependent inhibition of RAF-1 kinase activity *in vitro* and blocks RAF-1-dependent *in vivo* activation of ERK in COS cells. In addition, mutation of serine 43 to alanine blocked the ability of RAF-1 to bind RAS in response to cAMP increases. Thus, proper organization of cAMP signaling to PKA in the correct subcellular compartment may be critical in preventing other mitogenic pathways such as the PKC or ERK cascades from becoming overactive and promoting inappropriate cell proliferation.

Arrestin

G-protein coupled receptors are phosphorylated by the G-protein-coupled receptor kinase (GRK) family of proteins after ligand binding [118]. The scaffolding protein arrestin subsequently interacts physically with heptahelical receptors. Although this molecule has been primarily implicated in targeting GPCRs for endocytosis, arrestin has been also shown to couple GPCRs to the activation of Src-like kinases. This process apparently involves the formation of large multiprotein complexes that can include components of the MAPK and JNK cassettes [119,120]. For example, an arrestin-tethered multimolecular complex has been described in the Rac/Cdc42 independent activation of the JNK cassette MAPKKK ASK1 by $G\alpha_{12}$ [68]. It is interesting that arrestin only binds the neural-specific JNK3 isoform, suggesting that this scaffold enforces cell-type specificity in the activation of the JNK pathway by heptahelical receptors.

Conclusion: GPCR Biology Requires Both Signal Integration and Separation

The essential molecular events that GPCRs use to govern such a wide range of biological events seem elusive because of their apparent complexity. Classical second messenger–generating systems are now understood to be only a subset of the mechanisms that GPCRs use in physiological and pathological contexts. At first glance, this "brave new world" of complexity looks like a highly interconnected meshwork wherein signals derived from a GPCR can travel to any of a wide variety of end-points (Fig. 1). From our current standpoint, we cannot predict with any confidence which signaling pathway, of the many possible routes, will be activated by a heptahelical receptor. Yet this situation is not the case in biology: usually GPCR stimulation in a given context will

produce a repeatable result. Moreover, an emerging concept derived from recent studies with MAP kinases and other targets of GPCRs is that scaffold proteins are organizers and keepers of specificity. It may be as important to keep signals separate between closely related cascades, such as the MAP kinases, as it is to integrate them in a coordinated response.

Although physiological functions of GPCRs, including phenotypic differentiation and cell survival or death, most likely result from the integration of a complex network of signaling cassettes, it is probable that pathology induced by GPCRs, such as cancer or tissue hypertrophy, results from the breakdown of signal separation between targets downstream of these receptors. Recent advances in our understanding of GPCR-driven intracellular signaling networks and how they are organized by scaffolding proteins will provide a more global view of the general systems by which these receptors exert their numerous physiological roles and will elucidate their role in many pathological conditions. This new understanding may also point to novel approaches for pharmacological treatment of a variety of disease processes.

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CHAPTER 219

Signaling through G_z

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General Properties

Tissue Distribution

G_z is a member of the G_i subfamily that contains a 41 kDa α subunit $G\alpha_z$ that possesses several properties that set it apart from other $G\alpha_i$ proteins [1–4]. First, the tissue distribution of $G\alpha_z$ is quite restricted, being found primarily in brain, retina, adrenal medulla, and platelets; expression is virtually undetectable in other tissues [4,5]. In adult rat brain, $G\alpha_z$ is expressed mainly in the hippocampus and, at modest levels, in the cerebellum and neocortex. $G\alpha_z$ is expressed in large neurons, such as cholinergic interneurons, but not striatal neurons [6]. During development of the mouse peripheral nervous system, $G\alpha_z$ is expressed at high levels in the superior cervical ganglion, dorsal root ganglion, and trigeminal ganglion; however, expression appears to be downregulated when mice reach the age of three weeks, except in the superior cervical ganglion. In the mouse central nervous system, expression of $G\alpha_z$ peaks at around the third postnatal week in whole brain; however, in the cerebellum the peak is observed around birth [7]. These findings suggest a possible role for G_z signaling in development of the nervous system.

Biochemical Properties

One of the more intriguing properties of $G\alpha_z$ that was noted when the protein was first studied is that the intrinsic rate of GTP hydrolysis by $G\alpha_z$, 0.05 min^{-1}, is quite low compared with most other G-protein α subunits [3]. This relatively weak GTP hydrolysis activity may be a result of a Ser substitution of the second Gly in a conserved GAGES sequence among G-protein α subunits [8]. This slow rate of GTP hydrolysis by $G\alpha_z$ suggests that it may participate in longer-duration signaling events than other $G\alpha_i$ proteins. In addition, RGS (regulator of G-protein signaling) or RGS-like molecules (discussed in the section on RGS proteins) may play crucial roles in regulating G_z signaling due to their ability to enhance the slow GTP hydrolysis rate. The rate of GDP dissociation from $G\alpha_z$ is extremely slow and almost completely suppressed at Mg^{2+} concentrations greater than 100 μM [3]. Arachidonate and other unsaturated fatty acids selectively inactivate $G\alpha_z$ *in vitro* via a mechanism involving binding of negatively charged acidic lipid micelles to the nucleotide-free form of $G\alpha_z$ [9]. However, the biological significance of this unique lipid effect remains to be established.

Covalent Modifications

Unlike other members of the $G\alpha_i$ subfamily, $G\alpha_z$ lacks a consensus Cys residue near the carboxyl-terminus that is the site of modification by PTX-catalyzed ADP-ribosylation [3,4,10]. This makes $G\alpha_z$ a candidate for PTX-insensitive signaling processes. Another distinct property of $G\alpha_z$ is that $G\alpha_z$ can be phosphorylated both *in vitro* and in cells. Treatment of platelets with phorbol 12-myristate 13-acetate, thrombin, or a thromboxane A_2 analogue (U46619) activates protein kinase C (PKC). PKC activation both *in vitro* and *in situ* via permeabilized and intact platelets promotes rapid and stoichiometric phosphorylation of $G\alpha_z$ but has no effect on other members of the G_i subfamily, including $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_o$, or members of G_s subfamily [11]. Site-directed mutagenesis of potential PKC consensus sites revealed that Ser^{16} and Ser^{27} of $G\alpha_z$ are responsible for nearly 80 percent of the total phosphorylation [12]. This PKC-mediated phosphorylation is effectively inhibited by the presence of βγ complex, and phosphorylated $G\alpha_z$ has markedly reduced its ability to interact with the βγ complex [13], suggesting that a βγ contact site is around the amino-terminus of $G\alpha_z$,

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near Ser^{27}. RGSZ1 (discussed in the section on RGS) is a G_z-selective RGS. Phosphorylation of $G\alpha_z$ renders the α subunit much less susceptible to RGSZ1 action [14]. Taken together, PKC-mediated phosphorylation of $G\alpha_z$ is thought to increase the strength and duration of G_z signaling by both preventing RGSZ1-enhanced GTP hydrolysis and re-formation of an α-$\beta\gamma$ complex, although this hypothesis has not yet been directly tested.

Localization of $G\alpha_z$ to the plasma membrane requires two lipid modifications: myristoylation Gly^2 during protein translation and palmitoylation on Cys^3. Prevention of myristoylation by substitution of Ala for Gly^2 decreases palmitoylation on Cys^3, but palmitoylation can be rescued by overexpression of $\beta\gamma$ complex, suggesting that membrane association triggers a palmitoylation event [15,16]. Substitution of Ala for Cys^3, which prevents palmitoylation, does not affect myristoylation during translation, suggesting that myristoylation, an irreversible modification, directly affects the cellular localization of $G\alpha_z$, while palmitoylation, a reversible modification, is more important for the interaction between $G\alpha_z$ and its possible effectors and regulators [15,17].

Receptors That Couple to G_z

Many receptors that couple to G_i proteins can also activate G_z if the receptors are overexpressed in cells. In reconstituted lipid vesicles, nucleotide exchange of $G\alpha_z$ is stimulated when m2-muscarinic receptors are activated [18]. In HEK293 cells, expression of either A_1-adenosine, α_2-adrenergic, or D2-dopamine receptors together with wild-type $G\alpha_z$ confers PTX-insensitive inhibition of adenylyl cyclase in response to receptor activation [19,20]. Opioid receptors, including μ, δ, and κ opioid receptors, have also been reported to couple to several members of the G_i subfamily, including $G\alpha_z$ [21–23]. Clues to a receptor that preferentially couples to G_z have recently come from the analysis of $G\alpha_z$ knockout mice (discussed in the section on $G\alpha_z$ knockout mice). Mice lacking $G\alpha_z$ have decreased platelet aggregation and impaired inhibition of cAMP formation in response to epinephrine [24]. Since evidence indicates that the ability of epinephrine to promote platelet activation is mainly due to its ability to inhibit adenylyl cyclase via α_{2A}-adrenergic receptors [25], and the epinephrine response is lost even though the platelets still contain their normal complement of $G\alpha_i$ proteins, it seems that α_{2A}-adrenergic receptors preferentially couple to G_z in platelets. This preferential coupling was further confirmed in experiments with PC12 cells expressing wild-type $G\alpha_z$. When these cells are challenged with cAMP analogue or nerve growth factor (NGF), treatment with a specific agonist to α_{2A}-adrenergic receptors, UK14304, attenuates PC12 cell differentiation; such an effect is not observed in these cells in the absence of $G\alpha_z$ expression [26]. This pathway is discussed further in the section on effectors of G_z signaling.

Regulators of G_z Signaling: RGS Proteins

RGS proteins act as negative regulators of G-protein signaling by binding to, and enhancing GTP hydrolysis of, G-protein α subunits. Several RGS proteins that have been identified (at least 20 are known in mammals) selectively act on members of the G_i subfamily, including G_z. These include GAIP [27], RGS4 [28], and RGS10 [29]. In addition, an RGS protein identified through both biochemical and interaction-cloning approaches, termed RGSZ1, selectively acts on $G\alpha_z$ but not on other members of G_i subfamily [14,30]. It is not clear whether these specific RGS proteins function only as negative regulators of G-protein signaling, or whether they can also function as effectors of G proteins or as adaptors linking $G\alpha_z$ to other signaling pathways. Hints at such a process have arisen from studies indicating that RGSZ1 binds to the stathmin family member SCG10 and inhibits its microtubule disassembly functions, suggesting that RGS proteins have a broader role in cellular signaling [31].

Effectors of G_z Signaling

Although the cellular processes controlled by G_z activation are not well understood, activated $G\alpha_z$ does possess an ability to inhibit some subtypes of adenylyl cyclase, a property shared with other members of the G_i subfamily [20,32]. Since expression levels of other members of the $G\alpha_i$ subfamily are much higher than that of $G\alpha_z$, it is widely believed that the ability of $G\alpha_z$ to inhibit adenylyl cyclase activity is largely masked and $G\alpha_z$ has other unique, and presumably important, cellular functions. In this regard, stable expression of mutationally activated $G\alpha_z$ can transform Swiss3T3 and NIH3T3 cells by stimulating mitogenic pathways. It is interesting that this stimulation is apparently unrelated to the ability of $G\alpha_z$ to inhibit adenylyl cyclase [33].

Quite recently, potential clues to additional functions of $G\alpha_z$ have emerged from yeast two-hybrid screens such as the one that resulted in the identification of RGSZ1 as a specific regulator of $G\alpha_z$. Two additional molecules were also identified in this screen that selectively interacted with mutationally activated $G\alpha_z$. These molecules were Rap1GAP, a GTPase-activating protein for the Ras-like monomeric G protein Rap1, and a transcriptional coactivator termed Eya2. There is emerging evidence that the interaction of $G\alpha_z$ with each of these molecules has direct functional consequences. Eya2 interacts with transcription factors of Six family; this interaction results in a translocation of Eya2 from the cytosol to the nucleus. Expression of constitutively active $G\alpha_z$ blocks this process by competing with Six4 in an activation-dependent manner [34].

The physical interaction between $G\alpha_z$ and Rap1GAP blocks the ability of RGS proteins to stimulate GTP hydrolysis of the α subunit, and also attenuates the ability of activated $G\alpha_z$ to inhibit adenylyl cyclase. In addition, co-precipitation assays revealed that $G\alpha_z$, Rap1GAP, and Rap1 could form a stable complex [35]. In cell-based studies,

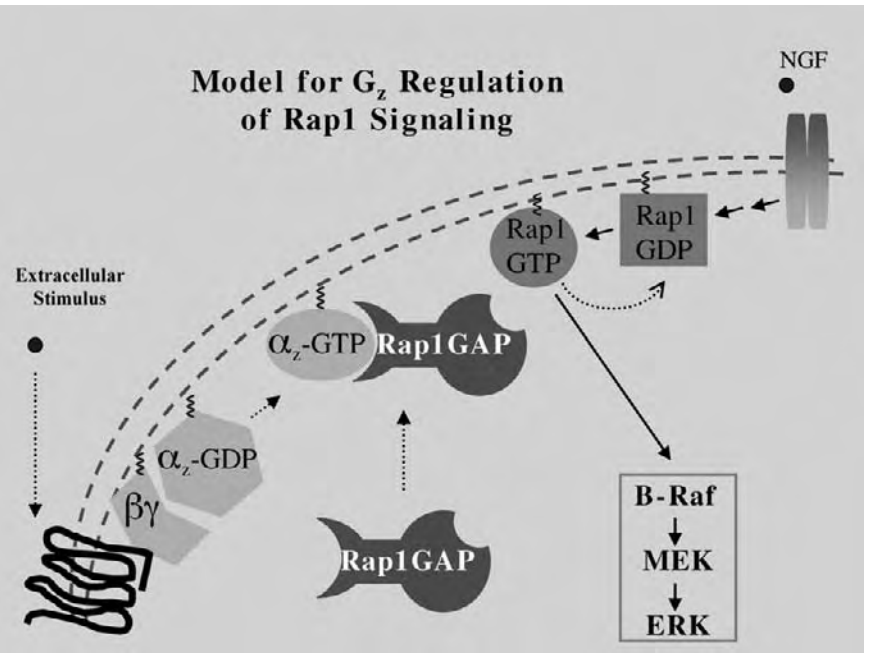


Figure 1 Model for Rap1GAP involvement in G_z signaling in neuronal cells. In the model shown, activation of Rap1 by elevated cAMP or NGF binding to its receptor leads to activation of a MAP kinase cascade that triggers cell differentiation. Concurrent activation of G_z by ligation of an appropriate G-protein-coupled receptor leads to recruitment of Rap1GAP to the membrane with subsequent downregulation of Rap1 signaling.

activated forms of $G\alpha_z$ were able to recruit Rap1GAP from a cytosolic location to the plasma membrane. Experiments in PC12 cells demonstrate that Rap1 activation, ERK phosphorylation, and cell differentiation induced by either cAMP analogue or NGF treatment were all blocked by either transfection of constitutively-activated $G\alpha_z$ or receptor-mediated G_z activation [26]. Based on these findings, a model has proposed in which receptor-mediated activation of G_z results in recruitment of Rap1GAP to the plasma membrane, where it can effectively downregulate Rap1 signaling (Fig. 1).

$G\alpha_z$ Knockout Mice

$G\alpha_z$ knockout mice have been generated by two independent groups [24,36]. $G\alpha_z$ null mice are viable and no major phenotypes associated with loss of $G\alpha_z$ have yet been described. As already noted in the section on receptors that couple to G_z, however, one group has reported that platelet aggregation in response to challenge with epinephrine and collagen is impaired in the $G\alpha_z$ knockouts [24]. Furthermore, both groups have reported that $G\alpha_z$ knockout mice exhibit abnormal responses to certain psychoactive drugs, including a pronounced increase in locomotor activity in response to cocaine administration and a reduction in the analgesic effects of morphine.

One particularly intriguing phenotype of the $G\alpha_z$ null mice was a complete loss of the antidepressant effects of catecholamine reuptake inhibitors, suggesting that the signaling pathways invoked by antidepressant drugs of the norepinephrine reuptake inhibitor class are mediated primarily through G_z [24]. The physiological effects of this class of drugs are thought to be mediated at least in part through increased neurogenesis at the expense of cell differentiation [37,38]. The afore-mentioned findings that activation of G_z attenuates PC12 cell differentiation may provide a link between these observations. It is tempting to speculate that G_z signaling plays an important role in cell fate determination of neuronal cells. In this scenario, treatment with antidepressant drugs leads to increases in circulating norepinephrine that binds to G_z-coupled receptors, and subsequent activation of G_z leads to suppression of signaling pathways that promote cell differentiation and thereby allow cell proliferation associated with neurogenesis. In $G\alpha_z$ knockout mice, treatment with this class of antidepressant drugs cannot lead to activation of G_z and its downstream effectors, resulting in increased cell differentiation without further neurogenesis.

Summary

Although the precise role(s) of G_z in cellular signaling remain to be established, accumulating evidence points to the involvement of G_z in platelet function and, what is especially interesting, neurogenesis. It is intriguing to speculate that G_z may play a crucial role in determining cell fate during early brain development and later in neurogenesis in adulthood. Through subtle but extremely important actions,

G_z could participate in such critical brain functions as learning and memory [39,40].

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CHAPTER 220

Effects of $G\alpha_o$ Signaling

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Introduction

Heterotrimeric G proteins transduce signals through a wide variety of intracellular signaling pathways. Signaling pathways activated by G proteins vary depending on the identity of the specific alpha subunits. Direct effectors of the $G\alpha_o$ subunit are largely unknown, but recent work using yeast two-hybrid analysis [1] and screening a cDNA expression library [2] has yielded exciting new data and helped identify proteins that directly interact with $G\alpha_o$ and may serve as effectors. Some of the direct effectors identified by these methods include the GTPase activating protein for the small G protein Rap (Rap1-GAP), the GAP from the large G protein $G\alpha_z$ (G_z-GAP), the regulator of G-protein signaling 17 (RGS-17), and the G-protein regulated inducer of neurite outgrowth (GRIN). In addition to the direct effectors, some of the signaling pathways that are activated downstream from $G\alpha_o$ have also been identified. These include mitogen activated protein kinase 1,2 (MAPK 1,2) [3], the nonreceptor tyrosine kinase Src [4,5], signal transducer and activator of transcription 3 (Stat3) [5], and phospholipase C (PLC) [6]. In this chapter we will detail the direct effectors as well as the other downstream signaling pathways activated by $G\alpha_o$.

Heterotrimeric G proteins comprise α, β, and γ subunits. In the inactive state the $G\alpha$ subunit is bound to GDP and the $G\beta\gamma$ dimer, and this trimeric complex is associated with the receptor. Upon activation there is an exchange of GDP for GTP on the $G\alpha$ subunit and a disassociation from the $\beta\gamma$ dimer. The GTP bound $G\alpha$ is then able to bind and activate its effector molecules, as is the $\beta\gamma$ dimmer in certain instances. The downstream signal specificity is dependent on the identity of the $G\alpha$ subunit. There are currently four families of $G\alpha$ subunits: $G\alpha_s$, which activates adenlyl cyclase (AC); $G\alpha_i$, which inhibits AC of which $G\alpha_o$ is a member; $G\alpha_{q/11}$, which activates phospholipase C; and finally $G\alpha_{12/13}$. Although the α subunits may activate some overlapping downstream signaling pathways, there are substantial differences between the individual members. Within the family of subunits, whereas the α subunits share common downstream targets they also associate with the common receptors that lead to their activation. A more detailed review of the richness of diversity among G-protein-coupled receptors, downstream targets, and physiological processes regulated by the various families of $G\alpha$ subunits has recently been published [7].

Of all the members of the $G\alpha$ subunits one of the more difficult to understand functionally has been $G\alpha_o$. $G\alpha_o$ was identified from bovine brain extract and is one of the most abundantly expressed proteins in the brain, making up almost 1 percent of the membrane proteins [8]. $G\alpha_o$ is present at high levels in the growth cones of neurons, and observations suggest a role for $G\alpha_o$ in regulation of neurite outgrowth [9,10]. Although $G\alpha_o$ is most abundantly expressed in the brain, other tissues have also been shown to express $G\alpha_o$, including the adenohypophysis, heart, kidney medulla, olfactory epithelium, and chromaffin cells [11].

$G\alpha_o$ is a member of the $G\alpha_i$ family of G proteins and has about an 80 percent homology to $G\alpha_{i1}$, but it does not appear to inhibit several forms adenylyl cyclase activity [12]. Early studies suggested a role for $G\alpha_o$ in coupling opiate and dopaminergic signals to the inhibition of voltage-sensitive calcium channels in neurons [13]. $G\alpha_o$ has also been to shown to couple the f-met-leu-phe (FMLP) receptor to PLC in HL-60 leukemia cells [14], the muscarinic receptor to PLC in *Xenopus* oocytes [15], and the $\alpha 2$ receptor to MAPK 1,2 in CHO cells [3]. Most $G\alpha$ subunits contain an intrinsic GTPase domain that hydrolyses the GTP to GDP, thereby inactivating the GTP-bound $G\alpha$. Mutation of this intrinsic GTPase domain results in a constitutively active $G\alpha$ subunit. The GTPase deficient $G\alpha_o$ subunit (Q-205-L)

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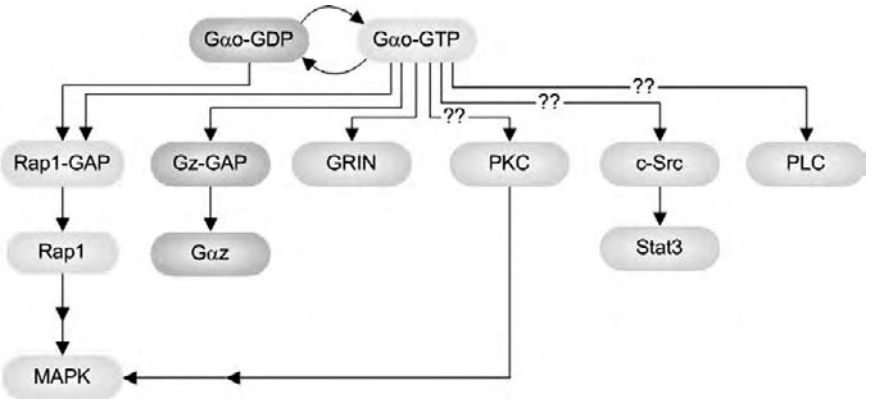


Figure 1 Effector pathways regulated by $G\alpha_o$. $G\alpha_o$ in the active GTP-bound or inactive GDP-bound state can regulate diverse signaling molecules. Not all pathways are likely to be present in all cell types. The regulation of multiple pathways allows for an increased complexity in the biological response to $G\alpha_o$ signaling.

has been used to identify physiological effects of $G\alpha_o$ (Q-L) expression in heterologous systems such as in *Xenopus* oocytes and mouse fibroblast cells [16,17]. This constitutively active mutant has also been a very useful tool to delineate and identify $G\alpha_o$-activated signaling pathways and putative direct effectors. The direct effectors and signaling pathways activated by $G\alpha_o$ are shown in Fig. 1.

By using yeast two hybrid we have identified proteins that can interact with $G\alpha_o$, and the concomitant use of either the inactive or the active $G\alpha_o$ subunit has allowed the identification of binding partners that can preferentially bind to either the inactive or the active $G\alpha_o$ subunit [1]. A chick dorsal root ganglia (DRG) neuron library was used for the two-hybrid analysis. The reason for using the chick DGR was that this is a model system in which the effects of activating $G\alpha_o$ have been well characterized [18]. The direct binding partners identified from the DRG neurons included Rap1GAP, Gz-GAP, RGS17, and GRIN3 [1]. One of the interesting aspects of this study was that Rap1-GAP binds the inactive or GDP-$G\alpha_o$ preferentially over the active or GTP-$G\alpha_o$. In addition, the interactions between $G\alpha_o$ and Rap1GAP lead to a change in Rap1 activity, whereby the binding of $G\alpha_o$ and Rap1GAP relieves the inhibitory effect of Rap1GAP on Rap1, leading to the activation of Rap1 [1]. The sequestration of a negative regulator to activate a third molecule was a novel finding in signaling via G-protein-coupled receptors.

Further studies in our lab have elucidated the mechanism for the negative regulation of Rap1GAP by $G\alpha_o$ [19]. The binding of $G\alpha_o$ to Rap1GAPII and RGS17 targets these proteins for degradation by the ubiquitin-dependent proteasome system. In rat hippocampal slices the induced degradation of Rap1GAPII could be stimulated by the addition of α_2 adrenergic agonist, which could be specifically blocked by inhibiting the proteasome. This novel mechanism for the regulation of a G-protein effector would allow for long-term modulation of signaling pathways. By selectively degrading negative regulators the intrinsic signaling properties would be altered, thus changing the required input for signal activation as well as signal duration.

A study to elucidate direct interactors for the $G\alpha_z$ subunit conducted by Kozasa and colleagues led to the identification of a protein that directly binds $G\alpha_o$ [2]. The approach taken in this study was to screen a mouse embryo expression library by using GTPγ-$G\alpha_o$. The novel $G\alpha_o$ interacting protein identified by this method was termed GRIN [2]. Expression of $G\alpha_o$ and GRIN in Neuro2A (N2A) mouse neuroblastoma cells leads to their differentiation marked by outgrowth of neurite-like fine processes. There are two isoforms of GRIN—GRIN1 and GRIN2—and both bind directly to $G\alpha_o$. One of the main differences between the two isoforms is their expression in different tissues: whereas GRIN1 is widely expressed in neuronal tissue with highest levels in the spinal cord, GRIN2 is only expressed in the cerebellum.

In addition to these direct interactors of $G\alpha_o$, several laboratories have identified signaling molecules and pathways that are activated by $G\alpha_o$. The signaling pathways that are activated by $G\alpha_o$ include c-Src, MAPK1,2, Stat3, and PLC. These pathways are also cell-type specific and are not activated in all cell types. Activation of MAPK 1,2 via ligand activation of $G\alpha_o$ occurs in CHO cells but not in COS-7 cells [3]. Further, this pathway is dependent on PKC activation, as depletion of PKC inhibited $G\alpha_o$-induced activation of MAPK 1,2. Experiments in chick DRG neurons show that the GABA-mediated inhibition of N-type calcium channels by $G\alpha_o$ occurs via activation of Src or a Src family kinase [4]. Src activation by $G\alpha_o$ has also been observed in NIH-3T3 mouse fibroblast cells [5]. Further characterization of the $G\alpha_o$ activation of Src showed that this leads to an increase in Stat3 tyrosine phosphorylation and activation [5].

Conclusions

It is still not clear how many signaling pathways are regulated by the $G\alpha_o$ subunit. However, what is increasingly

clear is that in contrast to the $G\alpha_s$ and $G\alpha_q$ pathways, which are ubiquitous, the $G\alpha_o$ and $G\alpha_i$ pathways are likely to be cell-type dependent. Defining which pathways operate in which cell type is an immediate goal in many laboratories.

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CHAPTER 221

Phosphorylation of G Proteins

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Introduction

In normal cells, heterotrimeric G-protein subunits are known to undergo several forms of co-translational or post-translational modification. Lipid modifications, including *N*-myristoylation and palmitoylation of Gα subunits and prenylation of Gγ subunits, are required for plasma membrane targeting and contribute to the interaction of Gα with Gβγ subunits, effectors, and regulators of G-protein signaling (RGS proteins) [1,2]. Gα subunits, including members of each of the four major families of Gα (Gαs, Gαi, Gαq, and Gα12), also undergo phosphorylation on serine or tyrosine residues. In addition, one Gγ subunit is serine phosphorylated. Here we review what is known about G-protein phosphoryation and its subsequent effects on G-protein function. (See Table I).

Serine Phosphorylation

Several heterotrimeric G-protein subunits are phosphorylated on serine residues *in vivo* and *in vitro*. The kinases responsible include various protein kinase C (PKC) isoforms, the p21-activated protein kinase (PAK), and cGMP-dependent protein kinase. In most cases, serine phosphoryation of Gα inhibits binding to Gβγ subunits and RGS proteins, which may lead either to prolonged Gα-subunit activation or to feedback inhibition of G-protein signaling.

Protein Kinase C

PKC-mediated phosphorylation of Gαi family (Gαz, Gαi, Gαt), Gαq family (Gα16), and Gα12 family (Gα12, Gα13) Gα subunits, along with one Gγ subunit (Gγ12), has been described. Of these, PKC phosphorylation of Gαz and Gα12 are the most thoroughly studied. In human platelets, exposure to phorbol ester, which directly activates classical PKC isoforms, or thrombin, which stimulates a Gq/11-coupled heptahelical receptor, results in rapid and nearly stoichiometric phosphorylation of endogenous Gαz [3,4]. Ser 27 has been identified as the major site of PKC phosphorylation both *in vivo* and *in vitro*, while Ser 16 serves as a secondary site [5,6]. The preferred substrate for PKC is the monomeric Gα subunit, since the addition of Gβγ *in vitro* strongly inhibits the phosphorylation [6–8]. The data are less clear as to whether the inactive GDP-bound or active GTP-bound form of Gαz is a preferred substrate. Since the Gα N-terminus contains determinants for the binding of the Gβγ subunit heterodimer, PKC phosphorylation of Gαz blocks Gβγ subunit binding [6–8]. Similarly, phosphorylation inhibits Gαz interaction with the RGS proteins RGSZ1, RET-RGS1, and GAIP [9,10]. The effect of PKC phosphorylation on Gαz function is unclear, however. Inhibition of Gβγ subunit and RGS protein binding might be expected to prolong Gαz activation *in vivo*, but phosphorylation has no effect on the ability of Gαz-GTPγS to inhibit adenylyl cyclase in a reconstituted system [8].

Conflicting data exist for the PKC-mediated phosphorylation of other Gαi family members. Some authors have reported PKC-mediated phosphorylation of Gαi or Gαt *in vitro* or phorbol ester-mediated phosphorylation *in vivo* [11–14]. Others, however, have failed to detect PKC-mediated phosphorylation of Gαi subunits [4,8]. In NG-108 cells, phorbol ester-induced phosphorylation of Gαi2 correlates with a loss of the inhibitory effect of Gαi on adenylyl cyclase activation [14], suggesting that Gαi phosphorylation

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Table I Phosphorylation of Heterotrimeric G protein Subunits.

G protein subunit	Kinase	Phosphorylation site(s)	Functional effect	References
Serine phosphorylation				
Gαz	PKC	Ser27; Ser16	Inhibits Gβγ subunit and RGS protein binding	[4–10]
	PAK	Ser16	Inhibits Gβγ subunit and RGS protein binding	[6]
Gαi1, Gαi2, Gαi3	PKC	N.D.	Inhibits regulation of adenylyl cyclase	[11–14]
	CGMP-dependent Protein kinase	N.D.	Inhibits IGF-1 and IGF-2 stimulated Ca^{++} influx	[20]
Gα16	PKC	N.D.	Inhibits TRH-stimulated PI hydrolysis	[15]
Gα12, 13	PKC	N-terminal 50 amino acids	Inhibits Gβγ subunit binding	[8,16]
Gγ12	PKC	N-terminal SSK motif	Increases affinity for Gαo; Inhibits activation of type II adenyl cyclase; Increases binding to F-actin	[17–19]
Tyrosine Phosphorylation				
Gαs	c-Src	Tyr37; Tyr377	Increases GTP binding/hydrolysis; Enhances β2AR-stimulated cAMP production	[21–23]
	EGF receptor	N.D.	Enhances Gs activation	[29]
Gq/11	c-Src; c-Fyn; c-Yes; v-Src;	Tyr 356	Modulates R-G interaction; Enhances PI hydrolysis; Stimulates GLUT4 translocation	[24–26]
	Insulin Receptor	N.D.	Stimulates GLUT4 translocation	[28]
Gαi, Gαt	Insulin Receptor	N.D.	N.D.	[12,27]

might provide a mechanism for cross-talk between different G-protein pools.

When ectopically expressed in *Xenopus* oocytes, the Gαq family protein, Gα16, can be phosphorylated in response to phorbol ester treatment or stimulation of thyrotropin-releasing hormone (TRH) receptors [15]. Phosphorylation correlates with a loss of TRH responsiveness, suggesting Gα-subunit phosphorylation may represent part of a feedback inhibitory loop.

Gα12, like Gαz, undergoes PKC-mediated phosphorylation in platelets in response to phorbol ester or thrombin [8]. The site of phosphorylation has been mapped to the N-terminal 50 amino acids but has not been explicitly determined. As with Gαz, phosphorylation of Gα12 blocks Gβγ-subunit binding. Gα13 is also phosphorylated in platelets in response to phorbol ester [16]. However, in contrast to Gα12, attempts to phosphorylate Gα13 *in vitro* using purified PKCα, δ, ε, or ζ have been unsuccessful [8], suggesting that the effect of PKC on Gα13 may be indirect.

Gγ12 contains a Ser-Ser-Lys motif at its N-terminus that is not present in other Gγ subunits and that serves as a substrate for PKC *in vitro* [17,18]. Endogenous Gγ12 in Swiss 3T3 and aortic smooth muscle cells is phosphorylated in response to phorbol ester [17]. Phosphorylation of Gγ12 increases its affinity for Gαo, presumably allowing for more stable heterotrimer formation and allowing phosphorylated Gγ12 to enhance high-affinity agonist binding to A1 adenosine receptors [18]. Phosphorylation selectively affects the interaction of Gγ12 with effectors, impairing Gβγ subunit-dependent activation of type II adenylyl cyclase but having no effect on activation of phospholipase-Cβ. Phosphorylated Gγ12 also affects its interaction with F-actin, possibly allowing G-protein phosphorylation to regulate cell motility [19].

p21-Activated Protein Kinase

Gαz, but not Gαs, Gαi, Gαo, or Gαq, serves as a substrate for PAK1 *in vitro*, with a stoichiometry of phosphorylation of about 1:1 [6]. The preferred phosphorylation site is Ser 16, and the phosphorylation is independent of the activation state of Gαz. In transfected HEK-293 cells, Gαz is phosphorylated by coexpressed PAK1 to an extent similar to that observed with PKC. As with PKC phosphorylation of Ser27, PAK1 phosphorylation inhibits binding of Gβγ subunits and RGS proteins *in vitro*. The effect of PAK1-mediated phosphorylation on Gαz function *in vivo* remains to be determined but might be expected to resemble the effect of PKC phosphorylation.

cGMP-dependent Protein Kinase

In vitro, cGMP dependent protein kinase Iα phosphorylates the Gα subunits of heterotrimeric Gαi1, Gαi2, and Gαi3 to a stoichiometry of about 0.4:1 [20]. The site of phosphorylation is not known, but a potential cGMP kinase site (Arg-Lys-Asp-Thr-Lys) is present in the C-terminal effector region of the protein. In CHO cells, expression of cGMP kinase modestly increases phosphorylation of endogenous Gαi subunits and attenuates pertussis toxin-sensitive calcium influx following insulin-like growth factor stimulation, suggesting that phosphorylation of Gαi may interrupt the coupling of Gαi with effectors.

Tyrosine Phosphorylation

Tyrosine phosphorylation of Gα subunits may account for the ability of tyrosine kinases to enhance certain G-protein-mediated signals. Phosphorylation of Gαs, Gαi, and Gαq/11 by Src family nonreceptor tyrosine kinases, or in response to epidermal growth factor or insulin stimulation, has been reported. Specific phosphorylation sites have been determined only for Src-mediated phosphorylation of Gαs.

Src Family Tyrosine Kinases

Expression of avian c-Src in murine fibroblasts leads to a significant enhancement of adrenergic receptor-stimulated cAMP production, both in isolated plasma membranes and in intact cells [21]. *In vitro*, c-Src phosphorylates purified Gαs and Gαi subunits to a stoichiometry of 0.3–0.9:1 [22]. The inactive monomeric form of the Gα subunit appears to be the perferred substrate, since GTPγS and Gβγ subunits both inhibit phosphorylation. Phosphorylation of Gαs involves two tyrosine residues, Tyr37 and Tyr377 [23], and increases the rate of GTPγS binding and of β2 adrenergic receptor-stimulated GTP hydrolysis.

Similarly, v-*src* transformation of fibroblasts causes enhanced endothelin-1 receptor-stimulated phosphatidylinositol hydrolysis [24]. The effect correlates with tyrosine phosphorylation of Gαq/11 subunits. In a reconstituted system, Gαq/11 from v-*src* transformed cells exhibits increased AlF_4^--stimulated phospholipase C activity. Although the phosphorylation *in vivo* was sensitive to the tyrosine kinase inhibitor herbimycin A, the authors were unable to demonstrate phosphorylation of Gαq/11 by v-Src *in vitro*. Tyrosine phosphorylation of Gαq/11 may also be mediated by the Src family kinase, c-Yes. In a recent study, endothelin 1 receptor-stimulated tyrosine phosphorylation of Gαq/11 in 3T3-L1 adipocytes was blocked by the Src inhibitor PP2 by microinjection of a dominant negative c-Src mutant and by microinjection of antibodies against c-Yes but not c-Src or c-Fyn [25]. Recruitment of a Src family kinase to the endothelin-1 receptor through the formation of β-arrestin1-Src kinase complexes was proposed as the mechanism underlying endothelin-stimulated Gαq/11 phosphorylation. The phosphorylation of Gαq/11 appears to play a role in the regulation of glucose transport by endothelin receptors, since inhibition of endothelin-stimulated Gαq/11 phosphorylation correlated with a marked decrease in of endothelin-stimulated translocation of the glucose transporter GLUT4.

Tyrosine phosphorylation of Gαq and Gα11 in response to M1 muscarinic receptor stimulation has also been reported [26]. In this case, the phosphorylation appears to involve Tyr 356, located in the C-terminus in a position analogous to Tyr 377 of Gαs. The responsible kinase has not been identified, but the effect can be mimicked by expression of c-Fyn, suggesting that it is mediated by a Src family kinase.

Insulin Receptor

Early work demonstrated that several Gα subunits, including Gαt, Gαi, and Gαo, can serve as substrates for the insulin receptor *in vitro* [12,27]. As is the case with Src kinases, the monomeric GDP-bound form of the Gα subunit is the preferred substrate.

Tyrosine phosphorylation of Gαq/11 subunits has been proposed to play a role in insulin-stimulated GLUT4 translocation [28]. Insulin stimulation is associated with a rapid and transient increase in tyrosine phosphorylation of Gαq/11 subunits in 3T3-L1 adiopcytes. In these cells, microinjection of RGS2 protein or antibodies against Gαq inhibited insulin-stimulated GLUT4 translocation, while expression of a consititutively active Gαq mutant stimulated GLUT4 translocation. Although the identity of the tyrosine kinase responsible for Gαq/11 phosphorylation was not determined in these studies, the data suggest that tyrosine phosphorylation of Gαq may play a role in regulating the translocation of glucose transporters to the plasma membrane.

Epidermal Growth Factor Receptor

Purified EGF receptors catalyze the tyrosine phosphorylation of Gαs subunits *in vitro*, with a stoichiometry of phosphorylation of about 2:1 [29]. In this case, the heterotrimeric form of the Gα subunit appears to be the preferred substrate. EGF receptor-phosphorylated Gαs exhibited increased GTPγS binding and GTPase activity and enhanced activation of adenylyl cyclase, suggesting that the phosphorylation leads to activation of Gαs.

Conclusions

There is convincing evidence that several heterotrimeric G-protein subunits are substrates for serine and/or tyrosine phosphorylation *in vitro* and in some cases, *in vivo*. The functional relevance of these phosphorylation events is less well established. Serine phosphorylation of Gα subunits by second messenger–dependent protein kinases is a potential mechanism of feedback regulation of G-protein-coupled receptor signaling. Tyrosine phosphorylation of Gα subunits may provide a mechanism for cross-talk between receptor and nonreceptor tyrosine kinases and G-protein-dependent signaling pathways.

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CHAPTER 222

Mono-ADP-Ribosylation of Heterotrimeric G Proteins

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Introduction

Heterotrimeric G proteins play a crucial role in determining the specificity of the cellular response to extracellular signals. Among the mechanisms controlling G-protein functions are a variety of covalent modifications, relevant in both normal and pathological conditions. The known post-translational modifications occurring on G protein α and $\beta\gamma$ subunits include lipid modifications, which are required for targeting to the plasma membrane of α and $\beta\gamma$ and for the interaction of α with either $\beta\gamma$ or effectors or RGS proteins [1]; phosphorylation, which affects the interactions between α and $\beta\gamma$, and between G proteins with either receptors or effectors [2]; and finally mono-ADP-ribosylation, whose function has not yet been fully defined. In the following, we will focus on the enzymatic mono-ADP-ribosylation of the G protein subunits, catalyzed by both bacterial toxins and eukaryotic enzymes, and discuss the mechanisms and potential role of this reaction in mammals.

The Mono-ADP-Ribosylation Reaction

The mono-ADP-ribosylation reaction is catalyzed by mono-ADP-ribosyltransferases (EC 2.4.2.31) that transfer an ADP-ribose residue from βNAD^+ to a specific amino acid of the acceptor proteins, via *N*- or *S*-glycosidic linkages, with the release of nicotinamide (Fig. 1). This reaction is easily distinguished from that catalyzed by poly(ADP-ribose)polymerases (PARPs), which are nuclear proteins involved in DNA repair, cell differentiation, and apoptosis and are able to transfer multiple ADP-ribose residues, and even branched polymers of ADP-ribose linked by *O*-glycosidic linkage, onto target proteins [3]. The mono-ADP-ribosylation reaction is also different from the nonenzymatic binding of ADP-ribose to acceptor proteins that forms a thiazolidine, instead of thioglycoside (typical of the enzymatic reaction), bond [4]. ADP-ribose can be released from βNAD^+ by cellular NAD^+-glycohydrolases (NADases), of particular interest among which is CD38, an ectoenzyme that catalyzes the formation and hydrolysis of cyclic ADP-ribose, a potent intracellular Ca^{2+}-mobilizing agent [5]. It is interesting that CD38 also catalyzes both auto-ADP-ribosylation on cysteine and ADP-ribosylation of unidentified proteins on the cell surface [6], thus displaying both ADP-ribosyltransferase and NADase activities [7], as seen with other enzymes such as cholera toxin (CT) [8].

ADP-ribosyltransferase activities have been reported in different organisms, including viruses and prokaryotic and eukaryotic cells. The pionering studies by Rappuoli and colleagues [9] showed that ADP-ribosyltransferases fall into two groups: the first is characterized by a conserved histidine in the catalytic domain and includes diphtheria toxin, ART3, and PARPs; the second is characterized by a conserved arginine and includes pertussis toxin (PT), CT, heat-labile enterotoxins, and other ADP-ribosyltransferases.

The mono-ADP-ribosylation of proteins can be reverted by cellular ADP-ribosylhydrolases, which hydrolyze the protein-ADP-ribose linkage, thus releasing ADP-ribose [10,11]. So far three genes have been cloned from rat, mouse, and human, which encodes a soluble, intracellular protein that specifically hydrolyzes the ADP-ribose-arginine bond [12a].

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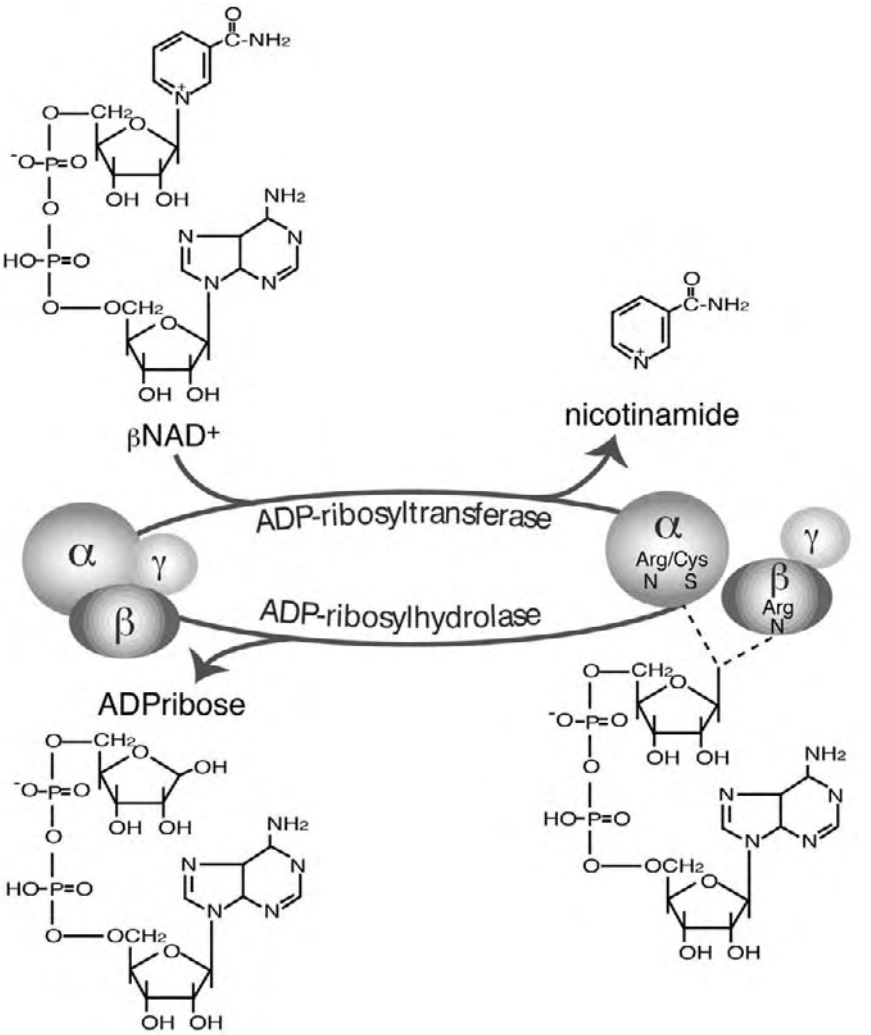


Figure 1 The mono-ADP-ribosylation cycle. The mono-ADP-ribosyltransferase transfers an ADP-ribose residue from βNAD+ to an arginine (αs and β) or to a cysteine (αi, αo, and αt) of the target G protein, via *N*- or *S*-glycosidic linkages, respectively, with the release of nicotinamide. Specific ADP-ribosylhydrolases hydrolyze the amino acid-ADP-ribose glycosidic linkage, thus regenerating free arginine or cysteine and releasing ADP-ribose.

The presence of ADP-ribosyltransferase and ADP-ribosylhydrolase activities in the cell is highly suggestive of a potential regulatory mechanism acting on the specific substrates of this reaction.

Bacterial Toxin-Induced ADP-Ribosylation

The mono-ADP-ribosylation of the G protein subunits was originally described as the mechanism of cell intoxication by bacteria, such as *Vibrio cholerae* (producing CT) and *Bordetella pertussis* (producing PT) [13,14]. These toxins, which specifically act on G proteins (see below), rapidly became important tools in the identification of these proteins and in investigations into their functional roles in cells (Table I). Other well-characterized bacterial ADP-ribosyltransferases are the diphtheria and clostridial toxins, which act by modifying crucial host cell proteins, such as the monomeric GTPases of the Rho family, monomeric actin, and elongation factor-2, permanently inactivating the cellular functions modulated by these proteins (reviewed by [15]).

The CT and heat-labile enterotoxins LT-1 and LT-2 from *Escherichia coli* are arginine-specific ADP-ribosyltransferases that are able to modify the α subunit of the stimulatory G protein (G_s) and to irreversibly inhibit its GTPase activity; this results in the activation of adenylyl cyclase and in the increase in intracellular cyclic AMP [13]. CT is an 84 kDa oligomeric protein that consists of the monomeric A subunit, a 29 kDa polypeptide that exhibits the ADP-ribosyltransferase activity, and the homopentamer B subunit, a complex of five 11 kDa polypeptides, which itself binds stoichiometrically with high affinity and specificity to five GM1 molecules on the plasma membrane [16]. To cause disease, both CT and LT coopt molecular machineries of the

Table I ADP-ribosylating/deribosylating Enzymes Acting on Heterotrimeric G Proteins

Enzyme/Source	Subunit structure/ Localization	Target/s	Effects	Ref.
Toxin ADPRT				
Cholera toxin	AB_5	G_s, G_t	Inhibition of G protein GTPase activity	13,18
Escherichia coli LT	AB_5	G_s, G_t	Inhibition of G protein GTPase activity	13
Pertussis toxin	AB_5	G_i, G_o, G_t	Uncoupling of G proteins from receptors	13,14
Cellular ADPRT				
Human erythrocytes	Membranes	G_i	Decrease in epinephrine-mediated AC inhibition	51,52
Human platelets	Membranes	G_i	Decrease in epinephrine-mediated AC inhibition	51,52
Rabbit ventricles	Membranes	G_s	Increase in AC activity	41
Human platelets	–	G_s	Increase in AC activity	42
Rat brain	Homogenate	G_s, G_o	–	43,44
Rabbit luteal	Membranes	G_s	Increase in AC activity	45
Chicken spleen	Membranes	G_s, actin	Increase in AC activity	46
NG108-15	Membranes	G_s	Increase in AC activity	47
CHO cells	Plasma Membranes	G_β	Inhibition of $\beta\gamma$-mediated function	55
Cellular Hydrolase				
Rat and human tissue	Cytosol	G_s	–	12
Human erythrocytes	Cytosol	G_i	–	11
CHO cells	Cytosol	G_β	–	55

This table lists the ADP-ribosylating activities (ADPRT) identified in different systems and related to G protein modification. The ADP-ribosylhydrolases (hydrolase) catalyzing the reverse reaction are also indicated. See the text for other enzymes for which the action on G proteins has not been proven directly, such as the mammalian ART family (including ART 1-5) and the mammalian Sirt family (including Sirtuin 1-7). AC, adenylyl cyclase; – not determined.

host cell; the toxin enters by endocytosis of the toxin-receptor complexes, follows retrograde transport to the Golgi cisternae or to the endoplasmic reticulum (ER), and finally translocates to its site of action on the inner surface of the plasma membrane [17]. The ADP-ribosyltransferase activity of the A subunit requires proteolysis for activation with the release of a smaller, carboxyl-terminus 7 kDa fragment (CTA2) and of a larger 22 kDa catalytically active fragment (CTA1) whose activity is promoted by a family of 20 kDa GTPases, the ADP-ribosylation factors (ARFs) [18].

PT, a cysteine-specific ADP-ribosyltransferase, ADP-ribosylates the α subunit of G_i, G_o, and G_t. Unlike CT, PT-catalyzed mono-ADP-ribosylation occurs when the α subunit is associated with $\beta\gamma$, thus generating a modified G protein unable to couple to activated receptors and to transduce signals [19]. PT is a 119 kDa toxin consisting of a 28 kDa monomeric A component, an S1 subunit expressing the ADP-ribosyltransferase activity, and a B component, a complex of five polypeptides (S2, S3, two S4s, S5) [20]. The function of the B component is to attach the native toxin to the cell-surface receptor, thus initiating toxin internalization by receptor-mediated endocytosis and its trafficking through early to late endosomes and the Golgi apparatus [21]. There is no evidence that the ADP-ribosyltransferase activity of the S1 subunit requires cofactors, such as the ARFs reported for CT.

Endogenous Mono-ADP-Ribosylation

Vertebrate ADP-ribosyltransferase activity was first detected in turkey erythrocytes [22,23], rat liver homogenates [24], and *Xenopus* tissues [25]. Specific enzymes have been cloned from different sources [26–31]. The family of mammalian ADP-ribosyltransferases includes five enzymes referred to as ART1 to 5 [32,33]. ART1, ART2, and most likely ART3 and ART4, are glycosylphosphatidylinositol (GPI)-anchored to the cell surface. ART5 possesses a hydrophobic N-terminal signal sequence and is likely to be a secretory protein but with an as yet unknown function [34]. ART1 and ART2 have roles in immune regulation: ART1 inhibits T-lymphocyte functions, such as their cytolytic activity and proliferation, by ADP-ribosylating arginines of cell surface molecules, such as the T-cell co-receptors [32,35]; ART2 is expressed on resting T cells and on natural killer cells, has not been found outside of the immune system, and exerts a regulatory role in rat models for autoimmune insulin-dependent diabetes mellitus, where its defective expression on T-cells has been associated with increased susceptibility to the disease [32,36]. ART3 is strongly expressed in human testis, and ART4 is preferentially expressed in human lymphatic tissue [31].

The catalytic domains of the mammalian ART enzymes are extracellular, and thus it is unlikely that members of this family are involved in mono-ADP-ribosylation of

intracellular targets, like heterotrimeric G proteins. In principle, ARTs could be internalized from the cell surface and transported to the site of action, as with the bacterial toxins. However, the sorting mechanism reported for GPI-anchored proteins, which involves endocytic vesicles, would result in a lumenal localization of the catalytic domain, segregating it from cellular substrates such as the G proteins [37]. Thus, the possibility that a distinct family of intracellular enzymes that have ADP-ribosyltransferase activity should be considered. In support of this possibility, it has been suggested that the yeast silencing protein syr2 possesses ADP-ribosyltransferase activity [38]; moreover, the cloning and characterization of seven human proteins (sirtuins 1 to 7) that are able to metabolize NAD^+ and possess ADP-ribosyltransferase activity has recently been reported [39]. These sir2-like proteins do not share any obvious sequence homology with the well-characterized ART family, and represent good candidates for the intracellular ADP-ribosyltransferases.

Cellular ADP-ribosyltransferase activities able to catalyze the modification of G-protein arginine residues in a way similar to CT have been described in many cells and tissues (Table I) [40–49]. Although they present either the identification of the enzymatic activity or of the G-protein substrates, several of the initial reports do not fully demonstrate the mono-ADP-ribosylation reaction (no discrimination between the enzymatic and non-enzymatic ADP-ribose linkages, or use of α-NAD^+ rather than β-NAD^+, the preferred-substrate of ADP-ribosyltransferases, in the ADP-ribosylation assays); moreover, the identification of the G-protein substrates remains elusive, since these reports were only based either on the determination of the molecular mass or on the comigration with the bacterial toxin substrates by polyacrilamide gel electrophoresis [40–46].

A series of more recent reports has led to a better understanding of the endogenous mono-ADP-ribosylation machinery. It has been shown that in highly purified canine cardiac sarcolemma, isoproterenol produces the selective ADP-ribosylation of a single 45 kDa protein, identified as $G\alpha s$ by immunoprecipitation. This effect was correlated with the ability of NAD^+ to increase cyclic AMP production [48]. Similarly, a 52 kDa protein, identified as an αs isoform, is ADP-ribosylated in smooth muscle cells from bovine coronary arteries [49]. In this case, the $G\alpha s$ modification was related to the release of eicosanoids from the endothelium that stimulate the enzymatic reaction and thus activate K^+ channels [50].

Endogenous ADP-ribosylation of cysteine residues of G proteins that are comparable to the PT effect has been reported in human erythrocytes and platelets [51], where it attenuates the inhibition of adenylyl cyclase induced by epinephrine (Table I) [52]. Thus, the picture emerging from these observations is that the pathological modification of G proteins produced by bacterial toxins has an endogenous, possibly physiologically relevant, counterpart that could be looked upon as an additional mechanism of regulation of G-protein-mediated functions. In addition, there are indications in *in vitro* assays that not only the α but also the β subunit of G_t can be a substrate of an ADP-ribosyltransferase purified from the cytosol of turkey erythrocytes [53,54].

Recently, direct evidence of the functional, enzymatic modification of the G protein β subunit has been reported both in isolated plasma membranes and in intact cells [55]. The arginine-specific mono-ADP-ribosyltransferase that catalyzes this reaction is a plasma-membrane-associated, but not GPI-anchored, protein that acts intracellularly and specifically modifies residue 129 of the β subunit. It is interesting that the modified β subunit can be de-ADP-ribosylated by a cytosolic ADP-ribosylhydrolase, thus revealing a cellular ADP-ribosylation/de-ADP-ribosylation cycle that might parallel a functional activation-inactivation cycle of the $\beta\gamma$ dimer. It is worth mentioning that under resting conditions, the β subunit mono-ADP-ribosylation takes place also in intact cells, thus corroborating the potential physiological role of this reaction [55].

The modified β subunit loses its ability to modulate effector enzymes such as calmodulin-stimulated type 1 adenylyl cyclase and phospholipase C, indicating that the modification of arginine 129, a critical residue located in the β common-effector-binding surface [56], can indeed impair β subunit activities [55]. Mono-ADP-ribosylation can thus be considered to be a signal termination mechanism for $\beta\gamma$-mediated functions. In principle, the ADP-ribosylation of the β subunit could also affect the function of the α subunits, since it can lead to a sustained activation of α-subunit-dependent functions by sequestering the $\beta\gamma$ subunit from the signal cascade and preventing the reassociation of the heterotrimer.

Among the substrates of mono-ADP-ribosylating enzymes, there are other cellular proteins involved in signaling and cell organization, such as GRP78/BIP, p33, integrin $\alpha 7$, desmin, actin, and CtBP/BARS [57–64]. It is interesting, and in line with the data summarized above for the modification of the G-protein α and β subunits, that in all these cases ADP-ribosylation has been shown to cause protein inactivation.

Thus, although the regulation of mono-ADP-ribosylation in whole cells remains to be fully clarified, the information obtained to date indicates that this reaction has the characteristics of a fundamental regulatory process that is potentially relevant not only in G-protein-mediated signaling, but also in other cellular functions.

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CHAPTER 223

Using Receptor-G-Protein Chimeras to Screen for Drugs

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Receptor-G-Protein Chimeras: An Introduction

Direct measures of the levels of expression of the α subunits of heterotrimeric G proteins indicate that they are generally in considerable excess over the levels of any particular G-protein-coupled receptor (GPCR) that might activate them. Despite this, an emerging strategy that has been used to address both basic questions on the details of GPCR-G protein interactions and to allow screens to be designed for agonist ligands at GPCRs has been to generate chimeric GPCR-G protein constructs that define a 1:1 stoichiometry of the two partner polypeptides. Such constructs provide a convenient means to assess the effects of mutations and polymorphisms in GPCRs on signal transduction effectiveness without altering the ratio of expression of GPCR to G protein. They have also allowed effective means of developing direct assays of GPCR-mediated guanine nucleotide exchange on G proteins of the G_s and G_q families that historically has been difficult to monitor. Such assays have allowed direct analysis of the extent of constitutive activity of different GPCRs and the detection of inverse agonists.

Construction of a GPCR-G protein chimera involves linking the 5′ end of a G protein α subunit cDNA to the 3′ end of a GPCR cDNA or DNA from which the stop codon has been eliminated. This results in the production of a single open reading frame containing the sequences of both partner proteins. Despite the artificial nature of such constructs all available data indicate that the expressed polypeptides are properly folded and functional. The α subunit in such chimeras is clearly able to interact with G protein β/γ complexes and the basic pharmacology of the GPCR is preserved. A number of recent reviews on the production and analysis of function of such chimeras are available [1–4] and should be consulted to expand on the information provided herein.

Defining the Signal

In the vast majority of cases analysis of GPCR-G protein chimeras takes place following expression in cell systems that also express endogenously the G protein of interest. A range of techniques has been developed to ensure that observed signals represent activation of the G protein within the chimera. For members of the G_i-family of pertussis toxin-sensitive G proteins, the cysteine residue that is the target for toxin catalyzed ADP-ribosylation is within a key receptor contact site but is not inherently required for function [5]. Amino acids with high hydrophobicity can substitute at least as effectively [5,6]. Thus, replacement of this cysteine in the GPCR-fused G protein ensures that the chimera is resistant to the actions of pertussis toxin. Prior treatment of cells with this toxin to modify the endogenously expressed G_i population thus prevents any possibility of agonist-induced signal deriving from interaction with these G proteins [7,8]. This idea has been adapted effectively to construct a ligand screen. The members of the edg family of GPCRs respond to lipid-derived products such as lysophosphatidic acid and sphingosine-1-phosphate [9]. However, at least eight GPCRs of this family are known and they display wide-ranging and complex patterns of expression. Virtually all cell lines respond to these ligands due to endogenously expressed edg family members, and such effects are generally transduced

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via G_i family G proteins. A screening assay for ligands at the human edg2 receptor was thus established by expressing a GPCR-G protein chimera in which a pertussis toxin-resistant mutant of $G_{i1}\alpha$ was linked to this receptor [10]. Following pertussis toxin treatment, guanine nucleotide exchange assays provided a specific and direct assay for ligand activation of edg2 [10].

Because it has been well established that GPCRs within certain chimeras can also activate endogenously expressed G proteins [8], the functionality of GPCR-G protein chimeras has also been tested in cells that lack expression of certain G proteins. Elevation of intracellular [Ca^{2+}] in cells derived from a mouse embryo in which the genes encoding both $G_q\alpha$ and $G_{11}\alpha$ were inactivated requires co-expression of both a GPCR and an appropriate G protein [11]. A chimeric α_{1b}-adrenoceptor-$G_{11}\alpha$ fusion protein is able to elevate [Ca^{2+}] in response to agonist and thus must be functional [11]. This signal is in fact a monitor of release of β/γ complex from the chimeric protein and provides further confirmation that such GPCR-G protein chimeras function in the expected manner [11]. Introduction of mutations that block β/γ binding to the G protein α subunit or prevent α and β/γ dissociation also prevent agonist-induced elevation of [Ca^{2+}] [12].

Guanine Nucleotide Exchange Assays

The initial steps in GPCR activation of a G protein are acceleration of GDP release from the G protein α subunit and its replacement with GTP. Deactivation requires hydrolysis of the terminal phosphate of the GTP. These processes provide conceptually easy assay endpoints. However, the low rates of basal guanine nucleotide exchange by G_s and G_q family G proteins has made this difficult to measure in membranes of mammalian cells because of the relatively high rates of exchange occurring on G_i family G proteins and on other non-signal transducing but GTP exchange-dependent polypeptides. Thus, in terms of assay screens for ligands, the signal-to-noise is generally too low to be acceptable. However, immunoprecipitation of such GPCR-G protein chimeras at the termination of a [^{35}S]GTPγS binding assay can produce data where signal-to-noise is between 20–40 to 1 [11–13]. This reflects that in the absence of agonist many GPCRs have a very low capacity to activate the G protein. Thus, when the chimeric protein is immunoprecipitated very little [^{35}S]GTPγS is associated with it. This is increased greatly by agonist occupation of the GPCR. Again, introduction of mutations that prevent guanine nucleotide exchange on the G protein can be used to confirm that the signal reflects activation of the G protein of the chimera [12,13]. Adaptation of this into a homogenous assay involving, for example, secondary antibody coated scintillation proximity assay beads offers an excellent opportunity to use this approach in ligand screening programs.

Although far less popular than [^{35}S]GTPγS binding assays, analysis of agonist-stimulated GTPase activity provides a highly attractive assay point for activation of GPCR-G protein chimeras. This is particularly so in two regards. First, the defined 1:1 stoichiometry of the two components means that combinations of analysis of the effects of agonist at Vmax and ligand binding studies means that direct measurement of GTP turnover and the catalytic efficiency of different ligands to activate the G protein can be obtained [7]. Second, the mechanisms of action of G protein interacting proteins can be deduced from their effects on enzyme kinetic parameters of GTP hydrolysis. These have been employed to examine the relative efficacy of different agonists and to demonstrate how regulators of G protein signaling (RGS) proteins function [14,15].

Constitutive Activity and Inverse Agonism

Many mutations have been described that introduce or enhance agonist-independent (also called constitutive) activity to GPCRs [16,17]. Such mutations may provide insights into the mechanisms of action of agonists and the conformational states of activated GPCRs. Such mutations have been introduced into GPCR-G protein chimeras. Using the [^{35}S]GTPγS binding assay in parallel with immunoprecipitation, direct measures of the extent of constitutive activity introduced by different mutations have been obtained for each of the α_{1b}-adrenoceptor when activating $G_{11}\alpha$ [13] and for the β_1-adrenoceptor to activate $G_s\alpha$ [18]. Equally, the very low levels of [^{35}S]GTPγS present in immunoprecipitates of the chimeras containing the wild-type forms of these GPCRs has allowed easy demonstration that a number of wild-type GPCRs display high levels of constitutive activity. For example when the melanocortin MC_4 receptor is fused to $G_s\alpha$ and a [^{35}S]GTPγS binding assay followed by immunoprecipitation, a large number of counts are present even in the absence of ligand. Addition of α MSH enhances this further but to a much smaller extent than produced by isoprenaline at an equivalent β_1-adrenoceptor-$G_s\alpha$ fusion. This is not inherently surprising because the agouti-related peptide acts as an endogenously expressed inverse agonist at the MC_4 receptor [19] and this ligand does indeed greatly reduce the levels of [^{35}S]GTPγS binding to the melanocortin MC_4 receptor-$G_s\alpha$ fusion protein. This approach is thus likely to be valuable in assessment of the extent of inherent constitutive activity in many GPCRs and could clearly be applied effectively to GPCRs encoded by viral genomes, at least some of which are thought to display high levels of ligand-independent activity [20]. It also offers excellent potential for development for screens for ligands that have inverse agonist activity. A limitation in the development of screens for ligands at orphan GPCRs has been to decide the G-protein coupling specificity of the orphan [21], but combinations of the use of so-called "universal" [22] and "chimeric" [23,24] G proteins may overcome this.

A second useful approach to improving the sensitivity of detection of ligands at GPCR-G protein chimeras takes advantage of constitutive activity but now monitors GTPase activity.

RGS proteins act as GTPase activating proteins for heterotrimeric G proteins [25]. Addition of a recombinant RGS to a GTPase assay in membranes expressing a chimera of the 5HT-1A receptor and $G_{o1}\alpha$ greatly enhances the responses to agonist ligands [26]. More usefully, however, the RGS protein also acts as a GAP for GTP loaded by the constitutive activity of the GPCR. As this increases the basal activity markedly, it greatly increases the dynamic range available for detection of decreased spontaneous loading of GTP produced by addition of an inverse agonist [26]. Adaptations of this whereby a GPCR that couples traditionally to $G_s\alpha$ is incorporated into a fusion protein containing a chimeric G protein that has the receptor recognition elements of $G_s\alpha$ and the guanine nucleotide exchange and RGS interaction sites of a $G_i\alpha$ [27] will allow this enhanced sensitivity to be used in screens for inverse agonists at $G_s\alpha$-coupled receptors.

Conclusions

Overall, GPCR-G protein chimeras display all the key properties of the two isolated polypeptides. However, due to the proximity produced by their fusion, they display enhanced effectiveness of interaction. Their defined stoichiometry also allows both detailed examination of basic research questions and the development of novel screens for GPCR ligands.

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CHAPTER 224

Specificity of G Protein βγ Dimer Signaling

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Introduction

The G protein βγ dimer interacts with multiple partners to perform numerous functions in the signal transduction process (for reviews see [1–4]). First, the βγ dimer interacts with the α subunit to form the G-protein heterotrimer. In this capacity, the βγ dimer functions to target the α subunit to the membrane and acts as a guanine nucleotide dissociation inhibitor for the α subunit. Second, the βγ dimer interacts with the receptor. In this capacity, the βγ dimer may be required for the receptor to activate the G protein; and the βγ dimer composition may contribute to recognition of the receptor. Third, the βγ dimer interacts with the effector, such as adenylyl cyclases (AC), phospholipases (PLC), ion channels, and kinases. In this capacity, the βγ dimer regulates the activity of the effector until re-association with the α subunit terminates the signal. Finally, the βγ dimer interacts with accessory proteins, including the cytoskeleton, phosducin, regulators of G protein signaling (RGS) proteins, and receptor kinases. In this capacity, the βγ dimer influences the magnitude and duration of the signal transduction process.

In the foregoing, the functions of "the βγ dimer" are discussed. In fact, there is the potential to generate a large number of different βγ dimers given the known existence of five β and twelve γ subtypes. However, their functional significance remains enigmatic. *In vitro* approaches have shown little specificity among the various βγ dimers, whereas *in vivo* approaches indicate greater specificity. Thus, a major question revolves around to what extent the G-protein βγ dimers are functionally interchangeable in the cell. The answer to this question has major implications for how these components are assembled into signaling pathways and how the fidelity of these signaling pathways are maintained. As discussed below, there is a growing recognition that the G-protein βγ dimers have specific functions *in vivo*.

Diversity of the β and γ Gene Families

The β-subunit family is highly conserved. At the genomic level, five genes encoding β subunits (GNB) have been identified [5]. Since their specific functions are not known, they have been named in order of cloning. The GNB1-GNB4 genes share a common intron-exon structure within the coding region, whereas the GNB5 gene does not [5]. The structure of the human GNB3 gene has been described in detail [6]. At the protein level, the β subunits display substantial sequence homology that is conserved across species.

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Notably, the β_1 through β_4 subtypes share 78 to 88 percent amino acid identity, whereas the β_5 subtype shows only 51 to 53 percent amino acid identity with the other β subunits.

By contrast, the γ subunit family is more divergent. At the genomic level, 12 genes encoding γ subunits (GNG) have been identified [5,7]. Again, since their functions are not known, they have been named in order of cloning. As shown in Fig. 1, all GNG genes have two coding exons and share a common splice site in the coding region, with the exception of the GNG13 gene [8]. In addition, most GNG genes have one or more 5′ noncoding exons, some of which are located more than 100 kb upstream of their coding regions.

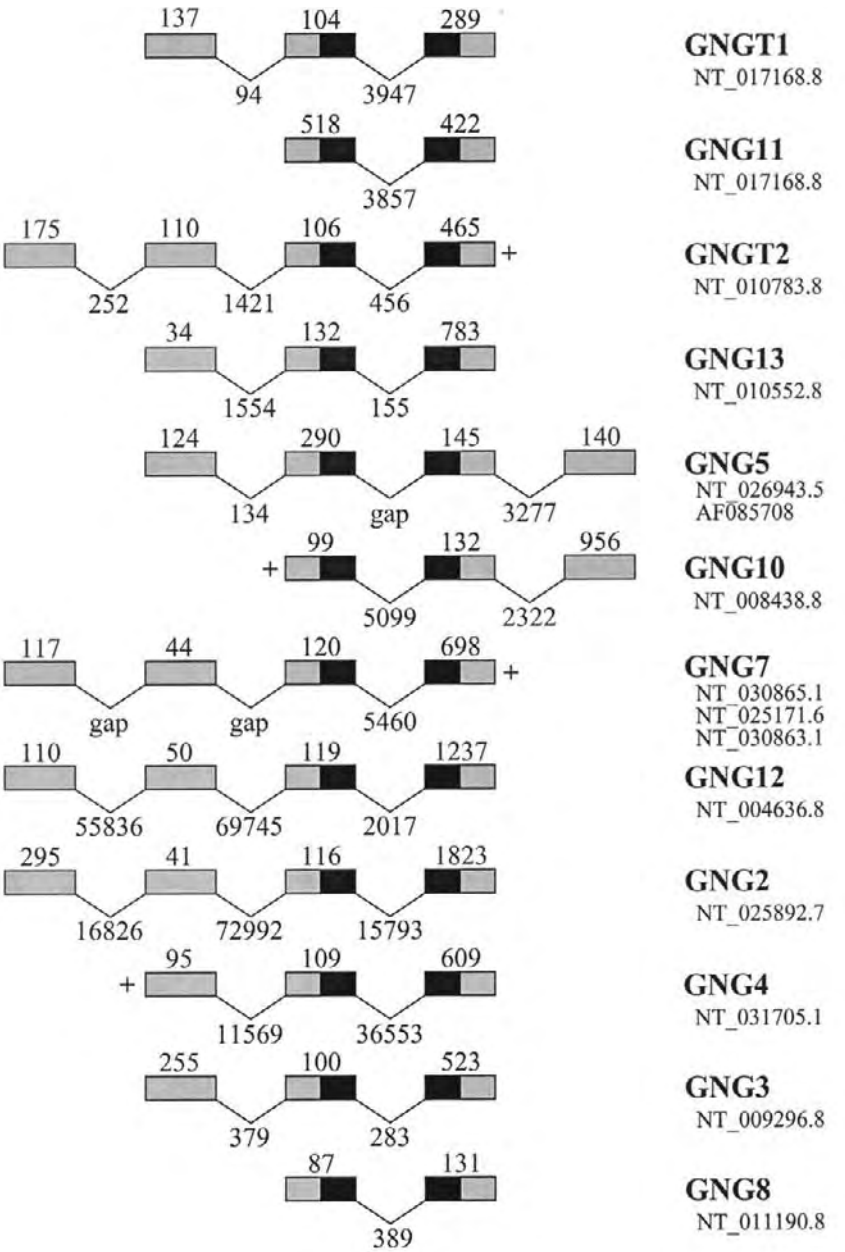


Figure 1 Structures of the genes encoding γ subunits, as predicted by comparing genomic sequences with mRNA and EST sequences via the data mining tools Evidence Viewer and Model Maker at the NCBI web site. Exons are diagrammed as shaded boxes, with size in bp indicated above each box. Dark shading indicates coding regions. Introns are diagrammed as V-shaped lines, with size in bp shown below each. The plus signs indicate the existence of ESTs with sequence beyond what is diagrammed, suggesting a possible additional exon or an extension of the indicated exon. At the right, the accession number for the working draft sequence containing each gene is shown below the gene name. Gaps were still present in the working draft sequences for the GNG5 gene and GNG7 genes. The coding portion of exon 2 of GNG5 was not identified in NT_026943.5 and was modeled based on AF085708. Two 5' noncoding exons of GNG7 were not identified in NT_030865.1, but were present in adjacent contigs.

Though the details remain to be worked out, these additional exons may allow for multiple promoters, coordinate regulation with other gene products, or alternate splicing of elements involved in regulation of translation. Finally, the GNG5 and GNG10 genes have one 3′ noncoding exon, which may be involved in mRNA stability or subcellular localization. This diversity in GNG gene structure suggests variability in gene expression that may underlie specificity in function. At the protein level, the γ subunits display a sequence diversity that is conserved across species, suggesting the individual subtypes have specific and distinct functions. Based on amino acid diversity, they can be arranged into five classes [2], with each group showing less than 50 percent homology to the other classes: (1) γ_7 and γ_{12} (76 percent identity); (2) γ_1, γ_{11}, and γ_{14} (62 to 73 percent identity); (3) γ_2, γ_3, γ_4, and γ_8 (56 to 75 percent identity); (4) γ_5 and γ_{10} (53 percent identity); and (5) γ_{13}. Analogous to the G-protein α subtypes, this pattern of structural diversity suggests that the γ subtypes may fall into functional classes that have yet to be characterized.

The genomic analysis can be extended to identify genes that are adjacent to GNG genes. Except for GNGT1 and GNG11, the genes encoding the γ subunits are widely dispersed throughout the genome [5,7]. The GNG2 gene is arranged head-to-head with an SH3 domain protein, NESH. The GNG3 gene is located head-to-head with the BSCL2 gene (murine Gng3-linked gene), which is responsible for Berardinelli-Seip congenital lipodystrophy [9]. Because of these arrangements, the promoter elements of these genes may overlap, suggesting the possibility of coordinate regulation. The bovine γ_5 subtype and chitobiase protein are alternatively spliced products of the same gene [10]; the human GNG5 and CTBS genes appear to be similarly linked.

Assembly of the βγ Dimer

Typically, the βγ subunits exist as a tightly associated complex that functions as a single unit. The only known exception is the β_5 subtype that interacts with certain RGS proteins [11]. Hence, an important step in deciphering which βγ subunit combinations can exist in intact cells is to understand the mechanics and determinants specifying their assembly. In this regard, there is some evidence suggesting that assembly of the βγ dimer occurs in a specific order: beginning with synthesis of β and γ in the cytosol, progressing to formation of the βγ dimer, to prenylation of the γ, to removal of the three terminal amino acids and carboxyl-methylation of the γ, and finally to translocation of the fully processed βγ dimer to the plasma membrane, perhaps in association with the α subunit [12,13]. Moreover, there is increasing evidence suggesting that spatial and structural constraints can contribute to selective assembly of specific βγ dimers.

Cellular Level

Just how β and γ subunits are brought together to form specific βγ dimers remains unresolved. At one level, the β and γ subtypes are expressed in cell-specific patterns [8,14–17], raising the possibility that each cell contains only a subset of all possible βγ dimers to participate in the signal transduction process at a given time. The mechanisms governing their expression have not been defined but may include differences in promoter function, mRNA stability, mRNA localization, translation efficiency, protein stability, or protein localization. Does this mean that βγ dimers assemble simply through random association in cells expressing multiple β and γ subtypes? *In vitro* studies indicate that structural constraints preclude a few βγ subunit combinations [18–20], most notably those involving β_2, β_3, and γ_1 subtypes. Moreover, *in vivo* studies suggest that spatial considerations contribute to the assembly of specific βγ subunit combinations [21–23]. For example, ribozyme-mediated suppression of the γ_7 subtype results in a coordinate reduction in the expression of the β_1 subtype but has no effect on the other three β subtypes expressed in human kidney cells [23]. Pulse-chase labeling studies show that there is a dramatic difference in the half-life of the β_1 monomer (20.8 min) compared to the $\beta_1\gamma_7$ dimer (14.2 hrs). Collectively, these results indicate that the β_1 protein is rapidly degraded when sufficient γ_7 protein is not available; and that other γ proteins are not able to randomly associate with the β_1 protein in these cells.

Subcellular Level

At another level, the γ subtypes exhibit distinct subcellular localizations [24,25], raising the possibility that they are involved in targeting the βγ dimers to specific locations within the cell membrane. For example, we have shown that the γ_7 subtype is present in the membrane overlying the actin fibers in the leading edge of the cell, the γ_5 subtype is highly enriched in the membrane overlying focal adhesions, and the γ_3 subtype is localized in a membrane site distinct from caveolae (J.D.R., unpublished; [24]). Other reports suggest that the γ subunit is not only associated with the membrane but also with intracellular components. For example, the γ_{12} subtype is present in a detergent-insoluble fraction in Swiss 3T3 cells in association with F-actin [25], and the βγ dimer associates with cytokeratin filaments in starfish oocytes [26]. These results suggest that differential subcellular localization of the γ subtypes may direct the specific assembly of βγ dimers and predicts that β subtypes may be found to share these patterns of expression.

The region(s) of the γ subunit responsible for their targeting includes the C-terminal tail and posttranslational modifications thereof. This raises the possibility that heterogeneity in their processing may account for their distinctive subcellular localizations. In this regard, the type of prenyl group may be important. The γ_1, γ_{11}, and γ_{14} subtypes are modified by the C15 farnesyl group, whereas the remaining subtypes are modified by the C20 geranylgeranyl group [17,19]. Though the prenyl group is important for targeting, the nature of this interaction is not clear. One possibility is that the prenyl group may interact as the result of a lipid-lipid interaction. In this case, the addition of the geranylgeranyl

rather than the farnesyl group may confer additional hydrophobicity to the protein [17,27]. This is consistent with the finding that the geranylgeranyl moiety is sufficient to target the βγ dimer to the membrane, but farnesyl-dependent association of the βγ dimer with the membrane requires additional modifications, including carboxyl-methylation [27]. Another possibility is that the prenyl group may associate as the result of protein-protein interactions. In this case, the presence of the geranylgeranyl or farnesyl group may act as part of a recognition target for specific "docking proteins." This notion is consistent with recent results showing that the geranylgeranyl group of the γ subunit preferentially inserts into a hydrophobic binding pocket on PLC-β [28]. However, the type of prenyl group cannot explain the differences in subcellular localization among the γ_3, γ_5, and γ_7 subtypes noted above, since all three are modified with a geranylgeranyl group. This suggests that variable processing of the C-terminal tail and/or additional upstream regions may be important. In this regard, an alternatively processed form of the γ_5 subtype has been identified as the predominant species in brain [29]. Notably, this protein is prenylated but retains the three terminal amino acids. Whether this pattern of processing determines the unique localization of the γ_5 subtype in focal adhesions is the subject of ongoing studies. Also, there is some evidence that the N-terminal region of the γ protein could be important for subcellular localization based on its extensive sequence divergence [2] and the finding that phosphorylation within this region of the γ_{12} subtype alters the nature of its association with cytoskeleton [30]. Collectively, these results support a growing body of evidence that the G-protein-dependent signaling pathways may be segregated [31,32] and that this is a critical factor in maintaining the specificity of the βγ signal, as discussed below.

Specificity of G Protein βγ Dimer Signaling

Combinatorial association creates the potential to assemble 60 different βγ dimers that can be utilized to direct the fidelity of signaling. However, specificity has been difficult to show in transfected and reconstituted settings where there is substantial overlap in terms of their functions. This is most likely due to the absence of critical factors that provide specificity only in the context of the intact cell setting. For this reason, investigators have begun to use reverse genetic approaches to ascribe specific functions to the β and γ subtypes. Such strategies clearly support the notion that the composition of the βγ dimer, in particular the γ component, has important ramifications for the specificity of signaling that begins at the receptor level and continues to the effector level.

Structural Constraints on Specificity

Reconstitution studies of purified proteins show that the βγ dimer interacts directly with the receptor and certain types of the effector. To identify the contact sites, chimeric and mutagenesis approaches have been used to identify multiple sites on the βγ dimer: some regions appear to stabilize the interaction, other sites appear to modulate the interaction, and a few regions appear to specify the interaction. These regions will be discussed in the context of the three-dimensional structure of the βγ dimer [33,34]. In this structure, the β subunit is composed of two major domains—an N-terminal α helix and a seven-bladed β-propeller—whereas the γ subunit is composed of three major domains—an N-terminal α helix that forms a parallel coiled-coil structure with the corresponding region of the β subunit, a middle domain that forms extensive contacts along the bottom surface of the seven-bladed β-propeller, and a C-terminal domain, including the prenyl group.

βγ DIMER–RECEPTOR

Recent evidence indicates that the βγ dimer directly interacts with the receptor [35], where it may actively participate in receptor-catalyzed nucelotide exchange of the α subunit [36]. Cross-linking studies confirm a receptor contact site within the C-terminal region of the β subunit [37], and reconstitution studies comparing different βγ subunit combinations reveal modest differences due to the β component [38–41]. Similar studies highlight even greater differences due to the γ component and point to the importance of the primary structure of the C-terminal domain and the type of prenyl group [38,41–45]. Collectively, these data indicate that the βγ-subunit composition contributes to the selectivity of receptor interaction. In addition, other results suggest that the βγ subunit composition may modulate the mechanism [46] and/or the duration [47] of receptor interaction. Nevertheless, the finding that the range of G protein βγ-receptor interactions *in vitro* is far greater than that observed *in vivo* suggests the presence of additional mechanisms for conferring specificity of signaling beyond direct receptor recognition of βγ subtypes.

βγ DIMER-EFFECTOR

Once released from the G-protein α subunit, the βγ dimer regulates a growing list of effectors. Multiple regions of the β subunit interact with the effector. One site lies within the N-terminal α-helix of the β subunit [48]. This region is exposed regardless of the activation state of the G-protein heterotrimer and hence may represent a region where the effector can remain bound to the βγ dimer even in the presence of the α subunit. Another region lies along the top surface of the β propeller of the β subunit (49). This region is exposed only upon activation of the G-protein heterotrimer and hence may be critical for modulation of the effector. Other regions include the sides of the β propeller, with each effector having its own characteristic set of contact points [49]. Despite the large number of sites on β, there is little evidence that any of these regions is involved in specifying the interaction with the effector consistent with the high homology of these domains among the β_1–β_4 subtypes. The apparent exception is the β_5 subtype [50,51].

Whereas the β subtypes are very similar, the γ subtypes are much more divergent, suggesting that functional specificity of

different βγ subunit combinations is more likely due to the γ component. In this regard, comparison of βγ dimers differing only in γ reveal moderate differences in their abilities to activate effectors. The differences are due to both the primary structure of the N-terminal domain and the nature of the prenyl group on the C-terminal tail. It is remarkable that the relative contribution of these two regions appears to vary depending on the identity of the effector. For example, the amino acid sequence and/or charge of the N-terminal region of γ appears to be the major determinant for the AC activation. In this regard, the γ_1 and γ_{11} subtypes, which are negatively charged, are poor activators [17,52]. Likewise, the γ_{12} subtype, which is negatively charged as a result of phosphorylation, is a poor activator [53]. By contrast, the C-terminal domain and the type of prenyl group appear to be the more critical factors for activation of PLC-β [27,28,52]. (In one model, the prenyl group inserts into the β subunit, thereby producing a more active βγ dimer for regulation of PLC-β [54]. In the other model, the prenyl group inserts directly into PLC-β, thereby accounting for activation of PLC-β [28]. Supporting the latter model, fluorescence-based assays reveal that the prenylated γ peptide interacts directly with a site on PLC-β. Providing a possible basis for selectivity, this site prefers the geranylgeranylated to the farnesylated form of γ [28]. Finally, mutagenesis studies to alter the putative prenyl binding pocket of β [54] or the C-terminal tail of γ [28] reveal that all such mutants are less effective than wild type for activation of PLC-β. Thus, the position of the prenyl group on the βγ dimer has a profound impact on its ability to interact with PLC-β regardless of the model. However, there are still many unresolved questions. For instance, if the prenyl group of γ inserts into PLC-β, how does this fit with the crystal structure of phosducin-βγ dimer, which has the prenyl group of γ inserting into a hydrophobic pocket of the β subunit [55]? Also, how does this fit with the previous finding that the C-terminal tail and the prenyl group of γ bind directly to the receptor [45,46]?

Specific Functions of Individual β and γ Subtypes

The limited functional specificity that has been observed *in vitro* presents a conundrum. If all βγ dimers display substantial overlap in their interactions *in vitro*, how is specificity of the βγ signal maintained such that different classes of G-protein-coupled receptors have specific functions *in vivo*? For example, the β-adrenergic and muscarinic receptors activate G proteins to produce opposing effects on the rate and force of contraction of cardiac myocytes. This indicates that these two receptors cause release of βγ dimers that are not functionally interchangeable, and implies the existence of critical factors for specificity that can only be provided in the context of the cell. Increasingly, reverse genetic approaches provide the most direct and compelling evidence for specific functions of the β and γ subtypes. Analogous to the α subunit, loss of the β or γ subunit is likely to have major functional consequences by compromising the assembly of a specific G-protein αβγ trimer required for upstream interaction with the receptor and downstream regulation of effector(s).

RNA Suppression

Early studies relied on anti-sense oligonucleotides to suppress translation of the mRNAs encoding the β or γ subtypes. In rat pituitary cells, this approach was used to study regulation of voltage-dependent calcium channels following nuclear injection of anti-sense oligonucleotides against the various G-protein subunits. The intriguing results suggest a specific role for the $\beta_1\gamma_3$ dimer in the somatostatin receptor pathway, with a similarly selective requirement for the $\beta_3\gamma_4$ dimer in the muscarinic receptor pathway [56,57]. More recently, other studies in the same cells predict roles for the $\beta_2\gamma_2$ dimer in coupling the vasoactive intestinal peptide receptor to stimulation of AC, the $\beta_1\gamma_3$ dimer in linking the somatostatin receptor to inhibition of AC, and the $\beta_4\gamma_2$ dimer in coupling the thyroid-releasing hormone receptor to activation of PLC-β [58]. Finally, studies in mice suggest a role for the γ_2 subtype in antinociception by the opioid receptor [59]. Though intriguing, these studies do not show that such functional consequences are due to loss of the targeted protein(s). Compared to anti-sense RNA, ribozymes offer significant advantages by acting as site-specific nucleases [60]. In human kidney cells, we have used this approach for the first time to show a specific role for the $\beta_1\gamma_7$ dimer in coupling the β-adrenergic receptor to stimulation of AC [23,61]. Ongoing studies in mouse lung cells suggest a similarly selective requirement for γ_{11} subtype in control of cell growth. In both cases, these defects are linked to specific loss of the targeted protein(s).

Gene Targeting

More recently, we have begun analysis of mice or ES cells carrying targeted knockouts of the γ subunit genes to identify specific functions. Of the known γ subtypes, the γ_3 and γ_7 subtypes are closely related with respect to their primary structures and high expression levels in brain. Nevertheless, the γ_3 and γ_7 knockout mice exhibit two distinctive phenotypes, indicating they perform nonredundant roles in separate signaling pathways in the context of the whole animal. Collectively, the results of these reverse genetic approaches support the notion that receptors associate with particular combinations of G protein α and βγ subunits in the cell or whole animal setting; and that this provides a basis for the specificity of signaling through these pathways.

Conclusion

An intriguing, growing body of evidence suggests that polymorphisms in β_3 may be linked to disease [6]; and that sequestration of βγ may alter the course of diseases, including tumor invasiveness [62], vascular re-stenosis [63], and cardiac hypertrophy [64]. Thus, knowledge of which distinct subsets of βγ are involved in these processes will provide the rationale for more selective design of therapeutic strategies for these diseases.

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CHAPTER 225

The RGS Protein Superfamily

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Introduction

A large family of seven transmembrane–domain receptors for hormones, neurotransmitters, growth factors, chemoattractants, light, odorants, and other extracellular stimuli promote intracellular signaling responses by activation of heterotrimeric G proteins. Agonist-activated G protein-coupled receptors (GPCRs) selectively interact with one (or more) of approximately 20 different heterotrimeric G proteins, promoting exchange of GTP for bound GDP, dissociation of the G protein into GTP-bound Gα and free Gβγ subunits, and ensuing activation of downsteam effectors. Hydrolysis of GTP by GTPase activity intrinsic to each Gα subunit returns the heterotrimer to its ground state of Gα-GDP bound to Gβγ. Thus, signal amplitude of G protein-mediated responses is governed by the prevailing balance of receptor-promoted guanine nucleotide exchange and GTP hydrolysis.

Whereas activation of heterotrimeric G proteins by GPCRs has been understood in kinetic terms for almost two decades, understanding of the regulation of the deactivation process has accrued much more slowly. Purified G proteins hydrolyze GTP at a rate much lower than the very rapid deactivation of these proteins observed under physiological conditions. This discrepancy between the rates of GTP hydrolysis measured *in vitro* and *in vivo* presaged the existence of other classes of proteins that accelerate the deactivation of GTP-bound Gα-subunits. Observation of marked enhancement of the GTPase-activity of Gαq in the presence of its specific effector PLC-β1 [1] provided the first evidence for GTPase-activating proteins (GAPs) for heterotrimeric G proteins. More recently, a family of approximately 30 proteins was identified that contain a signature domain that selectively binds to GTP-bound Gα-subunits and stimulates their intrinsic GTPase activity up to several thousand-fold [2–8]. These "regulator of G protein signaling" (RGS) proteins promote the deactivation step in the heterotrimeric G protein regulatory cycle, catalyzing GTPase rates *in vitro* that are consistent with the rates of deactivation of G-protein signaling *in vivo*. Interaction of RGS proteins with GTP-bound Gα subunits confers to this large class of regulatory proteins the ability to modulate signaling response kinetics, amplitude, and specificity. The physiological functions of these proteins are currently under investigation and, in the case of many RGS proteins, include additional functionalities beyond their hallmark capacity to act as GAPs for Gα subunits.

The Signature RGS-Box as a Gα GAP

The defining feature of all RGS family proteins is an ~120 amino-acid region, the "RGS-box," that directly binds Gα-GTP subunits and markedly accelerates GTP hydrolysis. The RGS-box has a "modular" character like that of other recurrent protein functional modules such as PDZ (PSD-95/Dlg/ZO-1), PTB (phosphotyrosine-binding), and SH2 (Src homology-2) domains—that is, the minimal polypeptide sequence of the RGS-box is sufficient in isolation for full Gα-binding and GAP activity [9,10]. Soon after discovery of the RGS-box, Tesmer and colleagues [11] solved the first crystal structure of an RGS-box/Gα complex, namely RGS4 bound to the adenylyl cyclase-inhibitory Gα subunit Gαi1. The RGS-box of RGS4 folds as a compact bundle of nine-alpha helical segments [11]. Other structures of isolated RGS-boxes have since been solved by both NMR and X-ray diffraction methods [12–16], including a trimeric complex of RGS9 bound to both Gα-transducin (Gαt) and the gamma subunit of cGMP-phosphodiesterase [17]. Each of these

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RGS-boxes presents the same global fold comprising predominantly alpha-helical secondary structure.

The atomic-resolution structures of RGS-boxes in complex with Gα partners [11,17] indicate that the RGS-box primarily interacts with the conformationally flexible "switch" regions in the GTP-binding domain of Gα subunits. RGS-box residues do not directly take part in the chemistry of the GTP hydrolytic cycle. Rather, GTP hydrolysis traverses through a bipyramidal transition state, and the crystal structure of the RGS4/Gαi1 complex revealed Gαi1 to exist in this transition state [11]. Gα-GDP subunits bound with the planar ion aluminum tetrafluoride (AlF_4^-) mimic the transition state of GTP hydrolysis, and therefore most RGS proteins bind most avidly to GDP/AlF_4^- complexed Gα subunits over the GTPγS- or GDP-bound states (for example Fig. 1A; [18]).

The multidomain structure of many RGS proteins confers a complexity to these proteins that makes it difficult to define unambiguously their biological activities *in situ* or *in vivo*. Nonetheless, their signature activity exists in a capacity to act as GAPs for heterotrimeric G proteins, and quantitation of the GTPase activities of Gα subunits is directly possible *in vitro* and is also feasible by indirect means *in vivo*.

Single-Turnover Assays of GAP Activity

The GAP activity of RGS proteins can be quantitated reliably in single-turnover assays with most purified Gα subunits and RGS proteins [19]. Such measurements require the binding of [γ^{32}P]GTP to Gα in sufficient stoichiometry and under conditions that minimize GTP hydrolysis. This is usually accomplished by incubation of purified Gα with [γ^{32}P]GTP in the absence of Mg^{2+}, followed by removal of free [γ^{32}P]GTP by gel filtration. Since GTP hydrolysis is Mg^{2+}-dependent, addition of Mg^{2+} allows quantitation of a single cycle of GTP hydrolysis in the basal state of the Gα subunit. Moreover, concomitant addition of an RGS protein with Mg^{2+} provides assessment of the extent of RGS protein-promoted increase in hydrolytic activity (for example, Fig. 1B). The monoexponential time courses for release of [γ^{32}P]Pi under these conditions allow calculation of the first-order rate constant for catalysis (k_{cat}) of the intrinisic GTPase activity and the rate constant (k_{gap}) for GAP-promoted hydrolysis. Bimolecular Michaelis-Menten enzyme kinetics are followed under assay conditions in which Gα-GTP concentration is in excess of RGS protein concentration.

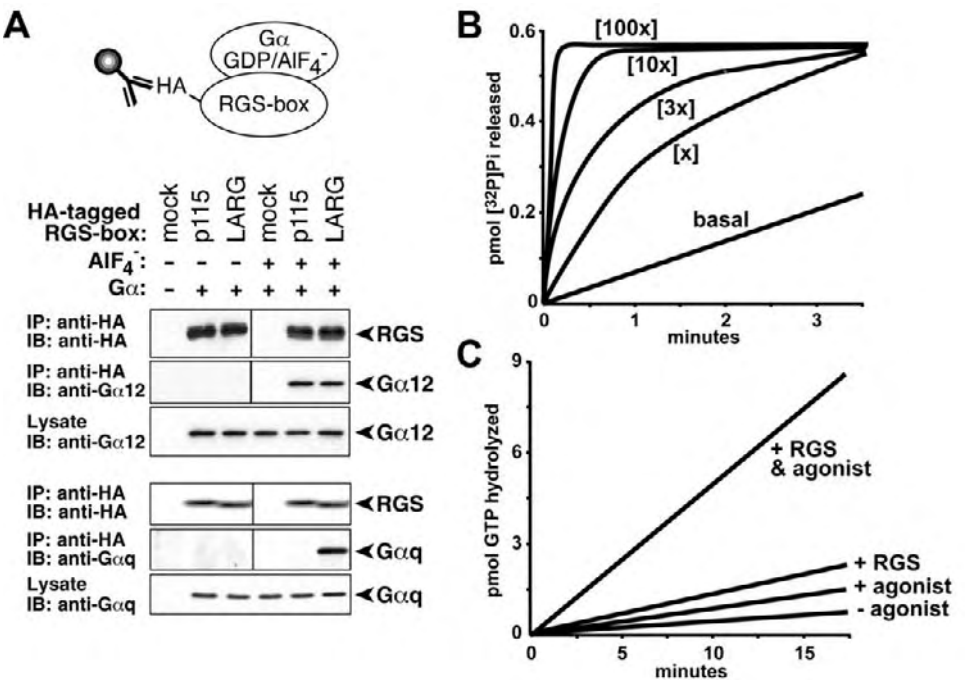


Figure 1 Representative functional data derived from assays of RGS-box activity. (A) Cellular co-transfection/co-immunoprecipitation assay to discern the Gα-binding specificity of hemagglutinin (HA)-epitope tagged RGS proteins is illustrated schematically above the immunoblot results. Binding of Gα subunits to the isolated RGS-boxes of p115-RhoGEF ("p115") or leukemia-associated RhoGEF ("LARG") is dependent on the addition to the cellular lysates of NaF and $AlCl_3$ (i.e., the components of aluminum tetrafluoride, AlF_4^-). IP, immunoprecipitation; IB, immunoblot. (B) Idealized results from a single-turnover assay measuring radioactive inorganic phosphate ([^{32}P]Pi) release (and, by inference, GTP hydrolysis) by [γ^{32}P]GTP-loaded Gα protein in the absence ("basal") or presence of increasing concentrations ([x] to [100x]) of RGS protein. (C) Idealized results from a steady-state GTP hydrolysis assay employing proteoliposomes reconstituted with purified GPCR, heterotrimeric G-protein Gα and Gβγ subunits, and incubated with [γ^{32}P]GTP in the absence or presence of agonist and/or RGS protein. As in part B, GTP hydrolysis activity is quantitated as the production of [^{32}P]Pi.

Such single-turnover GTPase assays are straightforward with Gα subunits that release GDP readily and therefore are readily labeled in solution by [γ^{32}P]GTP. Certain Gα subunits bind GDP tightly and are more difficult to label with GTP. This is particularly true with Gαq and Gα11. However, Ross and coworkers have experienced success in single-turnover assays with the Gαq mutant R183C [20], which exhibits reduced intrinsic GTPase activity and can thus be loaded (to relatively low stoichiometry) with [γ^{32}P]GTP by using prolonged incubations with [γ^{32}P]GTP.

A modification of the single-turnover assay takes advantage of the capacity of unlabeled Gα subunits to act as competitive inhibitors of the activity of RGS proteins against [γ^{32}P]GTP-bound Gα. That is, RGS protein-promoted increases in the rate of hydrolysis of [γ^{32}P]GTP-Gα are measured in the presence of AlF_4^- and a given GDP-bound Gα-subunit (for example, [21]). Interaction of an RGS protein with the transition state Gα (that is, GDP and AlF_4^- bound) will compete for the capacity of the RGS protein to act as a catalyst against the [γ^{32}P]GTP-bound Gα substrate. Thus, the selectivity of interaction of an RGS protein with a series of Gα subunits can be compared in measurements of their relative capacities to shift to the right the concentration effect curve for RGS protein-promoted release of [γ^{32}P]Pi.

Measurement of GTPase activity in single-turnover assays provides the most straightforward means of assessing RGS protein GAP activity. As discussed above, these assays are of limited use with Gα subunits that bind GDP tightly and are thus difficult to label with radioactive GTP. Since single-turnover GAP assays are carried out in solution, these measurements also ignore any role played by the lipid bilayer in concentrating an RGS protein in the milieu of its site of action. This is an increasingly important concern given the growing evidence that membrane-binding domains and important sites of posttranslational modification exist in many RGS proteins.

Steady-State Measurements of GAP Activity

GTPase activity of Gα subunits measured during multiple cycles of GTP hydrolysis depends on both GTP hydrolysis and guanine nucleotide exchange. Thus, steady-state GTPase activity can only be measured under assay conditions wherein GDP release and GTP binding are sufficiently rapid. In practice, it is the exchange rate that is usually rate-limiting, and increases in GTP catalysis by the presence of RGS proteins results in only small increases in measured GTPase activity (for example, Fig. 1C). Again, this also is particularly true for steady-state GTPase measurements with Gα subunits such as Gαq that bind GDP tightly in the basal state. In contrast, if guanine nucleotide exchange is promoted by GPCR activation, GTP hydrolysis now becomes rate-limiting and remarkably large increases in GTPase activity are observed due to the catalytic activity of RGS proteins (Fig. 1C). Indeed, up to 2000-fold stimulation of steady-state GTPase activity of Gαq has been observed in a system in which guanine nucleotide exchange was promoted by carbachol-mediated activation of the M1 muscarinic cholinergic receptor and GTP hydrolysis was accelerated by RGS4 [22].

Steady-state GTPase measurements have been effectively carried out with membrane preparations from various tissues [23]. Thus, activation of a native or heterologously expressed receptor with an appropriate agonist results in an increase in GTPase activity that may be enhanced by addition of a GAP for the involved G protein. Although such assays provide a readout for receptor activity, they are of limited value in assessing activities of RGS proteins per se. More elaborate assay systems have been developed that allow more quantitative analyses. For example, a useful test system is provided by heterologous expression of a GPCR in Sf9 insect cells (or a mammalian cell line) followed by membrane preparation and removal of endogenous G proteins by treatment with high concentrations of urea [24]. Purified G proteins then are reconstituted with the stripped membranes, and receptor-promoted G protein activation can be quantitated either by [^{35}S]GTPγS binding, which assesses guanine nucleotide exchange, or by steady-state GTPase assays. The activity of RGS proteins as GAPs for the reconstituted Gα subunit also can be assessed. Similarly, purified GPCRs and G proteins can be reconstituted in phospholipid vesicles so that steady-state GTPase activity can be measured as a function of receptor agonist and RGS protein concentration [22,25,26].

Steady-state GTPase activity of a Gα subunit under the concerted regulation by a GPCR and an RGS protein provides a complex signaling response that integrates many partial reactions. The work of Ross and coworkers in studying the GTPase activity of Gαq in proteolipomes reconstituted with the M1 muscarinic receptor, as a promoter of guanine nucleotide exchange, and PLC-β1, as a GAP for Gαq, provides an excellent example of the complexities that underlie such responses [27]. However, in its simplest application, such a system provides a reliable means to assess selectivity of RGS proteins (and other GAPs such as PLC-β isozymes) for Gα subunits. Thus, the relative activities of RGS proteins can be compared in a more physiologically relevant milieu of a lipid bilayer than is the case in single-turnover GAP assays in solution.

Heterologous overexpression of RGS proteins utilizing mammalian expression vectors results in attenuation of GPCR-promoted activation of signaling pathways. Although RGS protein-promoted inhibition of signaling through heterotrimeric G proteins is predictably a consequence of enhancing the GTPase activity of cellular Gα subunits, the physiological significance of such inhibition is often unclear. The Gα subunit selectivity of most RGS proteins *in vivo* is unknown and, under conditions of RGS protein overexpression, promiscuity of GAP activity against many, if not all, Gα subunits should be suspected. This may be a particular concern with overexpression of RGS protein constructs that include only the RGS-box. As discussed below, RGS proteins also exhibit many signaling modulatory activities, in addition to their Gα-GAP activity, that may influence their cellular actions. Moreover, certain RGS proteins directly

inhibit heterotrimeric G-protein-mediated signal transduction by other means. For example, RGS2 acts both as a GAP for GTP-bound Gαq and as an "effector antagonist"—that is, by inhibiting GTP-Gαq promoted stimulation of PLC-β [28].

Gα GAP and Other Signaling Regulatory Activities of RGS Family Members

The function of RGS proteins as multifaceted signaling regulators, rather than simply as Gα GAPs, is reflected in the observed structures of these proteins—the RGS-box that embodies GAP activity is rarely found in the absence of other regulatory protein modules (Fig. 2). Even for those RGS proteins in subfamilies A (RZ) and B (R4) that lack defined modules beyond the RGS-box, recent work in the field has discerned roles for non-RGS-box components in their subcellular localization and/or receptor-selective GAP activity, as outlined below.

A- or RZ-Subfamily (RGS17, RGS19, RGS20)

The founding member of the A- or RZ-subfamily, GAIP (G-alpha interacting protein, now known as RGS19), was the first mammalian RGS protein identified via the yeast two-hybrid system, with Gαi3 serving as the "bait" [2]. Additional family members, RGSZ1 (RGS20) and RGSZ2 (RGS17), were also isolated in yeast two-hybrid screens with Gαz and Gαo baits, respectively [29,30]; in addition, RGSZ1 was independently identified by purification and protein sequencing of a bovine brain Gαz-specific GAP activity [31]. It remains unclear whether the RGS-boxes of these RGS proteins exhibit strict selectivity toward Gαz or are indeed active *in vivo* as GAPs for other Gα subunits. PKC-mediated phosphorylation of Gαz has been reported to block RGS-box interaction [29,31].

All three subfamily members share a cysteine-rich cluster or "cysteine string" that is N-terminal of the RGS-box (Fig. 2). This polypeptide is believed to be heavily palmitoylated, leading to tight membrane association [32]. Other RGS proteins outside the RZ-family, such as RGS4, RGS7, RGS10, and RGS16, are thought to be targetted to the membrane by palmitoylation of N-terminal cysteine residues, although they lack a formal cysteine string [33–35].

B- or R4-Subfamily (RGS1, RGS2, RGS3, RGS4, RGS5, RGS8, RGS13, RGS16, RGS18)

The largest group of RGS proteins, the B- or R4-subfamily, comprise some of the smallest proteins that possess

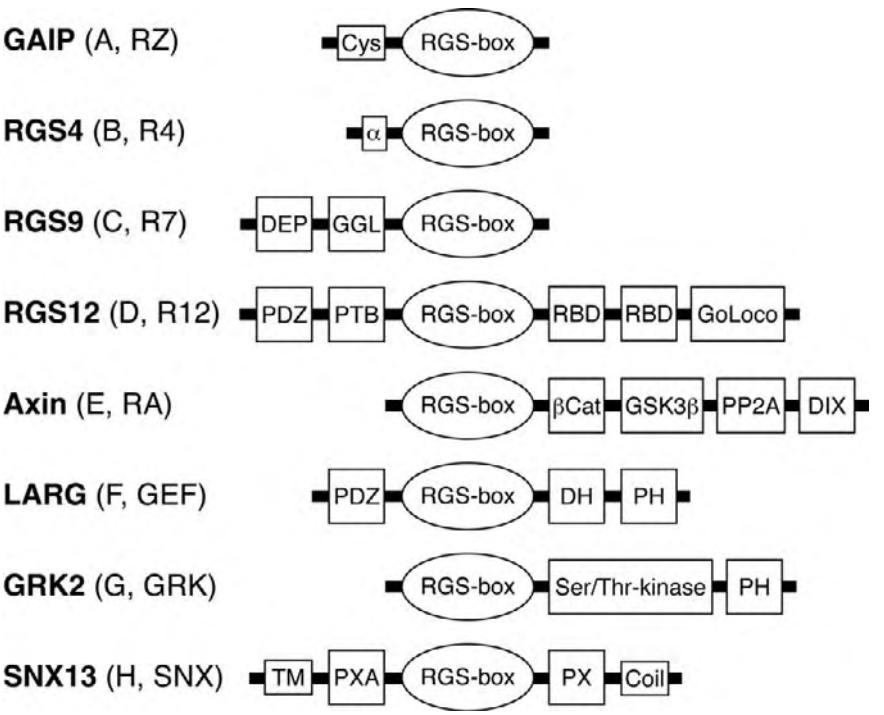


Figure 2 Protein domain architecture of representative members of the RGS protein subfamilies. Alternate nomenclatures for RGS protein subfamilies, as proposed independently by Zheng and Wilkie and coworkers [90,91], are denoted in brackets. Cys, cysteine-string motif; α, amphipathic alpha-helical region; DEP, dishevelled/Egl-10/pleckstrin domain; GGL, G-protein gamma subunit-like domain; PDZ, PSD-95/Dlg/ZO-1 domain; PTB, phosphotyrosine-binding domain; RBD, Ras-binding domain; GoLoco, Gαi/o-Loco interaction domain; βCat, β-catenin binding region; GSK3β, glycogen synthase kinase-3β binding region; PP2A, phosphatase PP2A binding region; DIX, domain of unknown function shared between Dishevelled and Axin proteins; DH, Dbl-homology domain; PH, pleckstrin-homology domain; Ser/Thr-kinase, protein kinase catalytic domain specific for serine and threonine residues; TM, putative transmembrane domain; PXA, "PX-associated" domain of unknown function; PX, Phox-homology domain; Coil, putative coiled-coil region.

RGS-boxes, with little in the way of non-RGS-box sequence.[1] Nevertheless, specific members of this subfamily exert receptor-selective inhibitory activity. Rat vascular smooth muscle cells, for example, express at least three R4-subfamily members capable of Gαq-directed GAP activity: RGS2, RGS3, and RGS5. However, ribozyme-mediated "knockdown" of the expression of each of these RGS proteins has a differential effect on cellular GPCR signaling coupled to Gαq activation. Neubig and colleagues [36] have observed that RGS3-ribozyme treatment selectively potentiates carbachol signaling via the M3 muscarinic receptor, whereas RGS5-ribozyme treatment only potentiates angiotensin II signaling via the AT1a receptor; RGS2 ribozyme treatment was without effect on either receptor signaling pathway.

Thus, determinants outside the RGS-box appear to play a role in targetting the Gα-GAP activity of these RGS proteins to particular receptors. In another example, RGS1, RGS4, and RGS16 exhibit large differences in their relative capacities to inhibit muscarinic-, bombesin-, or cholecystokinin-receptor-promoted Ca^{2+} signaling in permeabilized pancreatic acinar cells [37]. Since Ca^{2+} responses to all three GPCRs occur through Gq heterotrimers and all three RGS proteins exhibit similar activities *in vitro* as GAPs against Gαq, it was concluded that receptor-selective action of these RGS proteins contributes to their differential inhibitory activity *in vivo* [37]. Mutational analyses confirmed that selectivity did not involve the RGS-box per se, but rather, was contributed by sequences in a 33 amino-acid span of the RGS4 N-terminus [38]. This work has been extended by Wilkie, Muallem, and colleagues to propose a mechanism whereby RGS proteins play a central role in the Ca^{2+} oscillations that often accompany GPCR-promoted activation of phospholipase C [39]. Activation of Gq heterotrimers results in formation of GTP-Gαq, activation of phospholipase C-β, formation of $Ins(1,4,5)P_3$, and mobilization of intracellular Ca^{2+}. Gβγ also is released upon activation of Gq heterotrimers, which results in activation of phosphatidylinositol 3-kinase and formation of phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3 binds to and inhibits the GAP activity of RGS4 [40]. Since Ca^{2+}/calmodulin reverses this PIP_3- mediated inhibition, Wilkie and coworkers have proposed that competition between PIP_3 and Ca^{2+}/calmodulin for inhibition and disinhibition of RGS4 may provide a feedback regulation that accounts for Ca^{2+} oscillations occurring in the constant presence of GPCR-activating agonists [39]. Kurachi and coworkers [41] recently proposed a similar role for PIP_3, Ca^{2+}/calmodulin, and RGS4 in the voltage-dependent regulation of the activation state of cardiac G_i heterotrimers, which in turn regulates potassium channel activity in rat heart.

[1]RGS3, while clearly a member of the R4-subfamily by virtue of its RGS-box sequence, is an outlier with respect to the overall length of its polypeptide sequence. RGS3 isoforms with considerable N-terminal extensions are known to exist; one extended RGS3 isoform contains an N-terminal PDZ domain that binds the ephrin-B cytoplasmic tail and mediates "reverse signaling" through these transmembrane-tethered ligands of Eph receptors (Schmucker, D., and Zipursky, S.L. (2001). Signaling downstream of Eph receptors and ephrin ligands. *Cell* **105**, 701–704.)

C- or R7-Subfamily (RGS6, RGS7, RGS9, RGS11)

The central structural features of the C- or R7-subfamily of RGS proteins are DEP and GGL domains N-terminal to the RGS-box (Fig. 2). The DEP domain [42] is a conserved ~80 amino-acid sequence found in *D*ishevelled (an intracellular component of Wnt/Frizzled signaling), *E*GL-10 (the first RGS protein discovered in *Caenorhabditis elegans* [3]), and *P*leckstrin (a major PKC substrate in platelets). DEP domains are also found in an N-terminal tandem repeat within Sst2, the archetypal RGS protein of the yeast *Saccharomyces cerevisiae* [43]. The role of the DEP domain within RGS proteins is assumed to be membrane localization [3,44], but the intracellular target(s) of the DEP domain remain undefined. A recent report by Dohlman and colleagues suggests that the N-terminal DEP domains of Sst2 modulate the yeast stress response pathway [45].

The discovery of the GGL or "G-protein gamma subunit-like" domain, present within all members of the R7-subfamily [21,46–48], has led to a radical departure from previous assumptions regarding the coupling of heptahelical receptors to heterotrimeric G-protein complexes [49]. In the standard model of heterotrimer assembly, conventional Gγ subunits exist as short, isoprenylated, alpha-helical polypeptides that form obligate heterodimers with the conventional Gβ subunits Gβ1 through Gβ4 [50]. In contrast, the GGL domains of R7-RGS proteins serve as obligate binding partners for the more distantly-related Gβ subunit, Gβ5 [21,46–48], thus supplanting the requirement for a conventional Gγ subunit [49]. This novel GGL/Gβ5 association implies a role for R7-RGS proteins in signaling functions akin to those well-characterized for conventional Gβγ subunits—namely, functional coupling of Gα subunits to GPCRs and modulation of downstream effectors [51]. With respect to the latter activity, screens of conventional Gβγ effects (for example, modulation of phospholipase C-β and adenylyl cyclase activity) using recombinant Gβ5/R7-RGS protein dimers have, to date, failed to identify any clear-cut effector activity by GGL/Gβ5 dimers [21,52]. However, a role for GGL/Gβ5 dimers in facilitating receptor/Gα coupling is suggested by recent findings that the Gβ5/RGS9 heterodimer can support agonist-stimulated guanine nucleotide exchange on Gαo by recombinant M2-muscarinic acetylcholine receptors (M2-mAChR) in proteoliposome reconstitution assays (Harden, T.K. *et al.*, unpublished observations). Such receptor-coupling activity could help to explain the accelerated, rather than attenuated, kinetics of GIRK channel activation by M2-mAChRs observed upon co-expression of Gβ5 with RGS7 or RGS9 in a *Xenopus* oocyte reconstitution system [53].

The best-characterized R7-RGS protein is RGS9-1, the retinal-specific isoform of RGS9 that represents the long-sought GTPase-activating protein for Gαt within the phototransduction cascade [54]. Inactivation of the mouse *Rgs9* gene leads to a greatly slowed inactivation of photon-induced signaling in both rod and cone photoreceptors [55,56]. RGS6, RGS7, and RGS11 all have been shown *in vitro* to accelerate GTP hydrolysis by Gαo subunits [21,52,57],

but the physiological roles of these RGS/Gβ5 dimers remain to be elucidated.

D- or R12-Subfamily (RGS10, RGS12, RGS14)

All three of the D- or R12-subfamily RGS proteins, RGS10, RGS12, and RGS14, act as GAPs for Gαi-family Gα subunits *in vitro* [8, 58–60]. PKA-mediated phosphorylation of RGS10 at serine-168 attenuates its cellular function at the plasma membrane, not by reducing its intrinsic GAP activity, but by inducing its sequestration in the nucleus [61]. RGS10 belongs to the R12-subfamily given its RGS-box sequence similarity, but as a "small" RGS protein RGS10 is structurally similar to R4-subfamily members that lack discrete accessory modules.

In contrast, RGS12 and RGS14 share a multidomain organization C-terminal to the RGS-box that consists of tandem Ras-binding domains (RBDs) [62] and a GoLoco motif [63] (Fig. 2). Traver and colleagues [59] have identified the Ras-superfamily GTPases Rap1 and Rap2 as binding partners for the RBD region of RGS14; this interaction is dependent on the GTPases being in their GTP-bound or "activated" state, suggestive of a potential role for RGS14 as an effector for activated Rap proteins. The GoLoco motifs of RGS12 and RGS14 interact selectively with GDP-bound Gαi-family Gα subunits and prevent guanine nucleotide dissociation [60,64]. The X-ray crystal structure of the RGS14 GoLoco-motif region bound to Gαi1/GDP [65] indicates clear roles for both the Gα all-helical domain and polypeptide sequence C-terminal to the GoLoco motif in engendering binding specificity; guanine nucleotide dissociation inhibitor (GDI) activity is thought to result, in part, from direct contacts between a highly conserved arginine in the GoLoco motif and the phosphate groups of the bound GDP.

Unlike RGS14, RGS12 isoforms can also contain an N-terminal domain cassette consisting of a PDZ (PSD-95/Dlg/ZO-1) domain and a PTB (phosphotyrosine-binding) domain. The PDZ domain has a "group I" binding specificity (that is, C-terminal Ala/Ser-Thr-Xaa-Leu/Val motifs) and has been shown *in vitro* to interact with GPCR C-termini, including those of the interleukin-8 receptor CXCR2 [58] and the corticotrophin-releasing factor receptor CRF-R1 (D. P. Siderovski, *et al.*, unpublished observations). In chick dorsal root ganglia neurons, RGS12 controls desensitization of $GABA_B$-receptor coupling to calcium channel inhibition by virtue of its agonist-dependent recruitment to the phosphorylated N-type calcium channel via its PTB domain [66]. With the ability to bind phosphotyrosine-containing proteins, Gα subunits, and Ras superfamily GTPases, RGS12 appears to represent a signaling nexus that facilitates convergence and cross-regulation of receptor tyrosine-kinase, heterotrimeric G-protein, and Ras-superfamily GTPase signaling. Some as-yet undefined aspect of this cross-regulatory function must underlie the ability of RGS12 and RGS14 to inhibit G12/13-heterotrimer signaling; overexpression of either RGS12 or RGS14 is reported to block G12/13-dependent transcriptional readouts [67,68], yet neither RGS protein acts as a GAP for Gα12/13 subunits in solution-based assays [58,68].

E- or RA-Subfamily (Axin, Axil)

Axin ("*ax*is *in*hibition") and Axil ("*axi*n-*l*ike") proteins, negative regulators of Wnt signaling through Frizzled receptors, constitute the E- or RA-subfamily of RGS proteins. Axin and Axil are scaffold proteins that coordinately bind β-catenin, glycogen synthase kinase-3β (GSK3β), and APC (the *a*denomatous *p*olyposis *c*oli tumor-suppressor protein), facilitating the effects of GSK3β and APC on β-catenin destruction, which is antagonistic to Wnt-dependent transcriptional upregulation [69]. The RGS-boxes of Axin and Axil have, as yet, not been found to associate with Gα subunits; rather, the RGS-box represents the binding site for APC [70,71]. The crystal structure of Axin in complex with APC indicates that this interaction occurs on the opposing face of the RGS-box, leaving the Gα-interaction face unhindered [14]. Thus, the possibility exists that a Gα partner may yet be discovered for the RGS-boxes of Axin and Axil. Additional circumstantial evidence for the involvement of Gα subunits in the Wnt/Frizzled signaling pathway includes several reports suggesting that Frizzled receptors act as canonical GPCRs (for example, [72,73]) and the recent finding that activated Gα12 and Gα13 subunits can release β-catenin from cadherins [74].

F- or GEF-Subfamily (p115-RhoGEF, PDZ-RhoGEF, LARG)

The three members of the F- or GEF-subfamily (p115-RhoGEF/Lsc, PDZ-RhoGEF/GTRAP48, and leukemia-associated RhoGEF [LARG]) represent the clearest examples of RGS proteins as *positive* regulators of GPCR signaling—that is, as effectors coupling the activation of Gαq, Gα12, and Gα13 subunits to the activation of the small GTPase RhoA. All three RhoGEF proteins share a common structure of an N-terminal RGS-box and C-terminal Dbl-homology (DH) and pleckstrin-homology (PH) domains that collectively encode RhoA-specific guanine nucleotide exchange factor (GEF) activity [75]; the latter two proteins (PDZ-RhoGEF and LARG) also possess an N-terminal PDZ domain (Fig. 2). Kozasa and colleagues first demonstrated that the p115-RhoGEF RGS-box can accelerate GTP hydrolysis by Gα12 and Gα13 subunits [76]. Moreover, binding of activated Gα13, but not Gα12, to the RGS-box was shown to derepress the RhoGEF activity of the C-terminal DH/PH tandem [77]. LARG acts as a Gα-responsive RhoGEF not only for Gα12 and Gα13 subunits [78], but also for Gαq [18]. In addition, LARG associates with the C-terminal tail of the insulin-like growth factor (IGF)-1 receptor tyrosine kinase via its PDZ domain [79] and its RhoGEF activity can be modulated by tyrosine phosphorylation [80]. These recent findings suggest that LARG, and potentially other members of this subfamily, can participate in cross-talk between tyrosine kinase, GPCR, and monomeric GTPase signaling pathways.

G- or GRK-Subfamily (GRK1, GRK2, GRK3, GRK4, GRK5, GRK6, GRK7)

Among the original set of papers [2–5] heralding the discovery of the RGS superfamily, one report identified the conserved RGS-box sequence within a known family of GPCR signaling regulators, the G-protein-coupled receptor kinases or GRKs [4]. These serine/threonine kinases phosphorylate activated GPCRs, thereby allowing the binding of arrestin proteins, functional uncoupling from G proteins, and endocytosis of the phosphorylated receptor [81,82]. Three groups [83–85] have since shown that, at least for GRK2, the N-terminal RGS-box can act to inhibit Gαq signaling; this phosphorylation-independent inhibitory activity of GRK2 is thought not to result from RGS-mediated GAP activity (which is barely detectable *in vitro* [83]), but rather by sequestration of activated Gαq by the N-terminal RGS-box.

H- or SNX-Subfamily (SNX13, SNX14, SNX25)

The RGS-box sequence is also present in at least three of the sorting nexins or SNXs, a growing family of proteins involved in vesicular trafficking between cellular compartments [86]. Sorting nexins invariably contain Phox-homology (PX) domains that generally bind phosphatidylinositol-3-phosphate (PtdIns(3)P); the SNX-subfamily of RGS proteins also contain a poorly-defined PX-associated (PXA) domain N-terminal to the RGS-box and C-terminal to a putative transmembrane region (Fig. 2). The RGS-box of SNX13 was purported to have Gαs-directed GAP activity and inhibit Gαs-dependent signaling pathways upon overexpression [87], but the Gα specificities of the other two known subfamily members (SNX14 [88] and SNX25) have not been characterized to-date. SNX1, the founding member of the sorting nexins, is a binding partner of the epidermal growth factor receptor (EGF-R) and, upon overexpression, enhances EGF-R degradation [89]. Conversely, SNX13 overexpression delays EGF-R degradation [87]. A direct role for SNX13 or other SNX-subfamily RGS proteins in the trafficking of GPCRs and/or heterotrimeric G-protein subunits remains to be determined.

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CHAPTER 226

Mechanism of Gβγ Effector Interaction

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Introduction

The Gβγ subunit was initially recognized as an inactive subunit in G-protein-mediated signal transduction. Its main function was considered to form a high-affinity complex with GDP-bound Gα to generate an inactive heterotrimer. Only GTP-bound Gα subunits were thought to be responsible for the regulation of effectors. In 1987, Logothersis *et al.* presented the first clear evidence that βγ subunit free from Gα can activate K^+ channels in cardiac atrial cells [1]. Activated Gα subunit did not have an effect on K^+ channel activity. The following year, Whiteway *et al.* showed that βγ, but not Gα, is the responsible subunit to mediate mating-factor response in yeast [2]. Since then, various effectors or regulatory proteins have been identified as direct targets for the βγ subunit. It is now clear that receptor activation of G proteins generate two active signal mediators, GTP-bound Gα and free βγ subunit (reviewed in [3–5]).

Structure of βγ Subunits

cDNAs for five β subunits and 12 γ subunits have been isolated so far. Among five β subunits, β1, β2, β3, and β4 are highly homologous (about 80 percent identical to each other). β5 is distantly related (about 50 percent identical with other βs) and is expressed only in brain [6]. γ subunits are divergent in amino acid sequences and tissue distributions. The carboxyl terminus of the γ subunit contains a CAAX motif that directs prenylation of the molecule. Most of γ subunits are geranyl-geranylated except for γ1 and γ11, which are farnesylated. β1 to β4 subunits form a tightly complex with γs. They can only be separated under denaturing conditions. The interaction of β5 with γs is weak and sensitive to detergent condition [7].

The Gβ subunit is made up of two structurally distinct regions, an amino terminal α–helical segment, followed by seven WD-repeat sequences. The role of the WD repeat (about 40 amino acids in length) in cell signaling is not clearly understood, but many WD repeat-containing proteins participate in large macromolecular assemblies [8]. The crystal structures of βγ subunit, free or complexed with GDP-bound Gα, were solved recently [9–11]. The core WD repeat provides a rigid scaffold of a seven-bladed β-propeller structure. Each blade is made up of four anti-parallel β strands. The amino terminal region of γ forms an α-helical coiled-coil structure with the amino terminus of β. The rest of γ subunit extends along the wider bottom surface of the propeller through multiple interaction sites. Gα is mainly interacting with the opposite narrow surface of the propeller through its switch regions. The amino terminus of Gα is interacting with the outer strand of blade 2. It is important to note that the βγ subunit does not change its conformation when it dissociates from α subunit.

Effectors Interacting with βγ Subunits

GIRK Channel

G-protein-gated inwardly rectifying potassium channels (GIRKs) play an important role in regulating membrane excitability through G-protein-coupled receptors, especially in heart and brain [12, 13]. To date five mammalian cDNAs for GIRKs (GIRK1–5) have been isolated. GIRK functions as the heterotetramer, such as GIRK1 and 2 in heart and brain or GIRK1 and 4 in brain [12]. βγ subunits activate

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hetero- or homotetramer of GIRKs. It was shown that βγ subunits bind directly to the N- and C-terminal intracellular domains of GIRKs [14–16]. EC_{50} of β1γ2 for GIRK activation is about 30 nM [17,18]. βγ subunits with β1–β4 showed similar potency to activate GIRKs. It was shown recently that β5γ2 inhibited basal or agonist-induced GIRK1 and 4 activity by competing with β1γ2 [19]. The interaction of Giα with N-terminal region of GIRK was reported, but the regulatory function of this interaction is unknown [15].

PLCβ

Numerous G-protein-coupled receptors stimulate phosphoinositide phospholipaseCβ activity to induce intracellular Ca^{2+} release [20]. Both Gα subunits of Gq subfamily and βγ subunits can stimulate PLCβ activity *in vitro* and *in vivo*. Four isozymes of PLCβ are isolated, and all of them are activated by Gαq. The stimulation by βγ subunit was demonstrated for PLCβ1, β2, and β3 (with the potency of β3 > β2 > β1) but not for PLCβ4 [21–23]. Gqα and βγ directly bind to PLCβ with separate binding regions. In PLCβ2, the binding site for βγ has been mapped within the first half of the Y-domain of the catalytic region [24]. The stimulation of PLC activity by Gαq and βγ is additive for PLCβ3 but not for PLCβ2· [23,25]. The potency of βγ subunit to activate these PLCβs is much lower than Gαq. EC_{50} of βγ for PLCβ activation is 30–300 nM; in contrast, EC_{50} of Gαq is in the sub to low nanomolar range [23,25]. βγ-mediated PLCβ activation mainly contributes to pertussis-toxin sensitive PLC activation that is mediated through Gi/o. Recently, *in vitro* reconstitution experiments showed that β5γ2 could stimulate PLCβ1 and β2 but not PLCβ3 [26].

Adenylyl Cyclase

Isoforms of adenylyl cyclase (at least eight) are activated by Gαs and are differentially regulated by βγ subunits, Ca^{2+}, and phosphorylation [27]. AC-I is inhibited by βγ [28]. This inhibition by βγ is independent of Gαs stimulation. However, βγ activates AC-II and AC-IV synergistically with Gαs [28,29]. This activation of AC-II or AC-IV by βγ requires the activation by Gαs. Without Gαs stimulation, βγ has no effect on these adenylyl cyclases. Thus, in cells that are expressing AC-II or AC-IV, receptors that normally do not regulate cAMP production, such as receptors that couple to Gi or Gq, can further potentiate cAMP production. The site of βγ binding on adenylyl cyclase is not precisely known. For AC-I, the βγ binding region is separated from the Gαs binding region. The amino terminal part of the first large cytosolic domain of AC-I appears to include a βγ binding site [30]. For AC-II, a 14-amino acid peptide containing a QXXER motif derived from the second cytoplasmic domain blocks interaction with βγ· [31]. It is interesting to note that the same peptide can block interaction of βγ with several other effectors, including PLCβ and GIRK channels. In reconstitution experiments, β5γ2 was markedly less effective than β1γ2 to activate AC-II (EC_{50} of ~700 nM versus 25 nM) [7].

N- and P/Q-type Calcium Channels

It has been shown that several neurotransmitters inhibit N-type and P/Q type calcium channels through pertussis-toxin sensitive G proteins. This effect is mediated by the direct binding of βγ subunit to calcium channels [32,33]. The βγ binding site was mapped at the intracellular loop and the C terminus of alpha1 subunit [34,35]. QXXER motif in loop I was shown to be important for the modulation by βγ subunit [35].

PI3K

Phosphoinositide 3-kinase (PI3K) catalyzes phosphorylation of phosphoinositide at the 3 position and plays a key role in a variety of cellular functions. It is stimulated by both G-protein-coupled receptors and receptor tyrosine kinases. PI3Kγ is specifically activated through the direct interaction with βγ subunit [36,37]. This activation involves the interaction of βγ subunit with both p110 catalytic subunit and p101 regulatory subunit (EC_{50} about 20 nM). The presence of p101 enhances the sensitivity of PI3Kγ to βγ subunit [38]. β5γ2 does not activate PI3Kγ but can bind to the molecule [26]. PI3Kβ is synergistically activated by βγ subunit and phosphotyrosine-containing peptide [39]. PI3K α and δ are activated by tyrosine kinase pathway but not by G-protein-mediated pathway.

MAP Kinase Pathway

It has been shown that the yeast homologue of βγ subunit (STE4 and STE18) is the active mediator for the pheromone-induced MAP kinase pathway to control cell cycle [2]. STE4 interacts with serine-threonine kinase STE20/PAK and scafolding protein STE5 [40,41]. These interactions are critical to mediate pheromone-mediated MAP kinase activation. G-protein-linked pathways can also initiate MAP kinase pathways in mammalian cells [42]. Some of these pathways are pertussis toxin-sensitive and some are pertussis toxin-insensitive. βγ subunit released from Gi or Go appears to initiate Ras-dependent MAP kinase activation in pertussis toxin-sensitive pathways. It was shown that βγ could activate several MAP kinase pathways, including the JNK/SAPK pathway and p38MAPK pathway [43,44]. Although the involvement of PI3K or nonreceptor tyrosine kinase has been suggested as a downstream component of βγ to MAPK activation, the precise biochemical mechanism to link βγ and MAP kinase activation has not been clearly understood. The complex of β5γ2 cannot activate either the MAPK or JNK pathway [45].

Other Effectors and Interacting Proteins

Btk Family Tyrosine Kinases

Btk family tyrosine kinases are nonreceptor tyrosine kinases with a unique N-terminal extension that contain

PH and TH (Tec homology) domains. The regulation of these kinases in cell has not yet been well characterized. βγ subunit binds to the PH-TH domain and catalytic domains of Btk to stimulate the catalytic activity of the kinase [46]. Tsk, another member of this family, was also activated by co-transfection with βγ subunits [47].

PLCε

PLCε is a recently identified PI-PLC that is unique in that it contains a CDC25 domain and Ras-associating domains [48]. Involvement of Gα12 for the regulation of PLCε activity has been demonstrated. In addition, co-expression of β1γ2 but not β5γ2 with PLCε potently stimulated PLC activity [49]. Although an *in vitro* reconstitution experiment was unsuccessful, the βγ stimulatory effect on PLCε seems to be independent of Ras activation and PI3K activation, suggesting the direct stimulation by βγ.

GRK

βγ binds directly to the C-terminal region of the pleckstrin homology domain of β adrenergic receptor kinases (GRK2 and GRK3) and enhances their kinase activity [50,51]. It is not clearly understood whether βγ stimulates catalytic activity of GRKs or βγ facilitates membrane translocation of GRKs for activation. The C-terminal region of GRK2 can sequester βγ in cells without interacting with other α subunits. Thus, it is a useful tool to differentiate between βγ-mediated effects and Gα-mediated effects.

PHOSDUCIN

Phosducin is a phosphoprotein mainly expressed in retina or pineal gland. Phosducin regulates Gt-mediated phototransduction through high-affinity binding to βγ subunit and prevents the reassociation of βγ with Gαt [52]. Phosducin-like molecule has also been found in brain [53]. The crystal structure of phosducin and βγ complex revealed that phosducin interacts with the Gα binding surface of the βγ propeller and the outer surfaces of blades 1 and 7 [54]. The phosphorylation of phosducin inhibits its affinity to βγ. This phosphrylation site is not on the interacting surface on this structure, suggesting allosteric regulation of the interaction by phosphorylation.

In addition to these molecules, βγ subunits can also interact with other regulatory proteins such as Dbl, Raf-1 kinase, RasGEF, Rho family GTPases, or ARF [55–59]. The functional significance of these interactions is not clearly understood yet.

Specificity of the Interaction between βγ Subunit and Effectors

With multiple β and γ subunit cDNAs, various combinations of βγ subunit were tested to examine the specificity of their interaction with effectors. Most of them could form functional dimers. However, the degree of specificity in reconstitution assays was minimal in general. Most of βγ pairs showed almost the same potency and efficacy for multiple effectors. β1γ1, βγ for retinal transducin, showed lower potency for several effectors compared with other combination of βγs, while showing better interaction with rhodopsin and phosducin. It was also shown recently that β5, which is the most distantly related member of β subunits, differentially couples to several effectors; β5γ2 activates PLC β1 and β2 but not β3. β5γ2 does not activate GIRK, PI3Kγ, or MAP kinase pathways, although it can interact with GIRK or PI3Kγ [26,45]. In addition, several groups suggested that the physiological binding partner of β5 is not γ subunit but RGS proteins with a G-protein γ-subunit-like (GGL) domain (RGS6, 7, 9, and 11) [60,61]. Although the homologues of β5-RGS have a regulatory function in the egg-laying signaling pathway in *C. elegans,* the biochemical function of the β5-RGS complex in mammalian cells has not been understood yet [62].

βγ-effector interaction requires C-terminus prenylation of the γ subunit. Although lipid modification does not affect βγ subunit formation, it is essential for the interactions of βγs with effectors. It is likely that the difference of the lipid modification on γ1 (farnesyl) versus other γs (geranyl-geralyl) contributes to the difference of effector interaction mentioned above. Another common feature of βγ-effector interaction is that βγ has to dissociate from the heterotrimer in order to interact with effectors. This indicates that effectors share the top surface of βγ with the Gα subunit. This was confirmed by mutagenesis studies [63,64]. However, it also became evident that the different effectors overlap with the Gα surface in different ways.

The effector-interacting region of the β subunit is not restricted to the Gα interacting surface. The N-terminal α helical region of STE4 is involved in the interaction with STE20 to mediate phromone signaling [41]. It was also shown that the N-terminal 100 amino acid of β (including an α helical region and blade 1 and 2) interacts with GIRK1, AC-II, and PLCβ2 [65]. The importance of the C-terminal region of β for activation of PLC, AC-II, or MAP kinase path ways was also demonstrated [45,66,67]. The study with chimeras between β1 and β5 revealed that amino acids 52 to 143 of β1, which spans blade 2 and 3, is critical for the activation of GIRK [68]. The outer surface of the β-propeller also contributes to the effector interaction. The crystal structure revealed that the C-terminal part of phosducin interacts with the outer surface of blades 1 and 7 [54]. And a mutational study demonstrated that outer strands of blade 2 and 3 are differentially involved in GIRK or PLCβ2 activation [18,69].

It is now evident that βγ subunits of G protein play a variety of functions in cell signaling. In order to understand the physiological role of the βγ subunit, including subunit specificity, further investigation will be required using methods such as antisense RNA, RNAi, or gene knockout mice. Also, the determination of the crystal structure of the complex of βγ with different effectors will be critically important in understanding the effector activation mechanism by Gβγ subunits.

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CHAPTER 227

βγ Signaling in Chemotaxis

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Introduction

Chemotaxis, the ability of a cell to sense and move up a chemical gradient, is important in various physiological processes and is involved in many disease states. Signal transduction during chemotaxis occurs through seven-transmembrane receptors that are coupled to heterotrimeric G proteins. The βγ subunits transmit the signal downstream, and although the direct effectors are unclear, increases in phosphoinositide 3,4,5-trisphosphate and phosphatidylinositide 3,4-bisphosphate occur at the leading edge of the migrating cell. The formation of these lipids results in the recruitment of a variety of signaling proteins. Recent work has implicated phosphatidylinositol 3-kinase and the lipid phosphatases, PTEN and SHIP in chemotaxis. This review discusses the role of G proteins in chemotaxis of *Dictyostelium discoideum* and neutrophils, with an emphasis on the possible role of proteins involved in lipid production and degradation.

Directed movement of cells toward soluble attractants (chemotaxis) is critical in immunity, wound healing, embryogenesis, and neuron guidance. Also, abnormalities in chemotaxis have been implicated in disease states such as tumor metastasis, atherosclerosis, arthritis, asthma, and multiple sclerosis [1]. The cell biology of chemotaxis has primarily been studied in two cell types, the social amoeba *Dictyostelium discoideum* and mammalian leukocytes. Due to the genetic manipulations available, *D. discoideum* is an excellent model system in which to study chemotactic signaling. Many observations originally made in these simple eukaryotic cells have subsequently held true in mammalian cells such as neutrophils.

Chemotaxis in amoebae and leukocytes is mediated through a heterotrimeric G protein that couples to a seven-transmembrane receptor on the plasma membrane. βγ dimers transduce the chemotactic signals downstream [2,3] to many known effectors ([reviewed in [4]). Recent work suggests that phosphatidylinositol-3-kinase (PI3K) is a critical effector involved in chemotaxis. βγ-signaling results in the accumulation of the lipids phosphoinositide 3, 4, 5-trisphosphate [PtdIns(3,4,5)P_3] and phosphatidylinositide 3,4-bisphosphate [PtdIns(3,4)P_2] at the leading edge of the cell (see below). Other downstream events include increases in actin polymerization, changes in cell shape, Ca^{2+} mobilization, myosin phosphorylation, and activation of adenylyl and guanylyl cyclases (Table I).

Evidence That G Proteins Are Involved in Chemotaxis

G-protein subunits were first implicated in chemotaxis by the generation of mutants in *D. discoideum*. Amoebae have at least 11 Gα subunits but only one β subunit and one γ subunit [5,6]. Mutation of the Gα2 or the Gα4 subunit [7–10] blocks chemotaxis to cAMP or folic acid, respectively. All chemotactic responses are defective in amoebae lacking the β subunit [11]. In mammalian systems, the scenario is more complicated, with many G protein subunits, receptors, and ligands involved (Table I). Early experiments showed that leukocyte chemotaxis was inhibited by pertussis toxin, indicating that many chemokine receptors couple to Gα subunits from the Gαi family. Studies using receptors that couple to Gαi, Gαq, or Gαs transfected into HEK293 cells or a

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Table I Proteins Involved in Chemotaxis of Amoebae and Neutrophils

	D. discoideum	Neutrophils
Ligands	cAMP [28]	*Classes**
	folic acid [29]	chemokines [30]
	other nutrients	complement factors [31]
		N-formylated peptides [31]
		leukotrienes [32]
		lysophosphatidic acid [33]
		platelet-activating factor [34]
		prostaglandins [35]
Receptors (R)	cAR1 [36]	*Classes**
	cAR3 [37]	Chemokine receptors [39]
All are coupled to eterotrimeric G proteins	folic acid/pterin receptor [38]	Complement receptors
		N-formylated peptide R
		BLTR [40]
		Endothelial Differentiation Genes (Edg) [33]
		Platelet-Activating Factor R [40]
		Prostanoid R [35]
G proteins	Gα2, βγ (cAMP, [6,7,11])	Gαi family, βγ [2,3,12]
	Gα4, βγ (folic acid, [6,10,11])	
PI3Ks	PI3K1, PI3K2 [19,41,42]	PI3Kγ [21,22,23]
Signaling proteins found at leading edge	CRAC [14]	Akt/PKB [16]
	Akt/PKB [15]	Rac1 [43,44]
	PhdA [19]	PAK1 [45]
Signaling proteins on lateral edges or back of cell	Myosin II [46]	Myosin II [48]
	PAKa [47]	
	PTEN [25]	
Cell responses	↑ PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 [14,42]	↑ PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 [16]
	↑ actin polymerization	↑ actin polymerization
	↑ Ca++ [49]	↑ Ca++
	Myosin phosphorylation [50,51]	Myosin phosphorylation
	↑ cAMP [52]	↑ inositol phosphates [54]
	↑ cGMP [53]	↑ cAMP [55]

*Due to space limitations the large number of ligands and receptors involved in neutrophil chemotaxis have been grouped into classes.

pre-B cell line have confirmed that Gαi is involved in chemotaxis [2,3]. In these same studies, chemotaxis was blocked when the βγ sequestering proteins (βARK or Gαt, transducin) were overexpressed, suggesting that the release of βγ is necessary for chemotaxis [2,3]. By expressing a chimeric Gα (which is activated by Gαi-coupled receptors but cannot inhibit adenylyl cyclase), Neptune *et al.* definitively showed that release of βγ subunits, not Gαi activation, was important for mediating the chemotactic signal downstream [12]. G-protein signaling, specifically the release of βγ, is critical for chemotaxis in amoebae and neutrophils.

Recently, it has been demonstrated that the G-protein heterotrimer disassociates upon chemoattractant stimulation in living cells. Using amoebae expressing Gα2 fused to cyan (CFP) and β fused to yellow (YFP) fluorescent proteins, Janetopoulous *et al.* observed fluorescence resonance energy transfer (FRET), which was lost when the cells were exposed to chemoattractant [13]. Upon removal of ligand, the heterotrimer quickly reassociated as indicated by the reappearance of the FRET signal. An important finding is that in the presence of continual stimulation, the FRET signal did not rebound, indicating that the heterotrimer remains disassociated in the presence of ligand and does not undergo desensitization [13]. Thus the key events that lead to desensitization are downstream or independent of G-protein activation.

PI3Ks—Role in Chemotaxis?

G-protein signaling activates the lipid kinase phosphatidylinositol-3-kinase (PI3K), which results in PtdIns(3,4,5)P_3

and PtdIns(3,4)P_2 being created at the leading edge. Studies on cytosolic regulator of adenylyl cyclase (CRAC), a pleckstrin-homology (PH) domain-containing protein in amoebae, provided the first clue to the involvement of phosphorylated lipids in the signaling pathway [14]. A class of PH-domains has been shown to associate with the lipids PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3. CRAC (or just its PH domain) fused to green fluorescent protein (GFP) localized to the leading edge in chemotaxing amoebae [14], suggesting that these lipids are generated there. Subsequently, it has been shown that other PH-domain containing proteins, such as Akt/PKB, also localize to the leading edge in amoebae and neutrophils [15,16]. PI3K is undoubtedly involved in the formation of these lipids. However, studies using pharmacological inhibitors of PI3K, such as wortmannin and LY294002, have yielded conflicting results [17,18], with some studies showing effects on chemotaxis and others with no effect. *D. discoideum* have three genes encoding putative PI3Ks. Knockouts of these genes have been performed, and disruption of two of the PI3Ks result in impaired chemotaxis [19,20]. Disruption of all three PI3Ks in amoebae has not been completed. The conflicting genetic and pharmacological evidence indicates that the role of PI3K in chemotaxis is still unclear.

In mammalian systems, neutrophils from a PI3Kγ null mouse line have been evaluated. These neutrophils did not create detectable PtdIns(3,4,5)P_3 when stimulated with specific chemoattractants, suggesting that PI3Kγ is the sole PI3K that is coupled to chemoattractant receptors in these cells [21]. This phenotype could not be attributed to a global defect in G-protein signaling because other G-protein-mediated responses were functional. It is important that migration of the mutant neutrophils to chemokines *in vitro* (Boyden assay) and *in vivo* (infiltration assays) was reduced up to 50 percent [21–23], suggesting that PI3Kγ activity is required for proper chemotaxis.

Lipid Phosphatases, PTEN and SHIP

Since PI3K is involved in chemotaxis, one would predict that lipid phosphatases also play an important role. Previous work has suggested that the lipid phosphatase PTEN plays a negative role in chemotaxis. PTEN is a phosphatidylinositide 3-phosphatase. Fibroblasts from *pten*$^{-/-}$ mice exhibited faster motility and an increase in the number of pseudopods formed; however, directional sensing was not evaluated [24]. Recently, Iijima and Devreotes disrupted PTEN function in *D. discoideum* [25]. In the absence of chemoattractant, the mutant cells produced random pseudopods and some cells constitutively localized PH-GFP to the membrane [25]. This is consistent with PTEN playing a role in negatively regulating chemotaxis. However, the *pten*$^{-}$ cells do not undergo constitutive development, indicating that loss of PTEN is not sufficient to result in chemotaxis. In the presence of chemoattractant, *pten*$^{-}$ cells formed many pseudopods along the length of the cell, instead of only at the leading edge [25]. Therefore, the mutant cells took a slower, circuitous route up the gradient. Their hypothesis is that directional sensing requires a regulated balance between the activities of PI3K and PTEN [25].

Another lipid phosphatase, SHIP, which hydrolyzes the 5′-phosphate from PtdIns(3,4,5)P_3 and inositol 1,3,4,5-tetraphosphate seems to be involved in negatively regulating chemotactic signaling. Various hematopoietic cells from SHIP-deficient mice exhibited enhanced chemotaxis toward SDF-1 and B-lymphocyte chemoattractant (BLC) [26], but not to another chemokine, CKβ-ll. SHIP's role in chemotaxis may be dependent upon the cell type or chemokine involved. The enhanced chemotaxis of cells toward SDF-1 and BLC is consistent with the hypothesis that SHIP negatively regulates chemotaxis by hydrolyzing the PtdIns (3,4,5)P_3 created by PI3K. Consistent with this, decreasing the level of this lipid in B cells inhibited PKB/Akt activation [27]. Both lipid phosphatases PTEN and SHIP appear to be involved in chemotaxis signaling pathways.

Cells can sense and move up chemoattractant gradients that vary by less than 2 percent across their length. Understanding how cells sense and respond to these shallow gradients is important to understanding the basic mechanism of chemotaxis. Chemotaxis is mediated by G-protein βγ subunits and appears to be evolutionarily conserved from amoebae to mammalian neutrophils. Further study is necessary to determine the direct effector(s) of βγ and establish whether the key to directional sensing is in the regulation of the PtdIns levels in the migrating cell.

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CHAPTER 228

Reversible Palmitoylation in G-Protein Signaling

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Introduction

Numerous proteins involved in cellular signaling undergo reversible palmitoylation [1–3]. This review will focus on reversible palmitoylation of G-protein α subunits (Gα), briefly discuss some more general aspects of palmitoylation and depalmitoylation, and highlight recent evidence that many regulator of G protein signaling (RGS) proteins are palmitoylated.

Palmitoylation is a covalent lipid modification in which the saturated, 16-carbon fatty acid, palmitate, is linked through a thioester bond to a cysteine. Palmitoylation is often referred to as S-acylation or thioacylation to indicate that radiolabeled fatty acids of varying lengths can be incorporated into a protein. Very few studies have addressed the identity and stoichiometry of the endogenous fatty acid covalently attached to a particular cellular protein. In fact, it is mostly unknown if palmitoylated proteins exist as isoforms with different attached fatty acids, if specific cellular mechanisms exist to dictate a preference for palmitate, or if palmitate is primarily used merely because of its relatively high abundance in cells.

Much of the interest in palmitoylation stems from its dynamic nature and the resulting idea that this covalent modification is more than just a static membrane anchor. Numerous studies have demonstrated that palmitoylation is a reversible modification, i.e., attached palmitate often has a much shorter half-life than the protein it modifies. Such reversibility implies the potential for important regulatory roles for palmitoylation.

Sites of Palmitoylation in Gα and RGS Proteins

Besides the modified cysteine(s) itself, no primary amino acid consensus sequence has been identified for palmitoylation. The only consensus is that an additional hydrophobic modification or membrane-targeting motif is often a prerequisite for palmitoylation [1–4]. Table I lists identified sites of palmitoylation from representative Gα and RGS proteins. Based on lipid modification, Gα can be divided into two subfamilies (Table I)—ones that are palmitoylated only and ones that undergo both myristoylation and palmitoylation. Recently, it was demonstrated that the yeast *Saccharomyces cerevisiae* G-protein γ subunit is palmitoylated at a cysteine adjacent to the prenylated cysteine at the extreme C-terminus [5,6]. However, none of the 12 human Gγ subunits or the 2 Gγ of the model organism *Caenorhabditis elegans* contain a potential C-terminal palmitoylation site.

Palmitoylation has been identified in some members of the RGS family (Table I). Interestingly, palmitoylation of RGS proteins can occur at one or two cysteines within short N-terminal extensions [7,8], multiple cysteines in a cysteine string motif in the N-terminus [9], or at a cysteine within the RGS domain itself [10] (Table I). Additional palmitoylated proteins involved in G-protein signaling pathways include G-protein-coupled receptors (GPCR) [11], G-protein-coupled receptor kinases (GRK) [12], and small GTPases of the ras and rho superfamily [2,3].

Activation-Regulated Palmitoylation of Gα

If palmitoylation functions as a regulatory modification, then the expectation is that appropriate cellular stimuli will cause changes in the level of palmitoylation of a particular protein. Indeed, regulated changes in palmitoylation appear to be a general phenomenon for Gα [1]. Regulated palmitoylation was first demonstrated for α_s. Palmitate attached to

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Table I Palmitoylation of Gα and RGS proteins[a]

Gα N-termini (myristoylated and palmitoylated)[b]			
α_{i1}		M **G** *C* T L S A E D K A A V E R S K M I D	
α_{o1}		M **G** *C* T L S A E E R A A L E R S K A I E	
α_z		M **G** *C* R Q S S E E K E A A R R S R R I D	
Gpa1		M **G** *C* T V S T Q T I G D E S D P F L Q N	
Gα N-termini (non-myristoylated)[c]			
α_s		M G *C* L G N S K T E D Q R N E E D A Q R	
α_q		M T L E S I M A *C C* L S E E A K E A R R	
α_{14}		M A G *C C C* L S A E E K E S Q R I S A E	
α_{16}		M A R S L R W R *C C* P W *C* L T E D E K A	
α_{12}		M S G V V R T L S R *C* L L P A E A G A R	
α_{13}		M A D F L P S R S V L S V *C* F P G *C* V L	
RGS N-termini[d]			
RGS4		M *C* K G L A G L P A S *C* L R S A K D M K	
RGS16		M *C* R T L A T F P N T *C* L E R A K E F K	
RGS cysteine-string motif[e]			
RGS-GAIP	35	S R N P *C C* L *C* W *C C C C* S *C* S W N Q E	54
RGS box palmitoylation[f]			
RGS4	86	E E N I D F W I S *C* E E Y K K I K S P S	105
RGS10	57	E E N V L F W L A *C* E D F K K M Q D K T	76

[a]Palmitoylated (or potentially palmitoylated) cysteines are in italics.

[b]Myristoylated glycines at position 2 are in bold. Myristoylation of Gα has been well-reviewed [1–3].

[c]Potential sites of palmitoylation have not been addressed for α_{14} and α_{16}.

[d]Similar N-terminal sequence found in RGS5.

[e]Cysteine-string motifs are found in other members of the RZ sub-family [63].

[f]Cysteine present at similar position in RGS box of most RGS proteins.

α_s turns over much more rapidly after activation by the β-adrenergic receptor (β-AR) agonist isoproterenol, activation by cholera toxin, or activation by a constitutively activating mutation in α_s [13–15]. Time courses of palmitate incorporation and pulse-chase analyses are consistent with a model [4,16] in which activation leads to both more rapid depalmitoylation and more rapid subsequent repalmitoylation of α_s. For Gα other than α_s, a thorough recent study showed that agonist activation of stably transfected 5-HT_{1A} receptors in CHO cells resulted in increased palmitate turnover on endogenous α_i [17]. In addition, stimulation of gonadotropin-releasing hormone (GnRH) receptors in pituitary cells caused increased palmitate incorporation into α_s, α_i, and α_q [18,19], stimulation of m1-muscarinic receptors in transfected cells increases the rate of palmitate incorporation into α_q (author's unpublished observations), stimulation of GPCRs in isolated membranes has been shown to increase palmitoylation of α_i, α_o, α_s, and α_q [18,20,21], and agonist-regulated palmitoylation has been observed for α_s and α_q even when they are directly fused to GPCRs [22,23]. A similar model of regulated palmitoylation/depalmitoylation in response to agonist activation has been well described for GPCRs [11,24,25]. Regulated changes in palmitoylation have not yet been described for RGS proteins.

Mechanisms of Reversible Palmitoylation

Regulated palmitoylation implies that changes in a protein's palmitoylation state are carried out by regulation of the palmitoylation and/or depalmitoylation machinery in the cell or by changes in the accessibility of the palmitoylated protein substrate to constitutive palmitoylating and depalmitoylating activities. Although intensely studied by many investigators, surprisingly little is understood regarding relevant enzymes and cellular pathways that regulate reversible palmitoylation.

Palmitoyl Transferases

A major unresolved question is whether palmitoylation occurs enzymatically or nonenzymatically. This controversial topic has been well discussed in recent reviews [2,3]. Compelling arguments for nonenzymatic palmitoylation are based on observations of transfer of palmitate to specific and appropriate cysteines in an *in vitro* reaction containing only palmitoyl CoA and a purified protein substrate, such as Gα [26]. On the other hand, others have argued that under physiological conditions, where most of a cell's palmitoyl CoA is bound to acyl-CoA binding proteins (ACBP), nonenzymatic palmitoylation would occur too slowly to be significant [27,28].

More importantly, the first convincing demonstrations of enzymatic palmitoylation have been described recently. Two different palmitoyl acyltransferases (PAT) were purified from yeast. One is composed of two proteins termed Erf2p and Erf4p and palmitoylates yeast Ras *in vitro* [29], while the other, Akr1p, was shown to palmitoylate the casein kinase Yck2p [30]. Both Erf2p and Akr1p contain a conserved Asp-His-His-Cys cysteine-rich domain (DHHC-CRD), and mutation of conserved residues in this domain abolishes PAT activity [29,30]. A large family of proteins exists that contains the DHHC-CRD domain, and clearly much research in the near future will be directed toward testing the possibility that the DHHC-CRD proteins are a family of PATs and identifying the relevant PAT(s) for heterotrimeric G-protein α subunits.

Palmitoyl Thioesterases

Recently, a candidate for a physiologically relevant palmitoyl thioesterase, termed an acyl-protein thioesterase (APT-I), was described [31]. Overexpression of APT in cultured cells increased the basal rate of depalmitoylation of co-expressed α_s. Further studies to substantiate a role for APT-I in depalmitoylation of signaling proteins are eagerly awaited.

Proteins and/or Pathways That Regulate Reversible Palmitoylation

G-protein βγ subunits appear to regulate both palmitoylation and depalmitoylation of Gα. βγ promotes palmitoylation of Gα *in vitro* [32] and allows palmitoylation of a non-myristoylated (G2A) mutant of α_i or α_z in transfected cells [33,34]. α_s or α_q containing mutations in N-terminal βγ interaction sites display greatly decreased palmitoylation [35,36], while a mutant α_o that has an increased affinity for βγ is

palmitoylated to a higher level than α_o wild type [37]. One way in which βγ can enhance palmitoylation of Gα is by promoting membrane targeting. Consistent with this, palmitoylation, and plasma membrane (PM) localization, of a βγ-binding-deficient α_q is recovered when it is engineered to undergo myristoylation [35], and βγ-binding mutations in α_o, a subunit that is normally myristoylated *and* palmitoylated (Table I), do not affect α_o palmitoylation [37]. Although myristoylation and binding to βγ appear, in some cases, to function interchangeably as membrane-targeting signals for Gα, they may have additional, more specific roles in promoting palmitoylation [1,32,37].

βγ can also inhibit depalmitoylation of α_s. A mutant α_s that binds tightly to βγ is refractory to activation-induced rapid depalmitoylation in cultured cells [13,15], and purified βγ inhibits depalmitoylation of α_s when assayed in cell extracts [15] or when using purified proteins [31,38]. These results suggest that activation-induced depalmitoylation of Gα is mediated, at least in part, by its dissociation from βγ.

Although studies of palmitate turnover on Gα and GPCRs suggest that palmitoylation and depalmitoylation are tightly coupled, a recent study utilizing a β-AR-α_s fusion protein was the first to separate these two activities [22]. The authors showed that palmitoylation occurred at appropriate sites in both the β-AR and α_s portions of the fusion protein, and isoproterenol induced rapid depalmitoylation, as seen with the separate proteins. However, isoproterenol-induced palmitoylation, likely re-palmitoylation, was blocked. This result suggests that, after rapid depalmitoylation, repalmitoylation requires dissociation of the G protein from the GPCR and/or later events that do not occur normally with this fusion protein, such as desensitization or internalization.

Tools for Studying Reversible Palmitoylation

As expected from our lack of knowledge of the proteins involved in palmitoylation and depalmitoylation, a dearth of tools exists for inhibiting these activities. Recently, the palmitate analog 2-bromopalmitate was used to inhibit palmitoylation of signaling proteins [39]. Another group synthesized analogs of cerulenin, a natural product inhibitor of fatty acid synthesis, and identified compounds that inhibited palmitoylation of H-ras without inhibiting a fatty acid synthase [40]. This interesting result suggests that not only might cerulenin analogs provide a powerful tool for studying palmitoylation of various proteins, but they might also provide a handle for identifying an elusive palmitoyl transferase [41].

Functions of Reversible Palmitoylation

Plasma Membrane Localization

An obvious role for palmitoylation is to tether a protein to cellular membranes, and thus regulation of this modification would allow changes in a protein's subcellular localization, either by dissociation off a cellular membrane or transfer to different membrane domains. Palmitoylation appears to function, in a poorly understood manner, as a specific membrane-targeting device that specifies localization to PM, and in some cases specialized PM microdomains [1–3]. This role of palmitoylation has been well documented and discussed in terms of a two-signal model for PM localization of signaling proteins [1–4, 42–44].

Although the consensus is that palmitoylation plays a critical role in PM localization of many proteins, including the α_i subfamily [4,34,45,46] of Gα (Table I), there is a surprising disagreement regarding a role for palmitoylation in the non-myristoylated Gα (Table I). Due to the lack of tools for perturbing palmitoylation in cells, virtually all localization studies have relied on preventing palmitoylation by mutating the relevant cysteines to serines or alanines. Subcellular fractionation and immunofluorescence localization of cells transiently or stably expressing nonpalmitoylated cysteine mutants of α_s, α_q, α_{11}, or α_{13} have demonstrated that such mutants are cytoplasmic and soluble [47–50], and, recently, these analyses were extended by the demonstration that a GFP-tagged, nonpalmitoylated mutant of α_q is cytoplasmic in living cells [51]. Moreover, overexpression of βγ with palmitoylation-defective α_s or α_q cannot restore their PM localization [36], providing further support for the critical importance of palmitoylation. On the other hand, some researchers have observed that nonpalmitoylated mutants of α_s, α_q, or α_{12} remain in a particulate fraction after subcellular fractionation of transiently transfected cells [14,52–55]. However, it is important to note that in the latter cases [14,52–55] immunofluorescence localization of the mutants has not been reported, and thus it is unknown whether these mutants arrive correctly at the PM or are mistargeted to intracellular locations. Lastly, mutation of the N terminally palmitoylated cysteines in RGS4 and RGS16 (Table I) had little effect on their localization [7,8,56]. Possibly, additional membrane-targeting signals may be favored in different cells or experimental conditions.

Can Reversible Palmitoylation Regulate Changes in Subcellular Localization?

Although palmitoylation is reversible and plenty of evidence indicates that nonpalmitoylated mutants of various proteins are defective in PM localization and/or localization to PM microdomains, actually demonstrating a relationship between changes in a protein's palmitoylation and movement of that protein within a cell is a difficult problem. Nonetheless, α_s provides the best example of a correlation between reversible palmitoylation and reversible subcellular localization. As described above, activation of α_s induces rapid turnover of its attached palmitate. Similarly, activation of α_s, by GPCRs, cholera toxin, or a constitutively activating mutation, can promote its redistribution from PM to cytoplasm [48,57–61]. Significantly, β-AR-induced redistribution of α_s appears to follow a similar time course as β-AR-induced depalmitoylation of α_s [15,48,57]. In addition, replacement of the N-terminal single site of palmitoylation of α_s with other membrane-targeting motifs results in mutant α_s subunits that are unable to translocate from PM to

cytoplasm upon activation [62], consistent with depalmitoylation of a single palmitate playing a critical role in activation-induced subcellular redistribution of α_s. Others have speculated that regulated palmitoylation is more relevant for allowing reversible movement of Gα within PM microdomains [2,16,54]. Possibly, reversible palmitoylation could help to regulate the availability of G proteins at diverse subcellular locations.

Palmitoylation Affects RGS-Gα Interactions

One of the most interesting recent advances is evidence that palmitoylation of certain Gα influences their interaction with RGS proteins, and, vice versa, palmitoylation of certain RGS proteins affects their interactions with Gα. *In vitro*, palmitoylation of α_z or α_{i1} greatly inhibited their sensitivity to the GTP-hydrolysis-stimulating activity of several RGS proteins [63]. This result implies that reversible palmitoylation of Gα functions as a key switch to regulate the ability of RGS proteins to "turn-off" signaling.

Palmitoylation of RGS4 or RGS10 was shown to either inhibit or accelerate their ability to stimulate GTP hydrolysis of α_z or α_{i1} [10]. The positive or negative effect of palmitoylation differed depending upon the *in vitro* GAP assay used and whether palmitoylation occurred at N-terminal sites or cysteine sites in the conserved RGS box (Table I). Consistent with an important role for palmitoylation of RGS proteins, when nonpalmitoylated RGS16, containing N-terminal cysteine mutations (Table I), was expressed in cultured cells it failed to effectively inhibit signaling mediated by G_q or G_i [7]. It remains to be determined whether effects of palmitoylation on RGS-Gα functional interactions are mediated through direct binding or via binding to membranes, although for RGS4 *in vitro* lipid vesicle binding experiments are consistent with the proposal that palmitoylation, in addition to an N-terminal amphipathic helix, is important for membrane binding and properly orienting the RGS protein for optimal activity [64].

Conclusion

Palmitoylation plays an important role in membrane binding and regulating interactions of signaling proteins. Future challenges include defining cellular pathways and enzymes that regulate reversible palmitoylation, and understanding how changes in a protein's palmitoylation are translated into functional changes inside the cell.

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CHAPTER 229

G Proteins Mediating Taste Transduction

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Introduction

The sense of taste in humans is comprised of five primary qualities: sweet, bitter, sour, salty, and *umami* (the taste of glutamate) (reviewed in [1,2]). Taste sensation is initiated in primary taste sensory cells located in papillae at the surface of the tongue and in the soft palate. The papillae contain one to several taste buds, each comprising approximately 100 cells, including taste receptor cells (TRCs), precursor, and support cells [3]. TRCs are specialized epithelial cells that typically respond to one or more tastants by undergoing depolarization, elevating intracellular calcium, releasing a neurotransmitter, and activating the afferent neurons that they synapse upon.

Based on electrophysiological, genetic, and biochemical studies, several mechanisms have been identified for signal transduction by taste cells. For salty and sour compounds, the main signaling pathway is initiated by passive diffusion of ions through apical ion channels, amiloride-sensitive sodium channels, and acid-sensing channels [4–10]. For bitter, sweet, and *umami* compounds, the main signaling mechanisms depend on seven transmembrane-helix receptors, their coupled G proteins, and downstream effector enzymes (reviewed in [1,2]).

Various molecular techniques (e.g. molecular cloning, ribonuclease protection, RT-PCR, *in situ* hybridization, and immunohistochemistry) have shown that α-gustducin, $G\alpha_{i\text{-}2}$, $G\alpha_{i\text{-}3}$, $G\alpha_s$, $G\alpha_{14}$, $G\alpha_{15}$, α-transducin, Gαq, $G\beta_1$, $G\beta_3$, and $G\gamma_{13}$ are expressed in rodent TRCs [11–14].

α-Gustducin

α-Gustducin, a G-protein α subunit that shares 80 percent identity with rod α-transducin, is expressed in ~25 percent of TRCs [12] and has been implicated in responses of mice to bitter and sweet compounds [15]. Behavioral and nerve-recording studies showed that α-gustducin null mice have a marked reduction in their responses to the bitter compounds denatonium benzoate and quinine sulfate, and to the sweet compounds sucrose and SC45647, whereas their responses to salty and sour compounds were identical to those of their wild-type littermates [15].

Trypsin sensitivity and GTPγS binding assays showed that α-gustducin can be activated by native bovine taste receptors in the presence of a variety of bitter compounds [16]. Molecularly cloned bitter-responsive taste receptors (the T_{2r}/T_{rb} receptors) have been shown to couple to and activate heterotrimeric gustducin in preference to heterotrimers containing $G\alpha_s$, $G\alpha_i$, $G\alpha_o$, or $G\alpha_q$ [17]. Recombinant α-gustducin has been shown capable of activating retinal phosphodiesterase (PDE) [18]. Specific PDE subtypes (PDE1a) have been identified in bovine taste tissue that can be activated by rod α-transducin ([11]; Bakre *et al.* 2003 submitted) and by α-gustducin or a peptide derived from the "effector-interaction" region of α-gustducin ([19]; Lupi and R.F.M., unpublished). Rapid quench-flow experiments have shown that anti-α-gustducin antibodies block a decrease in cAMP and cGMP levels in taste tissues elicited

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by bitter compounds [20]. Based on the structural and biochemical similarity of α-gustducin and α-transducin, and the above observations, it appears that many bitter responses involve α-gustducin activation by heptahelical T2R/TRB taste receptors followed by α-gustducin activation of PDE1a in the taste cell.

α-Transducin

Rod α-transducin mRNA and protein has been shown to be expressed in rat taste tissue and TRCs [11,21]. The level of expression and the number of expressing cells were much lower than found with α-gustducin, suggesting a more limited role for α-transducin in taste signal transduction. The α subunit of cone transducin was also amplified by PCR from rat taste tissue RNA but was undetectable by ribonuclease protection, suggesting a very low level of expression and/or expression in only a small number of TRCs [11].

Rod α-transducin, like α-gustducin, can be activated *in vitro* by bitter-stimulated native bovine taste receptors [11,16]. A peptide that competitively inhibits activation of rod α-transducin by rhodopsin also inhibited activation of rod α-transducin by native taste receptors [11]. Taste PDE can be activated by aluminum fluoride-activated rod α-transducin or a peptide corresponding to the region of rod α-transducin that interacts with retinal PDE ([11]; Bakre *et al.* 2003 submitted). A small subset of frog taste cells respond to the sweeteners saccharin and NC-01 with the generation of an inward current [22]. Injection of a peptide derived from the effector-interaction region of rod α-transducin also induced this inward whole-cell current [22], suggesting that rod α-transducin, or the similar α-gustducin, might elicit this response *in vivo*.

To determine the role of rod α-transducin in taste signal transduction, behavioral tests with rod α-transducin single knockout mice and rod α-transducin/α-gustducin double knockout mice were carried out [23]. No differences were found in the responses to denatonium benzoate, quinine sulfate, sucrose, and SC45647 between rod α-transducin knockout and wild-type mice or between rod α-transducin/α-gustducin double knockout and α-gustducin single knockout mice. However, the preference responses to monosodium glutamate (MSG) of rod α-transducin/α-gustducin double knockout mice were diminished compared to those of wild-type and α-gustducin single knockout mice [23]. At concentrations of MSG that are preferred strongly by wild-type mice, the double knockout mice were indifferent. These data show that rod α-transducin and α-gustducin are involved in *umami* signaling, but rod α-transducin, in contrast to α-gustducin, does not appear to contribute to sweet or bitter signaling.

Other G Protein α Subunits

The behavioral and gustatory nerve responses of α-gustducin knockout mice to bitter and sweet compounds were reduced, but not totally abolished, indicating that other G proteins and/or pathways are involved. Transgenic expression in the α-gustducin lineage of TRCs of a dominant-negative α-gustducin mutant that can bind to taste receptors and Gβγ subunits, but cannot be activated by receptors, further reduced the residual responses to sweet and bitter compounds of α-gustducin knockout mice [24]. This dominant-negative transgene also reduced responses to bitter and sweet compounds when introduced into α-gustducin-positive mice, demonstrating its effectiveness as a dominant-negative competitor [24]. $G\alpha_{i\text{-}2}$, $G\alpha_{i\text{-}3}$, $G\alpha_{s}$, $G\alpha_{14}$, and $G\alpha_{15}$ are possible candidates to mediate the residual responses in α-gustducin knockout mice; clearly these residual responses are not mediated by rod α-transducin based on results from the double knockouts. The precise role in taste transduction of G protein α subunits other than α-transducin and α-gustducin remains to be clarified.

βγ Subunits

A novel Gγ subunit, Gγ13, was found by screening cDNA libraries from individual α-gustducin-expressing versus nonexpressing TRCs [25]. Immunohistochemistry showed co-expression of Gγ13 and α-gustducin. Southern blot analysis of RT-PCR products from individual TRCs showed co-expression of α-gustducin and Gγ13 with Gβ3 (19/19 cells) and Gβ1 (15/19 cells) [25]. It was subsequently determined that α-gustducin is expressed in most, but not all, TRCs that express Gγ13, and that the Gγ13-expressing TRCs also express phospholipase Cβ2 and the type III inositol trisphosphate (IP_3) receptor [26–28]. The trypsin-sensitivity assay showed that Gγ13 can interact with α-gustducin and that α-gustducin/Gβ1/Gγ13 complexes can be activated by native taste receptors in the presence of the bitter compound denatonium benzoate [25]. Many bitter compounds elicit an increase in taste tissue levels of IP_3 and diacyl glycerol (DAG); this response was entirely blocked by antibodies directed against Gγ13, Gβ3 or PLCβ2 [14,25,29], implicating all three of these proteins in mediating this taste response. Control antibodies or antibodies directed against α-gustducin had no effect on IP_3 or DAG generation, while antibodies directed against Gβ1 had a slight effect. Gβ3Gγ13 appears to be the likely partner of α-gustducin and like α-gustducin is involved in TRC responses to bitter compounds.

G-Protein-Coupled Receptors

The T2r/TrB family of taste receptors contains approximately 25 members [30,31]. One member of this family, mT2r5, was shown *in vitro* to be activated by cycloheximide and to couple specifically to gustducin [17]; presumably the other T2r/TrB receptors are also bitter-responsive taste receptors. It was shown by *in situ* hybridization that multiple T2r/TrB receptors are co-expressed in a subset of the

α-gustducin-expressing TRCs in mice and rats, indicating that most if not all T2r/TrB receptors are expressed in the same subset of TRCs and that they most likely couple to gustducin.

The T1rs constitute a three-member family of taste receptors expressed in TRCs [32–37]. T1r1 is predominantly expressed in TRCs of the fungiform and foliate papillae and palate, whereas T1r2 is expressed in the circumvallate and foliate papillae and palate, but not in the fungiform papillae [32]. T1r3 is expressed in both anterior and posterior papillae of the tongue as well as in the palate [36]. Calcium-imaging studies showed that HEK-293 cells expressing T1r3 and T1r2 responded to several sweeteners and D-amino-acids, whereas cells expressing T1r3 and T1r1 responded to L-amino-acids [36,38,39]. Transgenic expression of T1r3 from a highly sweet-preferring "taster" strain into the less sweet-preferring "nontaster" background converted the nontaster phenotype to that of the taster [36]. The G protein(s) that couple to these receptors are currently unknown, but probably include G_s for sugars based on biochemical and electrophysiological experiments, implicating adenylyl cyclase and cAMP in TRC responses to sugars.

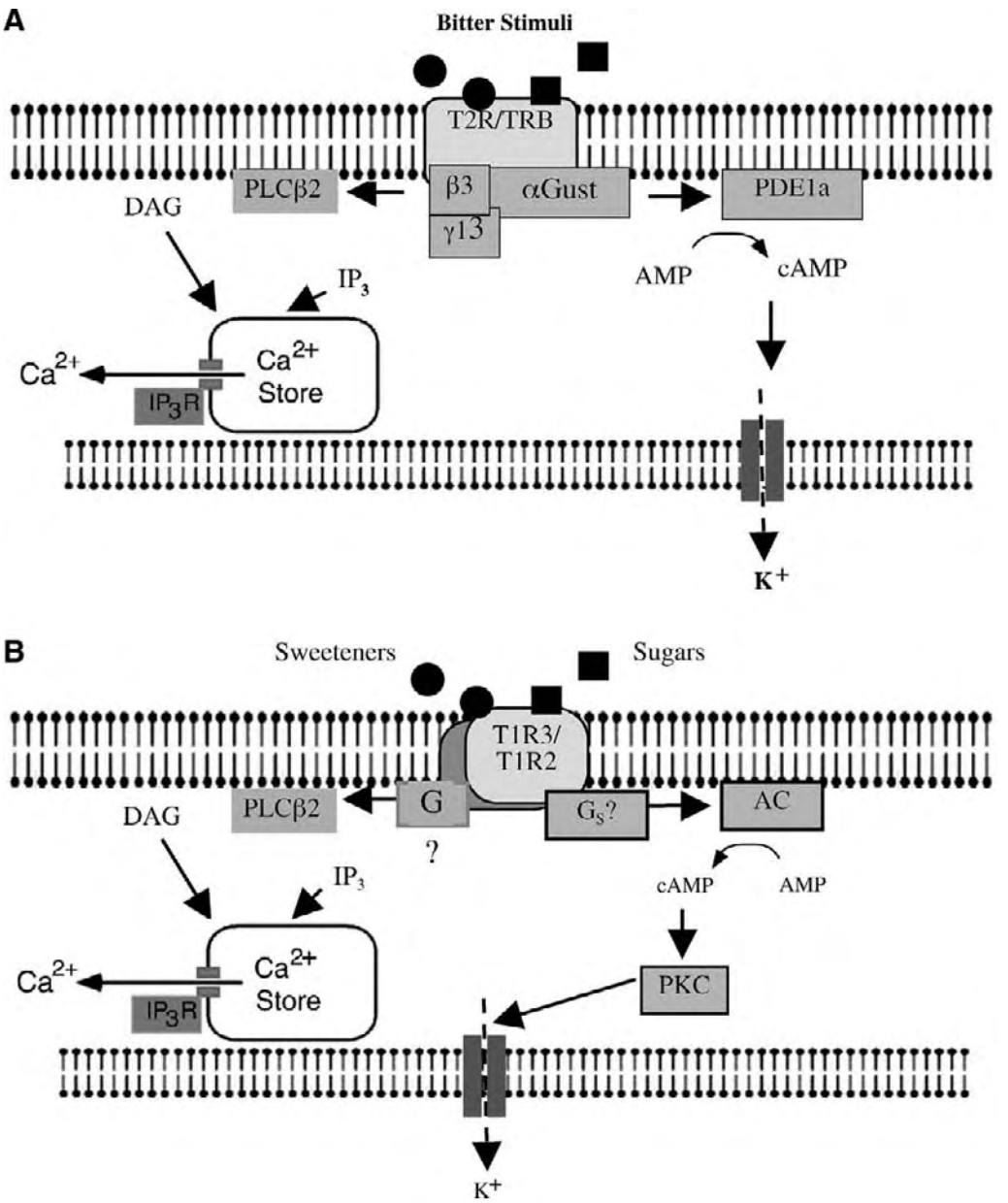


Figure 1 Schematic representation of the transduction pathways for bitter (A) and sweet (B) stimuli. One pathway, common to bitter compounds and artificial sweeteners, involves activation of PLCβ2, production of IP_3 and DAG, and release of Ca^{2+} from the ER. This pathway is activated by the β3γ13 subunits of gustducin for bitters and by unidentified G protein α and/or βγ subunits for sweeteners. The second main pathway for bitter compounds involves activation of PDE1a by the α subunit of gustducin and a drop in cAMP levels. For sugars the main pathway consists of activation of adenylyl cyclase (AC) by a G protein α-subunit, probably G_s, and a rise in cAMP.

Functional studies in heterologous cells, mRNA expression studies, and behavioral tests suggested that an mGluR4 variant, taste mGluR4, may function as an *umami* taste receptor [40,41]. It is currently not known whether taste mGluR4 couples to transducin and/or gustducin.

Second Messenger Pathways

The proposed main signaling pathways for bitter are

(1) T2r/TrB receptor → heterotrimeric gustducin → α-gustducin → activation of PDE1a → decrease in cAMP and cGMP → activation of a cyclic nucleotide suppressed cation channel → Ca^{2+} influx → neurotransmitter release;

(2) T2r/TrB receptor → heterotrimeric gustducin → βγ-gustducin (Gβ3/Gγ13) → PLC β2 → IP_3 + DAG → Ca^{2+} release from internal stores → neurotransmitter release (Figure 1a).

The two main pathways for sweetener signal transduction are believed to be

(1) T1r heterodimeric receptor → heterotrimeric G-protein (G_s?) → adenylyl cyclase → rise in [cAMP] → protein kinase phosphorlyation of K^+ channels → depolarization → Ca^{2+} influx → neurotransmitter release;

(2) T1r heterodimeric receptor→ heterotrimeric G protein →PLC β2 → IP_3 + DAG → Ca^{2+} release from internal stores → neurotransmitter release. Experiments using rat TRCs showed that natural sweeteners use the cAMP/Ca^{2+} influx pathway whereas artificial sweeteners use the IP_3 + DAG/Ca^{2+} release pathway [42] (Figure 1b).

For *umami* signaling, biochemical experiments have implicated both a rise and a drop in [cAMP] and a rise in [IP_3] [43].

Conclusion

Heterotrimeric gustducin plays a central role in the signal transduction of bitter compounds by coupling to T2r/TrB receptors and activating PDE and PLC β2. It also plays a central role in the signal transduction of sweeteners, although its coupling to specific taste receptors has not been experimentally demonstrated. The transduction of *umami* signals appears to use both gustducin and transducin. The role of the other G proteins expressed in taste tissue is still unclear. The expression of these G proteins in a wide range of tissues limits the usefulness of the conventional mouse knockout as a tool for elucidating their role in taste transduction. Many answers will probably come from taste tissue specific knockouts using the Cre/LoxP system.

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CHAPTER 230

Regulation of Synaptic Fusion by Heterotrimeric G Proteins

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Introduction

Exocytotic release of a neurotransmitter is necessary for interneuronal communication. In neurons, exocytosis, or the fusion of a synaptic vesicle with a specialized area in the plasma membrane, is a tightly regulated process that must be activated with strict temporal control. Ca^{2+} entry to the terminal is the trigger for exocytosis and neurotransmitter release, but the release of neurotransmitter evoked by Ca^{2+} is modified by receptors located at the presynaptic terminal. These receptors include G-protein-coupled receptors (GPCRs).

The Vesicle Fusion Machinery

To understand how G proteins modulate the release of neurotransmitter we must understand some basic principles of exocytosis. The best-characterized proteins involved in exocytotic fusion are those that comprise the core complex, the formation of which is absolutely necessary for fusion to occur. The core complex, or SNARE (soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor), is a bundle of four α-helices, approximately 65 amino acids in length, which is thought to bridge the synaptic vesicle and plasma membranes [1]. These α-helices are donated by three different proteins; a family member from the syntaxin and SNAP-25 families, both located in the synaptic active zone, and a VAMP (vesicle associated membrane protein, also known as synaptobrevin) family member, located in the synaptic vesicular membrane. The syntaxin family consists of integral membrane proteins around 300 amino acids in length, which have been shown to bind to many regulatory proteins [2]. Of these regulatory proteins, synaptotagmin is the most likely candidate for the Ca^{2+} sensor in synchronous release of neurotransmitter [3,4] The core complex is sufficient to mediate fusion of lipid micelles *in vitro* [5], and fusion of the synaptic vesicle with the plasma membrane requires the interaction of syntaxin, SNAP-25, and VAMP.

Vesicles containing neurotransmitter must be able to fuse with the plasma membrane in microsecond time scales. Consequently, synaptic vesicles are located very near the point of fusion, the active zone, at the presynaptic terminal. This organization is termed *docking*. Vesicular recruitment and docking requires the actin assembly network and ATP [6]. Multistep fusion reactions are ruled out due to the speed of release. For this reason, it is believed that there is a pool of ready-to-fuse synaptic vesicles that have undergone a further maturation step referred to as *priming* [7]. Priming in large dense-core vesicle requires ATP, submicromolar Ca^{2+} concentrations, and alterations in membrane lipids by lipid transferases and kinases [8]. Furthermore, the possible role in priming of the N-ethylmaleimide-sensitive factor (NSF) in synaptic vesicles may indicate similar requirements [9]. Evoked synaptic fusion is thought to require high (hundreds of μM) local concentrations of Ca^{2+} [10] following action potential invasion of the nerve terminal, although more recent work suggests that low μM increases in Ca^{2+} concentrations (~ 10 μM) may activate fusion in some neurons [11].

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G Protein-Coupled Receptor Mediated Modulation at the Presynaptic Terminal

Regulation of neurotransmitter release at the presynaptic terminal plays an important part in the plasticity of the nervous system [12]. Various neurotransmitters modulate release from presynaptic terminals, and many of these interactions involve the activation of a GPCR [13]. Modulation of exocytotic release by GPCRs is an important mechanism by which neurons are able to respond and adapt to changes in secretory requirements. Some GPCRs may couple to more than one G protein, while others show a great deal of specificity. Gβγ binding to Gα involves widespread contacts at two distinct interfaces. Following activation by a GPCR, the heterotrimeric G protein dissociates into an activated GαGTP subunit and a free Gβγ subunit [14]. Active Gα-GTP and free Gβγ may then activate many different signaling pathways [15].

Uncertainty over the mechanisms by which G proteins alter neurotransmitter release in part reflects the variety of G-protein effector targets and the difficulties in gaining experimental access to these small structures. Thus, most molecular studies of the detailed mechanisms come from either transfection of the relevant proteins into cultured cell lines and *Xenopus* oocytes or from electrophysiological measurements from neuronal cell bodies.

Possible Mechanisms of Presynaptic Inhibition by G Proteins

GPCRs that inhibit neurotransmitter release have perhaps been the mostly widely studied modulators of synaptic transmission. A consensus mechanism by which these transmitters may modulate synaptic transmitter release has been hypothesized to involve an alteration in action potential evoked Ca^{2+} entry to the presynaptic terminal.

1. This reduction in Ca^{2+} entry could occur by a direct action of G protein βγ subunit on the gating of voltage gated Ca^{2+} channels (VGCCs) [16,17]. If Gβγ inhibits VGCCs, less Ca^{2+} would enter the presynaptic terminal, and since neurotransmitter release is Ca^{2+} dependent, less neurotransmitter would be released. GPCR-mediated inhibition of release via a direct inhibition of VGCCs has been demonstrated at one presynaptic terminal [18] through multiple GPCRs [19,20]. Although, given the large number of neurotransmitters that inhibit release, many of which have very little effect on Ca^{2+} entry through VGCCs [20], it is unlikely that this is the only pathway involved in GPCR mediated inhibition at this terminal.

2. If G protein-coupled inwardly rectifying K^+ channels (GIRKs) were located at presynaptic terminals, activation by Gβγ could modulate action potential amplitudes, allowing fewer Ca^{2+} channels to open. Gβγ activates GIRKs in neuronal cell bodiestransfected cell lines, and *Xenopus* oocytes [21,22]. Although GIRKs have been histochemically localized to presynaptic terminals [23], physiological evidence for their presynaptic action is lacking. These channels are believed to be more important for postsynaptic modulation [24]. GPCR-mediated inhibition of voltage-gated K^+ channels at an autaptic presynaptic terminal has also been shown [25] to occur through activation of dopamine receptors, although it is not clear what G protein subunit, Gα or Gβγ, is responsible. At the reticulospinal-motoneuron synapse of the lamprey, both glutamate and 5-HT activate GPCRs, which modulate a K^+ current, although the channel subtype and G-protein subunit involved are unknown [26,27].

3. It has been suggested that G proteins may modulate voltage-gated Na^+ channels at the presynaptic terminal; however, no direct evidence has yet been presented [28]. Modulation of Na^+ channels could also indirectly affect the entry of Ca^{2+} into the presynaptic terminal.

These studies led to the idea that Gβγ-mediated inhibition of neurotransmission at the presynaptic terminal was through a direct or indirect effect on the amount of Ca^{2+} that enters the terminal during the action potential. However, there is growing evidence that Gβγ may also inhibit synaptic transmission by modulation distal to the point of Ca^{2+} entry. The ability of G proteins to inhibit neurotransmitter release by directly targeting the release apparatus was first demonstrated by Silinsky [29] in the neuromuscular junction. Spontaneous exocytotic events, where exocytosis occurs independently of Ca^{2+} entry, can be detected by recording miniature excitatory/inhibitory post-synaptic currents (mE/IPSCs). Measurements of mE/IPSCs allow the exocytotic modulatory processes that occur independently of Ca^{2+} entry to be isolated and studied, unlike evoked EPSCs. These mE/IPSCs have been shown to be regulated by many GPCRs [30].

In support of the hypothesis that G proteins inhibit vesicle fusion directly, G proteins can inhibit exocytosis after cell permeabilization, suggesting a role late in the exocytotic event [31]. In addition, exocytotic processes in pancreatic β cells, peritoneal mast cells, chromaffin cells, PC12 cells, and secretory granules are regulated independently of Ca^{2+} entry by G proteins [32]. Gβγ has been shown to interact directly with the fusion machinery in rat mast cells [33]. In the lamprey giant synapse 5-HT-mediated synaptic inhibition does not cause a reduction in Ca^{2+} entry to the synapse [27]. Furthermore, the actions of 5-HT at a GPCR are abolished by intracellular block of activated Gβγ [34]. A mechanism for a direct interaction between Gβγ and the core vesicle fusion machinery is suggested by the finding that Gβγ directly binds SNARE proteins syntaxin and SNAP-25 [34,35] as well as the cysteine string protein (CSP) [36].

Presynaptic Ca^{2+} Stores and Modulation of Neurotransmitter Release

Ca^{2+} release from internal stores located at the presynaptic terminal may lead to an enhancement of transmitter release. However, this Ca^{2+} may originate either from Ca^{2+} activated channels (that is, Ca^{2+}-induced Ca^{2+} release; CICRs) [37] or

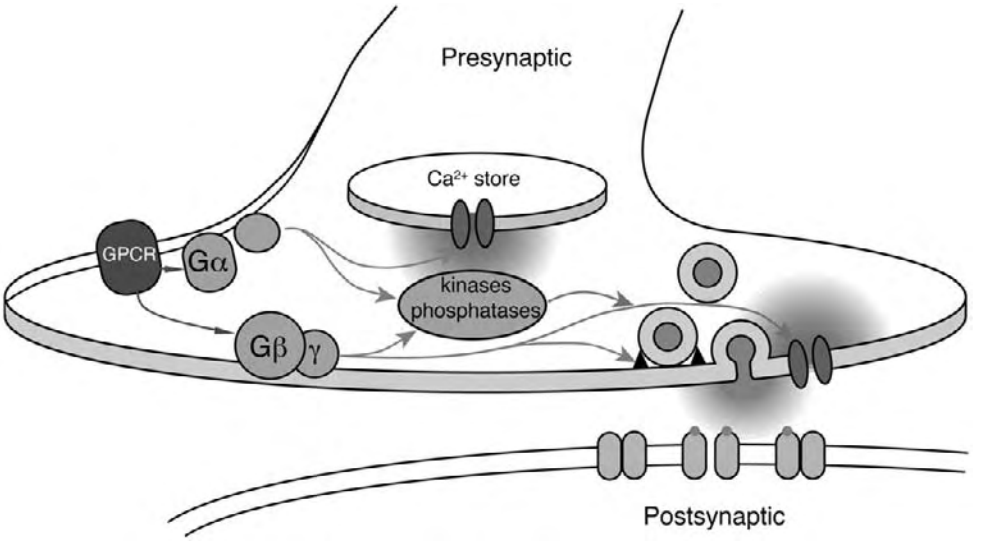


Figure 1 GPCRs at the presynaptic terminal (blue) may modulate transmission through either Gα or Gβγ. These pathways (green) may in turn alter the phosphorylation state of presynaptic proteins, cause the release of Ca^{2+} from presynaptic internal stores, or alter release through interactions with the release machinery or through actions at presynaptic ion channels.

following the activation of a presynaptic GPCR, leading to IP3 production and activation of presynaptic IP3 receptors [26,38]. It is also important to note that the activation of presynaptic receptors leading to IP3 production will also produce diacylglycerol in the nerve terminal, leading to the possible activation of PKC or direct effects on proteins associated with the release machinery, for example UNC13 [39].

G Proteins and Phosphorylation

G proteins may alter the efficacy of synaptic transmission either through phosphorylation or dephosphorylation of presynaptic components. Gβγ has been suggested to activate the calcineurin phosphatase pathway to inhibit release independent of Ca^{2+} entry [40] in neuroendocrine cells. In addition, the activation of both PKA and PKC have been implicated in the enhancement of synaptic transmission in the central nervous system. Indeed, tonic activation of PKA may be necessary for vesicle fusion to occur [41,42] and PKA phosphorylates CSP to alter its binding to syntaxin [43]. Metabotropic glutamate receptors in the mammalian CNS may activate either of these latter pathways [44,45].

Although relatively difficult to study, the presynaptic terminal may contain as rich an array of receptor-mediated mechanisms that modify information flow as has been identified at the postsynaptic side of the synapse. Figure 1 demonstrates some of the possible mechanisms by which presynaptic receptors may alter neurotransmitter release.

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CHAPTER 231

G Protein Regulation of Channels

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Stimulation of G-protein coupled receptor (GPCR) facilitates GTP exchange for GDP to Gα, leading to dissociation of $G\alpha^{GTP}$ from the Gβγ dimer and regulation of their effectors [1]. Ion channels as effectors allow neurotransmitters or hormones to elicit electric activity [2]. Measurements of ions that flow through the open channel pore by electrophysiological methods enable direct determination of the activity of a single channel protein at millisecond resolution *in vivo*. Different configurations of patch-clamp recording make it possible to control the composition of solutions on either side of the membrane.

There are numerous examples for modulation of channel activity by indirect means involving phosphorylation, second messengers (Ca^{2+}, cAMP), and regulators of G-protein activity such as RGS proteins [3]. In this chapter we will discuss modulation of channels due to their direct interaction with G proteins, and focus on the G-protein-gated inward rectifying potassium channels Kir3 (GIRK) [4], and the voltage-gated calcium channels [5,6]. This review is not comprehensive, as direct interaction of G proteins with other ion channels have been reported [7–10].

Interaction with K^+ Channels

The GIRK Channels

Opening of K channels allows outward flow of potassium ions down the concentration gradient, causing hyperpolarization. This way, the activation of GIRK channels by transmitter leads to calming of the heart rate and slowing of neuronal activity [4,11,12]. GIRK channels are tetramers of pore-lining subunits containing two transmembrane domains (TM1&2), and cytosolic N and C termini. The mammalian GIRK family has four members. They differ in the ability to form homotetrameric channels [13] and the traffic signals they possess [14]. Whereas midbrain dopamine neurons express GIRK2 channels, the brain and cortex contain primarily heterotetramers of GIRK1 and GIRK2 and the heart contains primarily GIRK1and GIRK4 [15].

GIRK channels have low basal activity, and are activated by GPCR, due to their direct binding to the βγ subunit of the G protein [16,17]. Mutations in the transmembrane domain may rescue mutant GIRK channels constitutively active in the absence of Gβγ, indicating that channel gating triggered by Gβγ binding is likely to involve conformational changes within the membrane [18,19].

The Gβγ Interacting Domain of GIRK

Almost all Gβγ combinations can activate GIRK [17]. Two-hybrid analysis showed interaction between the Gβ1 and the N-terminal domain of GIRK1 [20], but only Gβγ dimers can activate GIRK channels [21]. A recent study compared Gβ1 with Gβ5, which does not activated GIRK, and identified Gβ1 residues (S67, S98, and T128) important for basal activity [22]. Another study showed the importance of residues close or within the Gα binding interface of Gβ indicating a possible Gα competition with GIRK [1].

Direct binding of Gβγ to both the N terminal (amino acid 34–86) and C terminal (amino acid 273–462) domains of GIRK1 shown by *in vitro* binding assay may be reduced by peptides from those GIRK1 domains, which also partially inhibit channel activation [23]. Peptides from GIRK4 C-terminal domain also affected Gβγ binding and channel activation [24]. A chimerical Kir2 channel gained sensitivity to Gβγ by introduction of the two GIRK1 termini [25]. Further experiments have localized Gβγ binding region in the

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N- and C-terminal domains of GIRK1-4 [26]. Similar Gβγ activation has been shown for functional homotetramers of GIRK1 (F137S) and GIRK4 (S143T) [13]. Consistent with these findings, crucial GIRK1 (H57, L262) and GIRK4 (H64, L268) residues for Gβγ interaction have been identified at homologous sites [27].

Coupling of GIRK Activation to Specific Receptors

Remarkably, GIRK channels are activated specifically by Gi/o protein-coupled receptors *in vivo* and in mammalian HEK293 cells, even though GIRK channels are activated by Gβγ and in *Xenopus* oocytes, receptors coupled to different G proteins can all activate GIRK [28]. Chimeric Gα containing primarily Gαi except for the C-terminal residues allow Gs-coupled receptors to activate GIRK [29], indicating a pivotal role for Gα in G-protein/GIRK coupling. Conceivably, GIRK and Gi coupled receptors may be sequestered via scaffold proteins. If so, overexpression may saturate the scaffold protein that confers G-protein specificity *in vivo*. This may account for the observed GIRK activation by Gs coupled β1 adrenergic receptors [30]. It is also of interest to note that the receptor kinase GRK2 [31], which has Gβγ binding domains, has been implicated in mediating receptor specificity.

The kinetics of GIRK channel activation in mammalian cells *in vivo* are very fast (<1 s) as opposed to the much slower activation when receptors and GIRK channels are expressed in *Xenopus* oocytes. One possible explanation for this is that in the native cell GIRK channels and Gi/o-coupled receptors are colocalized [23,32]. Indeed, expression in *Xenopus* oocytes of fused muscarinic receptor m2R with the PTX resistant $G\alpha_Z$ resulted in acetylcholine (carbachol) stimulation of reduced currents with much faster activation kinetics [33]. The N-terminal domain of GIRK1 may also bind trimeric Gαβγ [23,34,35]. The physical association of Gα, though not needed for GIRK activation, may have a role in the coupling specificity to a certain GPCR, either by generating macro protein scaffold or by facilitating compartmentalization and hence rapid and specific channel activation by receptors. Interestingly, the N- and C-terminal domains of the GIRKs were found to interact with each other, including the N-terminal domain of GIRK1 to the C-terminal domain of GIRK4, and exhibited much enhanced binding of Gβγ [26]. Four Gβγ binding domains per GIRK tetramer have been implicated by cross-linking experiments [36], but any association between the channel and Gα remain to be examined.

The GIRK basal activity is due to free Gβγ without receptor stimulation. However, qualitative differences have emerged between the basal activity and the receptor-induced GIRK currents. Studies of chimeras between the GIRK4 (S143T, used for expression of functional homotetramer) and IRK1 channels have identified a single site, namely GIRK4 (L339E), that when mutated reduced binding to Gβγ and impaired agonist-induced activity [37]. What might be different between the basal activity and the receptor-induced activity? One can speculate that at resting, Gα is bound to the receptors and both GIRK domains are free to exhibit high affinity to the low level of cytosolic-free Gβγ [1,37]. Upon receptor activation, both $G\alpha^{GTP}$ and Gβγ are separated from the receptors. Gα is then bound to the GIRK N terminal, reducing its affinity to Gβγ. Thus while the released Gβγ will bind GIRK and cause increase in GIRK currents above the basal activity, the reduction in GIRK affinity will result in a fast dissociation of Gβγ and hence the fast reduction in channel activity (the apparent fast desensitization or deactivation of GIRK currents). In another scenario, the $G\alpha^{GDP}$ alone or in a complex with βγ is bound to the N terminal and actively inhibiting the channel [35]. This inhibition will be relieved upon agonist application only if the receptor itself is in a complex with the same $G\alpha^{GDP}$ and can facilitate the exchange to GTP. The mechanism of specificity of GIRK activation *in vivo* as well as the possible modulation of GIRK gating by Gα required further study.

Calcium Channel Interaction with G Proteins

High-voltage-activated (HVA) Ca^{2+} channels provide the coupling from the action potential to transmitter release in nerve terminals and endocrine cell, and of the excitation-contraction of muscle cells. The channel contains at least three subunits. The main subunit, α1 has four homologous repeats (I–IV) of six transmembrane (TM) domains (S1–S6). Each repeat contains the voltage-sensitive S4 domain, and the S5–S6 pore-forming domain. There are two auxiliary subunits with regulatory roles: α2/δ has extracellular domain and a single TM domain, and β is a cytosolic subunit [38]. There are three subfamilies of HVA Ca^{2+} channels named Ca_V1–3. $Ca_V1.1$–1.4 mediate the L-type currents; $Ca_V2.1$–2.3 mediate the P/Q-, N-, and R-type currents, respectively; $Ca_V3.1$–3.3 mediate the T-type currents. HVA Ca^{2+} channels are negatively regulated by G proteins in various neuronal preparations [2,39]. This response appears to be controlled by a membrane-delimited mechanism [40] via pertussis toxin (PTX)-sensitive G proteins [41].

G Protein Interacting Domains

Overexpression or injection of G-protein subunits in sympathetic neurons indicated that Gβγ mediates the Ca^{2+} channel inhibition [42,43]. Alanine mutations of Gβ1 residues 55 and 80, which reside at the N-terminal interface with Gα, had enhanced ability to inhibit current through $Ca_V2.2$, while mutations within the switch interface eliminated current inhibition [1], indicating a Gα role in regulating Gβγ interaction with Ca^{2+} channels.

The Gβγ Interacting Domain of HVA $Ca^{2\pm}$ Channels

Gi/Go coupled receptors selectively inhibited N-, P/Q- and R-type calcium currents [44], due to Gβγ interaction with

the α1 subunit of $Ca_V2.1–2.3$, while the channel β subunit or strong depolarization (prepulse/voltage dependent facilitation), reduced this inhibition [45,46]. Multiple Gβγ interaction domains have been found, including the C-terminal region of CaV2 family [47–49], and the α1 cytoplasmic linker connecting S6 of the first repeat and S1 of the second repeat. Within this linker, Gβγ binding occurs both in the α1 interaction domain (AID), which also mediates the interaction with the channel β subunit, and in a second downstream sequence [49,50].

Modulation of Gβγ Inhibition

All channel β subunits antagonize Gβγ effect but not by direct displacement of Gβγ from the channel. The palmitoylation sites on subunit β2A are responsible for its unique modulation of the channel and the reduced competition with Gβγ [51]. Another putative modulator of Gβγ effect is the synaptic protein syntaxin 1A that may be involved in scaffolding Gβγ to the calcium channels [52]. RGS proteins interact with Gα and facilitate its GTPase activity. Such interaction may reduce calcium current inhibition by Gβγ [53,54].

Voltage-Independent G-protein-Mediated Inhibition of Calcium Channels

Functional studies in calyx-type nerve terminal [55] and in sympathetic neurons [56] have identified a rapid, PTX- and voltage-insensitive (VI) inhibition of calcium currents. The N terminus of $G\alpha_{q/11}$ is necessary for its binding to the C termini of $Ca_V2.1–2.2$ [49] and for mediating the VI inhibition [57]. Consistently, *in vitro* binding assay identified direct interaction of Gβγ with the N and C termini of $Ca_V1.2$, which mediates the L-type current. The mechanism of voltage-independent channel inhibition thus differs from that of voltage-dependent channel inhibition in several ways: instead of channel β subunit it involves $G\alpha_{q/11}$ binding to the same channel region as Gβγ, and it requires simultaneous interaction of calmodulin [58].

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CHAPTER 232

Ras and Cancer

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Introduction: Ras Activation in Cancer

It has been 20 years since H-ras mutations were identified in DNA from the bladder cancer cell line T-24. Since this seminal observation, rates of mutation in H-ras, N-ras, and K-ras have been measured in most types of human cancers [1]. The clonal nature of these mutations in tumors strongly suggests a causal role, a suggestion that has been amply verified by mouse models of Ras-induced cancer. A striking result of this comprehensive survey is the considerable variation in frequency in Ras mutation between different types of cancer. In pancreatic carcinoma, K-ras is activated by point mutation in almost every case, whereas Ras mutations are hardly ever detected in mammary carcinomas, to cite two extreme examples. The biological or molecular basis of these observations is not yet understood. One interpretation is that alternative mechanisms of activating the Ras pathway (receptor amplification, activation of downstream pathways) also occur at varying frequencies. Another interpretation is that different types of cancer vary in their dependence on the Ras pathway. Another unresolved issue is the predominance of K-ras mutations over N-ras and H-ras: this may reflect different levels of expression of these genes in different tissues and different levels of dependence on each type. Mouse knockout experiments show that K-ras is essential [2], whereas N-ras and H-ras are not, consistent with K-ras being the most important form and therefore the most likely to be directly involved in carcinogenesis. However, other models must be considered: for example, each type of Ras may signal through a different set of downstream effectors, and K-ras happens to provide a repertoire of signals that is consistent with malignant progression. Although most evidence points toward shared effectors among all three types of Ras, evidence for discrimination among effectors also exists [3].

In addition to mutations in Ras genes, gains and losses of Ras genes have been reported in human tumors [4–9]. In mouse tumors double minute chromosomes encoding H-ras have been identified [10]. Also in mouse models, progress increase of copy numbers of H-ras mutants appears to drive malignant progression, along with selective loss of the wild-type allele [11].

Pathways Downstream of Ras

Figure 1 shows pathways regulated by Ras. In addition to the well-established pathways that Ras activates, the Raf-MAP kinase cascade and the PI 3′ kinase pathway, Ras activates RalGDS and possibly other effector pathways that are not well characterized [12]. Raf and PI 3′ kinase pathways act synergistically to mediate Ras transformation, suggesting that inhibitors of either pathway have profound effects on Ras transformation [13]. This is an important issue in the context of drug development based on Ras pathways.

The precise molecular basis of synergy between effector pathways is not fully understood. However, there are multiple elements of these pathways that intersect and could contribute to synergistic interaction. For example, the cyclin D1 gene is a transcriptional target of the Raf-MAP kinase pathway, and cyclin D1 protein is stabilized by the PI 3′ kinase pathway [14].

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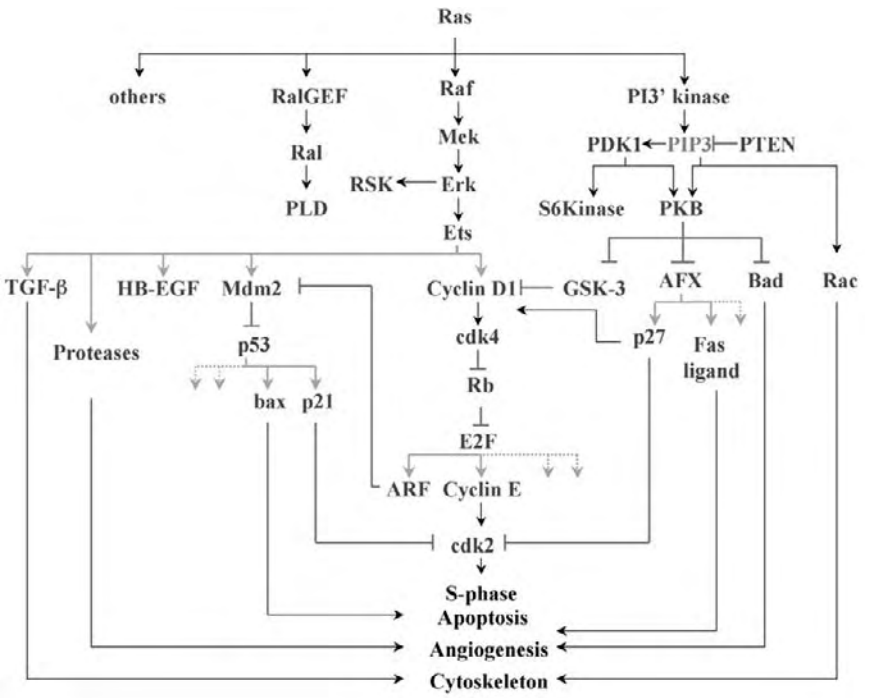


Figure 1

Until recently, there was little genetic evidence that the Raf-MAP kinase pathway is activated by mutation of gene copy number change in human cancer. However, the recent discovery that B-Raf is activated by mutation has changed this view dramatically. Two types of mutant have been described: one that renders B-ras independent of Ras and occurs in tumors in which Ras is wild type; another one that requires Ras interaction for full activity and occurs in tumors containing mutant Ras. It is conceivable that the high-throughput sequencing approach that identified these mutations may yet reveal other activating events in the Raf-MAP kinase pathway. Nonetheless, these new data imply that activation of the Raf effector pathway is the major selection for Ras mutation in these diseases.

In contrast, genetic changes activating the PI 3′ kinase have been well documented and are considered of major importance in human cancer. Loss of PTEN is by far the most frequent event that activates this pathway, but increases in copy number of Akt/PKB have been documented and implicate this arm of the pathway in PTEN-deficient tumors. In endometrial and cutaneous melanoma cancers, loss of PTEN and Ras activation are mutually exclusive, suggesting that in these conditions, the major selection for Ras mutation is activation of the PI 3′ kinase effector pathway [15,16].

Mouse Models of Cancer

Mouse models of cancer provide important clues relating to the role of Ras in cancer. Many have involved forced expression of mutant Ras proteins under tissue-specific promoters, revealing transforming power of the Ras oncogene in different physiological settings. An interesting aspect of these models is the sustained requirement for Ras expression even in advanced cancers: withdrawal of Ras expression causes complete regression of such tumors [17]. Recently a model has been developed in which mutant K-ras is activated sporadically: this appears to be an excellent model for sporadic human lung cancer [18].

Other informative rodent models have used mutagens to initiate cancers, followed by analysis of Ras activation and progression. The classic studies of Sukamar and Barbacid and coworkers proved conclusively that Ras mutation can be the initiating event in cancer and showed that mutations caused by an early chemical insult can persist in latent forms before progressing to cancer [19]. The skin cancer models of Balmain and coworkers have revealed a step-wise activation of H-ras during initiation and progression: mutant H-ras alleles created by exposure to carcinogen are selectively amplified in a step-wise manner as the tumors evolve. In parallel to increased ras activity, levels of cyclin D1 increase during progression. A role of cyclin D1 in Ras transformation in this model was confirmed by demonstration that tumors' progression is strongly retarded in mice lacking the cyclin D1 gene. Even more striking effects of cyclin D1 were demonstrated recently in a model of mammary carcinogenesis driven by Ras, erbB, wnt, or myc: the former two oncogenes were completely dependent on cyclin D1, whereas the latter were not [20]. This clear role of cyclin D1 points toward the importance of the Raf/MAP kinase effector pathway in Ras transformation, since this pathway activates transcription of cyclin D1 directly. However, a role of the PI kinase pathway cannot be ruled out, as this pathway stabilizes cyclin D1 through inhibition of GSK-3-mediated degradation.

Prospects for Cancer Therapy Based on Ras

Attempts to block Ras signaling in human cancers by inhibiting posttranslational farnesylation have been stalled by the fact that K-ras, the major form of ras involved in human cancer, can also be modified by geranylgeranylation. Thus allows continued K-ras activity in the presence of farnesyl transferase inhibitors. Such inhibitors may have clinical value through their action on other cellular targets, however [21]. More recent approaches to blocking Ras activity have targeted enzymes downstream of Ras. A Raf kinase inhibitor entered clinical trials recently [22], and a MEK inhibitor followed soon afterwards [23]. Attempts to block other enzymes downstream of Ras are also under way [24].

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CHAPTER 233

The Influence of Cellular Location on Ras Function

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Study of how cellular location influences the biological activity of signaling proteins is entering a second phase of important discoveries. Some of the very earliest studies on tyrosine kinases and Ras proteins recognized that these otherwise cytosolic proteins required membrane association in order to function. However, new work is providing a reminder that membrane structure is complicated and variable, and that the itineraries of Ras and other signaling proteins to and from membrane surfaces still contain mystery and controversy. The simple model of inactive cytosolic protein versus functional membrane-bound Ras has evolved into the greater challenge of mapping the topography of entire signaling pathways. As in the beginning, Ras proteins are teaching us many of these lessons.

Cytosolic Ras Is not Functional

The original work defining the membrane-binding domain of the vHRas protein discovered that if vHRas failed to achieve membrane binding it was completely and thoroughly transformation defective [1]. This crucial insight led to attempts to achieve similar potent interference with Ras function through pharmacological means, the results of which are described by A. D. Cox in another chapter of this volume. Further genetic and biochemical studies identified a farnesyl transferase (FTase) that attaches a farnesyl isoprenoid to the Ras C-terminus (see Fig. 1, Step 1; [2]). Two (and in most cases, three) additional modifications must also occur to convert Ras into a fully active protein at the plasma membrane [3]. However, the cellular site for these modifications came as a bit of a surprise. Both a unique farnesyl-directed protease and a farnesyl-cysteine methyl transferase were unexpectedly found to be integral proteins of the endoplasmic reticulum (ER) (Fig. 1, Steps 2 and 3;[4,5]). The speed and efficiency of these processing enzymes had kept the discrete visits of all four mammalian Ras proteins [6] to the endomembrane system from being noticed.

After Modifications by Endomembrane Enzymes, Ras Proteins Move Toward the Cell Surface

The KRas4B protein requires no further modifications, although it must contain a basic domain adjacent to its farnesylated cysteine to strengthen its eventual interaction with the plasma membrane [7]. The route through which KRas4B accesses the cell surface does not appear to be the traditional secretory pathway [8], but remains uncharacterized (Fig. 1, Step 5b). However, both HRas and NRas take similar journeys along the Golgi and onward to the cell surface, astride

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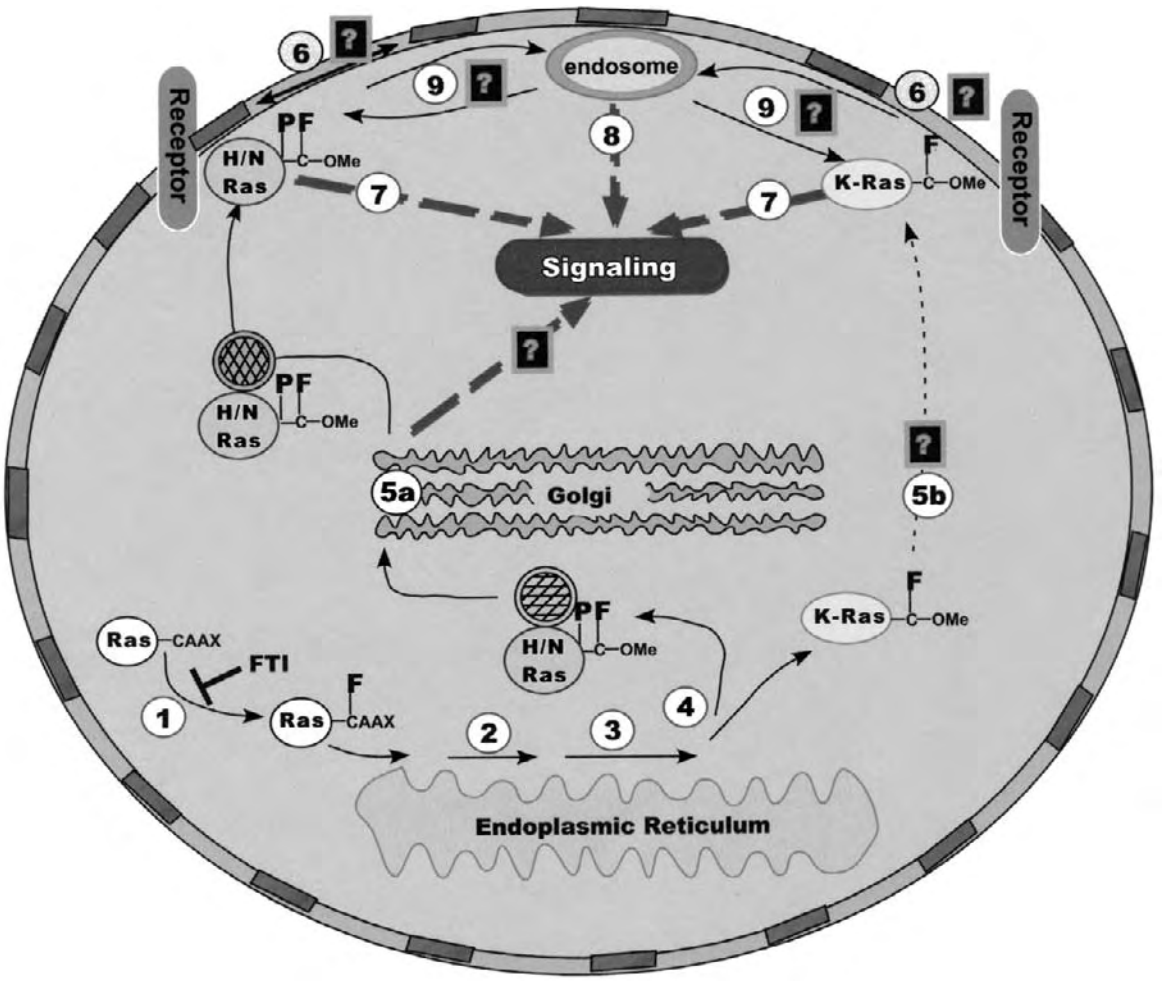


Figure 1 The life and times of Ras: a diagrammatic representation of Ras trafficking and localization. Newly synthesized Ras proteins are farnesylated in the cytoplasm (Step 1), then translocated to the cytoplasmic surface of the endoplasmic reticulum for further modification by a resident protease (Step 2) and carboxyl methyltransferase (Step 3). For H- and N-Ras, further modification occurs in the form of palmitoylation (Step 4). Processed Ras proteins then proceed to the plasma membrane and acquire functional activity. For H- and N-Ras, this occurs through vesicular transport through the endomembrane system (Step 5a). K-Ras4B is not palmitoylated and achieves plasma membrane localization via a separate, but undefined, mechanism (Step 5b). Once at the plasma membrane, H- and N-Ras (and perhaps K-Ras4B) partition into (and possibly out of) lipid rafts (shaded areas, Step 6). The primary events of Ras activation and signaling occur at the plasma membrane (Step 7). Endosomes (Step 8) may also be important sites for some Ras-mediated signaling events, but see text for questions on how Ras may be involved or recycled to the membrane (Step 9). Ras activity can be inhibited by preventing the first farnesylation step in this itinerary using farnesyl transferase inhibitors (FTI, Step 1). Steps where major unknowns remain in Ras trafficking and signaling are indicated with question marks.

intracellular vesicles [6]. Enroute both proteins claim a second modification—attachment of palmitoyl groups to C-terminal cysteines (Fig. 1, Step 4; [6,9]).

No enzyme for Ras palmitoylation has yet been identified. Two endomembrane proteins (ERF2 and ERF4/SHR5) that are required for palmitoylation of yeast Ras1 are known [10,11]. However, HRas palmitoylation also takes place, repeatedly, at the plasma membrane. A protein acyltransferase for G protein αsubunits has been partially purified from plasma membranes, but it acylates HRas poorly [12]. This leaves no current candidate for an enzyme with the crucial function of maintaining Ras plasma membrane attachment. Enzymes for Ras palmitate removal also await discovery. An acyl-protein thioesterase (APT1) that depalmitoylates G protein α subunits has been cloned [13] and can depalmitoylate HRas *in vitro*, but its activity toward HRas *in vivo* has not yet been confirmed.

Importantly, there are several studies showing that mutant HRas proteins that fail to be released from the ER or *cis*-Golgi [9,14] are almost completely transformation defective. A very new report suggests some intracellular HRas may be able to interact with Raf-1 even if endocytosis blocked [15]. Currently, there is almost nothing known about the fundamental processes or proteins that enable HRas and NRas to dock onto the surface of trafficking vesicles or move to the plasma membrane (Fig. 1, Step 5a). The first candidate is the PRA1 prenyl-dependent escort for Rab GTPases, which also interacts with Ras proteins in the Golgi [16]. Discovering these proteins will provide powerful new tools for controlling Ras activity.

Destination-Cell Surface: Ras Proteins Distribute Among Several Plasma Membrane Domains

A significant recent advance is the discovery that Ras proteins (and other signaling proteins as well) are not scattered evenly throughout the plasma membrane, but instead can reside in subdomains of the plasma membrane (Fig. 1, Step 6 [17]). These domains are popularly termed "rafts" and contain lipids that can coalesce into detergent-resistant, light density droplets within the more disordered phase of the general lipid bilayer [18,19]. Cells that express the protein caveolin have an additional raft subtype termed caveolae. It is notable that rafts are not a fixed entity. Microdomains can show dramatic variations in their lipid composition and size based on the lipids synthesized by a cell [20] and on rearrangements that occur during activation of the cell [21,22].

Because most immunofluorescence or gradient techniques utilize detergents or pH 11 carbonate buffers, it has proved difficult to study these dynamic, mobile microdomains without risking the release or intermixing of proteins that were separated in the native membrane. For proteins with a highly basic domain, such as KRas4B, pH 11 effectively neutralizes even the positive charge of lysines, and subsequent interactions may no longer reflect those initially present. For palmitoylated proteins, complete neutralization must occur rapidly to avoid hydrolysis of the delicate thioester-linked palmitates. The interaction of HRas with rafts appears to be tenuous, as the protein is easily dislodged by detergents [23,52], necessitating the use of nondetergent fractionation methods [24]. To complicate matters, although palmitates are readily accepted into the tightly packed core of a raft, farnesyl groups are poor inhabitants of raft-like domains [19,23,25]. HRas and NRas, which possess both palmitoyl and farnesyl modifications, therefore face a biophysical dilemma that is also a biological opportunity. The opposing effects of their lipids may poise these Ras proteins for easy transit into and out of raft domains. Such movement could then provide more than one location where regulatory interactions could occur. Given these caveats and speculations, optimism flavored with a good deal of skepticism should accompany current experimental conclusions.

Nevertheless, multiple techniques indicate that HRas proteins can reside graciously in raft domains [26,27]. Several reports indicate that some HRas is further localized within caveolae [28], where it may interact with caveolin-1 [29]. Whether this "some" represents a little or a lot of HRas is a matter of discussion. Discussions of how Ras proteins distribute among membrane domains are important, as HRas proteins with substitute membrane binding motifs can display aberrant signaling [30–32]. No consensus has yet been reached with the KRas4B protein. KRas4B has been reported to either avoid rafts [28,33], or to be concentrated in caveolin-rich (i.e., raft) domains [34]. Despite our poor understanding of the properties that control where or how tightly Ras proteins partition within the membrane, the underlying rationale that raft and nonraft membranes may each present unique environments for regulatory and signaling interactions is compelling.

Ras Proteins Finally Become Active at the Plasma Membrane

Normal Ras proteins gain functional competency at the plasma membrane. This is due largely to the requirement that in order for a cellular Ras protein to become GTPbound it must interact with a guanine nucleotide exchange factor (GEF) [35]. GEFs are themselves placed along the plasma membrane through interactions initiated by receptor-generated calcium signals, phosphatidyl inositols, and tyrosine phosphorylations. Thus coupling between Ras proteins and their GEFs may serve to (re)position *active* Ras at specific plasma membrane sites.

This possibility leads to two essential questions. Where are the active forms of each type of Ras located? Where do interactions of Ras-GTP with each of its effectors take place? A recent study suggests that HRas moves out of rafts when GTP-bound and that disrupting rafts diminishes the ability of HRas to activate Raf-1 [26,28]. In contrast, KRas4B is proposed to be in nonraft domains regardless of nucleotide state [28]. However, the question of whether Ras proteins are active when in raft domains (suggested by work in T and B cells and with EGF receptors in fibroblasts [21, 36–38]) or are inactive (especially if within caveolae) [27,39] remains an area of debate. One clear opportunity for discovery is to learn how GTP binding to HRas might be translated into lateral movement.

Although the mutations present in oncogenic Ras proteins cause constitutive elevations in GTP binding that occur prior to delivery to the cell surface, this does not seem to trigger premature signaling. In fact, in some situations, high levels of soluble oncogenic Ras proteins appear to dampen signaling [40]. An important unknown is whether membrane-bound oncogenic forms of Ras partition differently from the normal, cellular forms, or activate effectors in inappropriate locations.

Endocytosis—A New Stage for Ras Signaling

The plasma membrane has been considered the final destination for Ras, with the lipid-tethered protein undergoing repeated cycles of activation/inactivation and happily controlling signal passage through its territory. However, reports [41,42] that activation of the Raf-1/MEKK/ERK pathway may occur on early endosomes rather than the plasma membrane indicate that the tale of Ras location and signaling is not over [43,44]. During numerous signaling events, Raf-1 kinase is recruited to the plasma membrane where it interacts with GTP-bound Ras. However, this direct contact between Ras and Raf-1 does not seem to fully stimulate Raf-1 kinase activity [45]. Information from epidermal

growth factor (EGF)- or insulin-treated cells indicates that within minutes of its membrane recruitment [24], Raf-1 departs the plasma membrane aboard early endosomes (Fig. 1, Step 7), and it is there that the further steps of MEK and ERK phosphorylation may take place [42]. This finding immediately raises a plethora of questions. Do the endosomes form from rafts, caveolae, or more traditional coated pits [46]? Does Ras-GTP actually leave the plasma membrane and recycle immediately or simply break off the relationship before Raf-1 departs and remain available for interaction with additional effectors? If Ras enters the endosome, how does it reestablish its association with the plasma membrane (Fig. 1, Step 8)? At what location does a Ras protein interact with a GAP protein and return to the GDP state? This latter point is important as this site will then define when and where Ras signaling terminates. In neurons a complete and functionally competent signaling pathway, from the ligand NGF to Ras to ERK kinases, has been detected on clathrin-coated vesicles moving from the axon back to the cell body [47].

Drugs that Affect Ras Membrane Binding

For more than a decade it has been known that preventing Ras membrane binding can inhibit its oncogenicity. This has spurred the development of potent inhibitors of the farnesyltransferase enzyme (Fig. 1, Step 1) as novel cancer chemotherapeutics [48]. FTase inhibitors are not without their difficulties, however. Multiple other farnesylated proteins (e.g., lamins of the nuclear envelope, retinal transducin γsubunits, Rho GTPases [49]) are also targets of these compounds and may cause impairments in other important pathways. A significant frustration is that both NRas and KRas4B can be modified by a distinct prenyl transferase, GGTase I, that is not susceptible to FTase inhibitors [50]. An alternative strategy of dislodging already prenylated Ras proteins from membranes and accelerating their degradation, currently being explored with the prototype compound farnesylthiosalicylic acid, also appears to diminish Ras signaling [51]. As these studies demonstrate, manipulating the location of Ras proteins is not only possible, it is a tactic that can control Ras biological activity. The recent discoveries of Ras intracellular trafficking, partitioning in or out of rafts, and the link to endocytosis identify several new sites where novel approaches for controlling Ras location and its function can now be envisioned.

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CHAPTER 234

Role of R-Ras in Cell Growth

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Introduction

Ras proteins (H-Ras, K-Ras4A and 4B, and N-Ras) are regulators of signal transduction, mutated in 30% of human cancers, and targets for novel approaches for cancer treatment. Ras proteins are the founding members of a superfamily of small GTP binding and hydrolyzing proteins (GTPases). The small GTPases that share the greatest amino acid identity with Ras, such as R-Ras, Rap, and Ral, constitute members of the Ras family of proteins (Fig. 1). Within this family, the R-Ras subfamily proteins (R-Ras, TC21/R-Ras2, and M-Ras/R-Ras3) exhibit the strongest structural and biological similarities with Ras (Fig. 1). While studies in experimental model systems have shown that R-Ras proteins can promote oncogenic transformation, there is only limited evidence for aberrant R-Ras function in human cancers. Thus, are R-Ras proteins simply inferior versions of Ras proteins, or do they play distinct roles in normal cell physiology? In this review, we first summarize the general features of R-Ras proteins that are shared with Ras proteins, and then we highlight unique features of each R-Ras protein.

General Properties of R-Ras Proteins: Variations on Ras

Structure

R-Ras proteins are GTPases of approximately 200 amino acids that share significant primary, secondary, and tertiary structural characteristics with the 21-kDa Ras GTPases (Fig. 2). In addition, R-Ras proteins possess extended amino (10–26 residues) or carboxyl-terminal residues not present in Ras proteins that account for their larger size (approximately 25 kDa) and suggest functional differences with Ras proteins (Fig. 2). Therefore, the numbering of key R-Ras protein amino acid residues relative to Ras is by the addition of 26 (R-Ras), 11 (TC21), or 10 (M-Ras) to the Ras numbering system (Figs. 2 and 3).

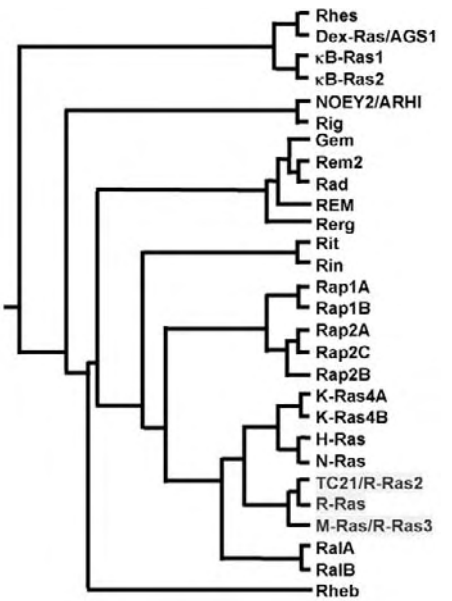


Figure 1 Ras family branch of the Ras superfamily. Small GTPases sharing strongest amino acid identity with Ras proteins are grouped together in the Ras branch. Among these, the three R-Ras proteins (highlighted) share the strongest overall amino acid identity with Ras. The dendrogram was generated by alignment of primary sequences of human proteins using ClustalW, a dynamic sequence alignment program. From the resulting multiple sequence alignment, a distance matrix was prepared and used to construct the dendrogram. Branch lengths are proportional to the estimated divergence along each branch.

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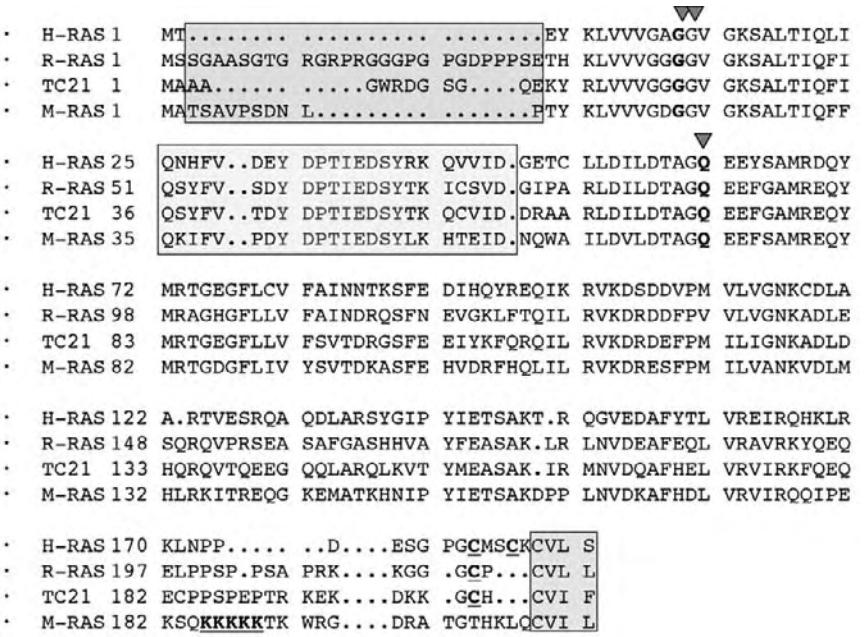


Figure 2 Amino acid sequence alignment of H-Ras and R-Ras proteins. R-Ras proteins are larger than Ras proteins due to additional amino (boxed) and carboxyl-terminal sequences. R-Ras proteins share complete sequence identity with the core Ras effector domain (H-Ras residues 32–40), but sequence differences in the extended effector domain (boxed; H-Ras residues 25–45) account for the different abilities of R-Ras proteins to interact with Ras effectors. Missense mutations at H-Ras residues 12, 13, and 61 (indicated by arrows) have been identified in human cancers and result in constitutively activated, transforming variants of Ras proteins. Mutation of the equivalent residues in R-Ras, TC21, or M-Ras results in the generation of constitutively activated mutants of the R-Ras proteins. The carboxyl-terminal CAAX motif (boxed) present in H-Ras and R-Ras proteins direct posttranslational modification by protein prenyltransferases. Whereas Ras proteins are modified by covalent addition of a C15 farnesyl isoprenoid by farnesyltransferase, R-Ras proteins are modified by covalent addition of a C20 geranylgeranyl isoprenoid by geranylgeranyltransferase I. This modification is followed by the proteolytic removal of the AAX residues and methylation of the now terminal isoprenylated cysteine residue. The CAAX-signaled modifications, together with specific sequences upstream of the CAAX motif, represent the two signals required to target Ras and R-Ras proteins to the plasma membrane. Like H-Ras, R-Ras and TC21 contain cysteine residues (bolded and underlined) that undergo posttranslational modification by the fatty acid palmitate which facilitates membrane association. Like K-Ras4B (not shown), M-Ras contains a lysine-rich sequence (bolded and underlined) in place of the palmitoylated cysteine that is important for membrane targeting.

Expression

The three *ras* genes are expressed ubiquitously, although at distinct tissue-specific levels. Similarly, the three R-*ras* genes are also expressed widely. R-*ras* transcripts showed a wide range of expression levels in a variety of cell types [1]. Human TC21 protein levels were found to be highest in adult human kidney, placenta, and ovary [2]. Interestingly, M-*ras* was isolated in two separate differential gene expression cloning strategies, reflecting its regulation at the level of gene expression [3,4]. M-*ras* gene expression is particularly high in brain and heart, with lower levels in skeletal muscle, ovary, and cells of hematopoietic origin [5–8].

Biochemistry

R-Ras proteins share 60–70% amino acid identity with each other and 50–60% amino acid identity to Ras, primarily in the consensus guanine nucleotide binding motifs and the switch I and II regions of Ras that alter conformation based on the bound guanine nucleotide (GTP or GDP), permitting function as GDP/GTP-regulated molecular switches (Fig. 2). These strong sequence similarities account for the fact that Ras and R-Ras proteins are regulated by an overlapping set of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (Table I). Like Ras, GTP-bound R-Ras is the active form, and mutations at positions analogous to those found in tumor-associated mutant Ras proteins (amino acids 12 and 61) also produce constitutively activated R-Ras GTPases (Figs. 2 and 3).

Like Ras, R-Ras proteins contain a carboxyl-terminal CAAX tetrapeptide motif that directs posttranslational modifications, including the covalent addition of an isoprenoid lipid, which promotes the association of R-Ras proteins with the inner face of the plasma membrane [9]. However, whereas Ras proteins are modified by a C15 farnesyl isoprenoid,

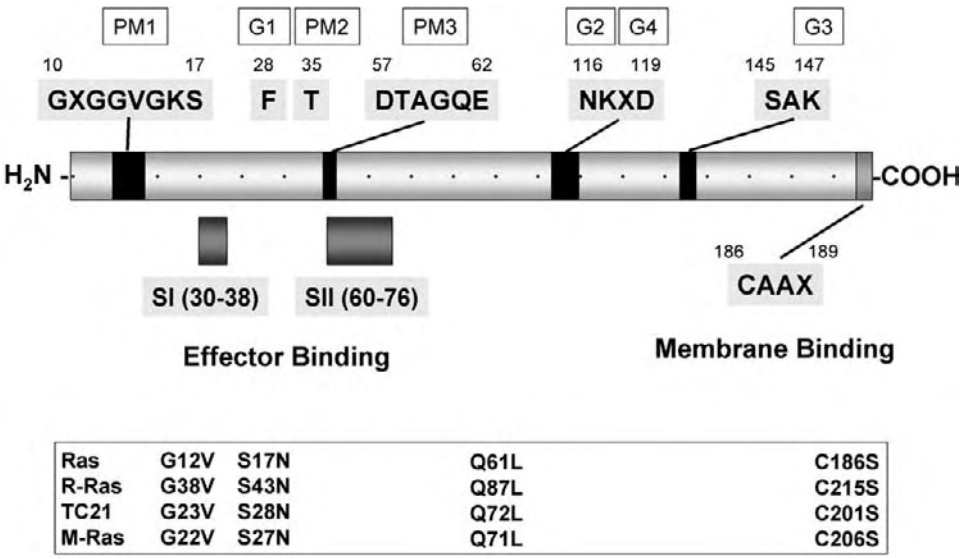


Figure 3 Functional amino acid sequence motifs of Ras and R-Ras proteins. R-Ras proteins possess a set of conserved sequence elements found in all small GTPases and are important for high-affinity binding of GDP and GTP guanine nucleotides, hydrolysis of GTP, or the conformational switch between the GDP- and GTP-bound states (numbers refer to positions in Ras proteins). These sequence elements are involved in binding to the phosphate and magnesium ions (PM) or to the guanine base (G) of the bound GDP or GTP nucleotide. By analogy to Ras proteins, the introduction of missense mutations in R-Ras proteins produces gain-of-function (e.g., Ras mutants G12V and Q61L), dominant-inhibitory (Ras mutant S17N), or loss-of-function cytosolic (Ras mutant C186S) mutants of R-Ras proteins that are useful reagents to study the signaling activities and biological functions of R-Ras proteins.

Table I Overlapping and Distinct Regulators and Effectors of R-Ras Proteins

GTPase	GAPs	GEFs[1]	Effectors[2]	Putative effectors[3]
Ras	P120 GAP, NF1-GAP, Gap1^m	Sos 1/2, RasGRF1/2, RasGRP1/2/3/4	Raf, PI3K, RalGEF, Rin1, PLCε	AF-6, Nore1, RASSF1, Rin1, PLCε
R-Ras	P120 GAP, NF1-GAP, Gap1^m, R-Ras GAP	RasGRF1, C3G, RasGRP1-3	PI3K	RalGEFs
TC21/R-Ras2	P120 GAP, NF1-GAP, Gap1^m, R-Ras GAP	Sos1, RasGRF1/2, RasGRP1-3, C3G	Raf*, PI3K, RalGEF*, PLCε	AF-6, Rin1
M-Ras/R-Ras3	P120 GAP, NF1-GAP, Gap1^m	Sos1, RasGRF1, RasGRP1/3	Raf, PI3K	RalGDS, RGL3, Norel, AF-6, Rin1, RapGEFs

[1]RasGRP proteins are also called: Ca1DAG-GEFI (RasGRP2), Ca1DAG-GEFII (RasGRP), Ca1DAG-GEFIII (RasGRP3);
[2]Functional interactions
[3]Interactions determined, but functional activation not established
*conflicting reports

R-Ras proteins are modified by the more hydrophobic C20 geranylgeranyl isoprenoids [10]. Isoprenoid modification of Ras and R-Ras proteins is essential for biological function. Consequently, inhibitors (FTIs) of the enzyme farnesyltransferase which catalyzes the modification of Ras proteins, have been developed as inhibitors of oncogenic Ras function and are currently under clinical evaluation as anti-neoplastic drugs. Similarly, inhibitors (GGTIs) of the enzyme geranylgeranyltransferase I which catalyzes the modification of R-Ras proteins, have also been developed and have shown anti-tumor activity in preclinical studies [11].

Signal Transduction and Cell Biology

Like Ras, R-Ras proteins serve as relay switches that transmit signals initiated by diverse extracellular ligands to cytoplasmic signaling pathways. However, unlike Ras, the specific upstream signals which promote activation of endogenous R-Ras proteins are less well characterized, perhaps due in part to technical limitations and due in part to limited experimental analyses. Because Ras and R-Ras proteins share common GEFs, presumably many of the same signals that cause Ras activation also cause R-Ras protein family

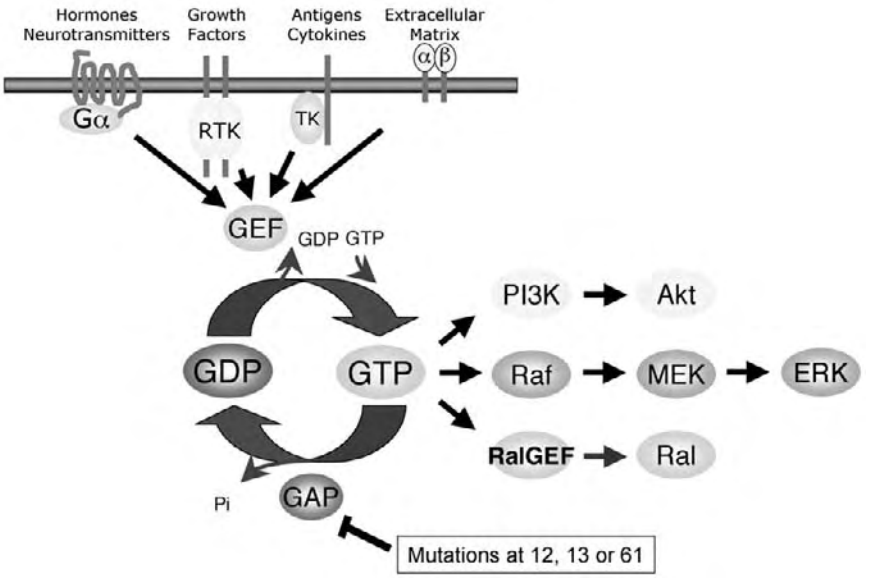


Figure 4 Ras family signaling pathways. Extracellular stimuli act on various cell surface receptors, including G-protein-coupled receptors, receptor tyrosine kinases (RTKs), tyrosine kinase (TK)-associated receptors, and integrins (α/β subunits). The activated receptors typically cause Ras and R-Ras GTP-binding via the activation of guanine nucleotide exchange factors (GEFs) specific for one or more Ras and/or R-Ras proteins. GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity of Ras and R-Ras proteins to return the proteins to the inactive, GDP-bound state. Mutations analogous to those at Ras residues 12, 13, or 61 render R-Ras proteins insensitive to GAP stimulation, and constitutively GTP-bound and active in the absence of extracellular stimuli. The GTP-bound protein preferentially binds to downstream effectors, such as phosphatidylinositol 3-kinases (PI3Ks), Raf serine/threonine kinases, or GEFs specific for Ral small GTPases. The PI3K lipid kinases phosphorylate phosphoinositides and promote formation of phosphatidylinositol 3,4,5-phosphate (PIP3), which in turn facilitates the activation of other cytoplasmic signaling proteins, including the serine/threonine kinase Akt/PKB. Raf phosphorylates the MEK1/2 dual specificity protein kinases, which in turn phosphorylate the ERK1/2 serine/threonine kinases.

activation (Fig. 4). Once activated, Ras protein signaling is mediated by interaction with a multitude of downstream effector targets (Table I). Among these, the Raf serine/threonine protein kinases, the phosphatidylinositol 3-kinase lipid kinases (PI3Ks), and the Ral small GTPase guanine nucleotide exchange factors (RalGEFs) have been established as key mediators of Ras-mediated transformation [12]. The contribution of each of these major Ras effectors to R-Ras family-mediated transformation is distinct, and will be discussed in the following sections. The core effector domain of Ras (amino acids 32–40) is critical for interactions with downstream signaling molecules, and R-Ras proteins share complete identity with this core effector domain. However, flanking residues within the extended effector domain (Ras amino acids 25–45) of R-Ras proteins differ more significantly, suggesting that R-Ras subfamily members may regulate a distinct set of effectors and cytoplasmic signaling pathways.

R-Ras subfamily GTPases are implicated in regulating biological functions both similar to and distinct from those controlled by Ras proteins. For example, like Ras, constitutively activated forms of all three R-Ras members have been shown to cause growth transformation of NIH 3T3 mouse fibroblasts. Similarly, both Ras and R-Ras family proteins regulate cell survival, actin cytoskeletal organization, and differentiation. However, R-Ras proteins also cause consequences opposing those of Ras, and the biological phenotypes of R-Ras function can be significantly different from those of TC21 and M-Ras. The following sections detail the distinct properties and roles of each R-Ras subfamily member in signal transduction and cell biology.

R-Ras

GDP/GTP Regulation

R-*ras* encodes a 218 amino acid protein that shares approximately 55% identity overall to Ras proteins (Fig. 2) [1]. While no GEFs have been shown to be specific for R-Ras, R-Ras GDP/GTP exchange is stimulated by GEFs for some Ras and/or Rap small GTPases (e.g., RasGRF, RasGRPs, C3G) (Table I) [13–19]. R-Ras interacts with some of the known Ras GAPs, and GTP hydrolysis is stimulated by p120GAP, GAP1^{m} and R-Ras GAP (Table I) [20–22].

Signal Transduction

R-Ras fails to activate the Raf > MEK > ERK mitogen-activated protein kinase (MAPK) cascade, which is the key effector pathway required for Ras-mediated transformation of rodent fibroblasts (Table I). However, R-Ras strongly activates the PI3K/Akt signaling pathway, perhaps the second most critical effector pathway for Ras-mediated transformation and survival [23–25]. This effector pathway has also been shown to be required for R-Ras-mediated cell survival and transformation [24,26,27]. R-Ras apparently does not utilize the RalGEF pathway [26], which contributes significantly to Ras-mediated transformation of human cells [28]. Nevertheless, the GTP-bound form of each R-Ras family member can bind to isolated Ras binding domains of Raf and RalGDS, which enables the selective detection of activated R-Ras proteins [29].

Cell Biology

Although mutated R-*ras* genes have not been identified in human tumors, introduction of point mutations analogous to those that cause mutational activation of Ras (38V, 87L) stimulate R-Ras transforming potential. Unlike Ras, in which mutations at positions 12 and 61 are similar in their transforming potency, in members of the R-Ras family, activating mutations analogous to Ras codon 61 (e.g., 87L, 72L, or 71L) are significantly more potent than those analogous to Ras codon 12 [8,26,30,31]. Although constitutively activated mutants of R-Ras cause tumorigenic transformation of NIH3T3 mouse fibroblasts, the strong morphological transformation observed in Ras-transformed rodent fibroblasts is not observed with activated mutants of R-Ras [30,32]. Further, constitutively activated R-Ras fails to rescue the block in growth caused by expression of dominant negative Ras [33], suggesting that R-Ras induces transformation by regulation of signaling pathways distinct from those utilized by Ras. Hence, the normal biological role of R-Ras may lie in processes distinct from those that contribute to cell transformation.

R-Ras has been shown to control and promote integrin-mediated cellular adhesion [34,35]. For example, expression of dominant negative R-Ras (S43N) in adherent cells reduced cell spreading and expression of activated R-Ras in suspension cells promoted spreading and fibronectin assembly, suggesting that R-Ras is required for integrin-mediated cell adhesion [34]. R-Ras also promoted retinal neural outgrowth on laminin, a process dependent on integrin function [36]. R-Ras induced integrin-mediated migration and invasion of breast epithelial cells on collagen, by signaling to $\alpha 2$, but not $\alpha 5$ integrin receptors, suggesting that R-Ras induces selective activation of integrins [37]. R-Ras-mediated control of adhesion may be linked to both Src- and Raf-mediated pathways [38,39]. R-Ras has also been shown to control apoptosis [33], although the effect on the apoptotic response varies depending on cell type. For example, R-Ras promoted myeloid cell apoptosis in response to growth factor withdrawal, an effect which is abrogated by overexpression of Bcl-2 [40]. In contrast, a greater pool of evidence from several model cell systems suggests that R-Ras blocks the apoptotic response. For example, constitutively activated R-Ras induced protection from cell death following withdrawal of anchorage or growth factors in RIE-1 rat intestinal epithelial cells, C2C12 skeletal myoblasts, and BaF3 cells, an IL-3-dependent mouse pro-B-cell line [24,27,41].

In summary, R-Ras is both similar to and distinct from Ras. R-Ras utilizes only some of the same effectors and signaling pathways, such as PI3K/Akt, and regulates only some of the same cellular functions, such as cell proliferation and survival, similar to Ras. Conversely, R-Ras also functions in opposing cellular regulatory roles, such as in cell adhesion. Thus, R-Ras effector utilization is expected to be distinct from that of Ras.

TC21/R-Ras-2

GDP/GTP Regulation

Human TC21/R-Ras-2 is a 204 amino acid protein that shares approximately 70% amino acid identity to R-Ras and approximately 60% amino acid identity to H-Ras (Fig. 2) [32,42]. TC21 is regulated by a set of GEFs and GAPs similar to those that regulate R-Ras GDP/GTP cycling, but responds to more Ras regulators than does R-Ras (Table I) [17]. TC21 intrinsic GTP/GDP cycling activity is activated by SOS, RasGRF1/2, C3G, and RasGRP [2,17,43]. Hydrolysis of TC21-bound GTP is enhanced by known Ras GAPs (p120 GAP, $Gap1^m$, neurofibromin) (Table I) [2,17]. Hence, extracellular signals that regulate Ras GDP/GTP cycling are also likely to cause concurrent regulation of TC21 activity.

Signal Transduction

Whether Raf is an important mediator of TC21-induced transformation remains unclear (Table I). One study found that TC21 failed to interact with and activate Raf kinases *in vivo* [2,44]. Consistent with this failure to activate Raf is the observation that, unlike Ras, TC21, as well as R-Ras, failed to induce senescence in primary fibroblasts [45]. Ras-mediated induction of senescence has been attributed to activation of the Raf/ERK pathway. However, other studies determined that TC21 interacts with and activates Raf kinases, and activates the Raf/MEK/ERK signaling pathway in transformation [43,46]. The basis for these different observations is currently not known. The recent observation that association of 14-3-3 proteins with Raf-1 prevented R-Ras- or TC21-, but not Ras-induced, activation of Raf-1 suggests that the presence of other Raf-1 interacting proteins may account for these different observations [47].

Using various screening approaches for protein-protein interactions, TC21 was found to interact with RalGDS and RalGDS-like proteins (RGL, RGL2) [48,49]. However, whether TC21 activates RalGEFs is unclear (Table I).

One study showed that although TC21 interacted with RalGDS proteins, in contrast to Ras, TC21 did not promote activation of RalA [49]. Thus, similar to observations with R-Ras [50,51], TC21 can bind but not activate RalGDS, suggesting that RalGDS may not be a key effector for mediating TC21-induced growth transformation. In contrast, another study [52] determined that activated TC21 stimulated activation of Ral. These differences may be attributed to experimental, cell type, or cell strain type differences [53] or to the utilization of different expression vectors for ectopic expression of TC21 proteins [54].

Aside from the conflicting observations regarding whether Raf or RalGDS are involved in TC21-mediated transformation, there is a strong consensus that TC21 binds the p110 catalytic subunit of PI3K and stimulates PI3K and PI3K-dependent Akt activities (Table I) [49,52,55]. Inhibition of PI3K function with pharmacological inhibitors or genetic manipulation reversed TC21-mediated transformation of rodent fibroblasts [49,52,55]. However, activated PI3K alone does not cause transformation of fibroblasts [56], suggesting that TC21 must also utilize other effectors to mediate transformation. Like Ras, TC21 also activates phospholipase C epsilon (PLCε) (Table I) [49]. Further study is clearly necessary to determine whether PLCε-mediated second messenger activation is important for TC21-mediated transformation.

Cell Biology

Of the R-Ras subfamily members, TC21 regulates biological functions most similarly to Ras. Unlike other R-Ras subfamily members, activating mutations in *tc21* or overexpression of wild-type TC21 protein have been observed in several human tumors and tumor-derived cell lines [32,43,46,57–59]. Similar to activated forms of Ras, activated TC21 causes strong morphologic growth and neoplastic transformation in several cell types, including rodent fibroblasts and human breast epithelial cells [31,43,57]. In addition, unlike R-Ras, activated TC21 can overcome the growth inhibitory actions of dominant negative Ras, indicating that TC21 and Ras share some common functions important for normal cell proliferation [33]. However, in contrast to Ras, neither TC21 nor R-Ras caused premature senescence of primary rodent fibroblasts [45]. In addition to regulating cell proliferation and transformation, TC21 promotes cell survival of matrix-deprived intestinal epithelial cells and chemotherapeutic drug-treated NIH3T3 cells [41,55]. Like Ras, TC21 also promoted PC12 pheochromocytoma cell differentiation and growth cessation and blocked serum deprivation-induced differentiation of C2 myoblasts [44]. In addition, like R-Ras, activated TC21 has been shown to induce migration and invasion of breast epithelial cells [37].

In summary, of the three R-Ras subfamily proteins, TC21 is regulated and functions most similarly to Ras to control processes such as cell proliferation and transformation. While controversy exists over the mechanism by which TC21 causes transformation, it is clear that chronically activated versions are potent oncogenes, and contribute to various aspects of the transformation phenotype and to tumor formation in humans. Whether the three *ras* genes are functionally distinct or redundant remains an issue of considerable debate. Therefore, whether *tc21* is functionally distinct or redundant with *ras* genes is an issue that also remains to be elucidated.

M-Ras/R-Ras-3

GDP/GTP Regulation

Human M-Ras is a 209 amino acid protein that shares approximately 60% amino acid identity to R-Ras, TC21, and Ras. M-Ras GTP/GDP cycling is regulated more similarly to Ras than to R-Ras or TC21 (Table I). For example, while M-Ras is activated by RasGRF and RasGRP, unlike R-Ras and TC21, it is also stimulated by SOS [8,17]. In addition, like Ras but unlike TC21 and R-Ras, C3G failed to upregulate GTP-bound M-Ras [17]. Similarly, M-Ras GTPase activity is stimulated by the same set of GAPs, such as p120 Ras GAP that enhances GTP hydrolysis on Ras [8,17].

Signal Transduction

The effectors and pathways stimulated by M-Ras to cause transformation may involve both known Ras effectors and novel mechanisms, but are poorly characterized (Table I). Compared to Ras proteins, M-Ras interacts only weakly with Raf kinases [8], and activated M-Ras is only a weak stimulator of the Raf > MEK > ERK MAPK cascade [3,6,8,60]. However, inhibition of ERK activation by pharmacological inhibitors caused a reduction in M-Ras-mediated fibroblast transformation, and Raf-1 cooperated with M-Ras to induce transformation, suggesting that Raf is a key player in M-Ras-mediated transformation [8].

In addition to Raf, M-Ras associates, to varying extents, with other known Ras effectors, such as RalGEFs, PI3K, and Rin1 [8]. Like Ras and TC21, M-Ras was found to interact strongly with RGL3, a RalGDS-like protein [61]. In addition, M-Ras bound strongly to and activated PI3K in fibroblasts [60].

M-Ras-GTP also interacts with newly identified Rap GEFs, MR-GEF, and RA-GEF-2, which possess a Ras association (RA) domain [62,63]. Co-expression of constitutively activated M-Ras with RA-GEF-2 resulted in upregulation of GTP-bound, plasma membrane-bound Rap1 [63]. In contrast, activated M-Ras blocked MR-GEF-mediated GTP loading of Rap1A [62].

Cell Biology

Of the R-Ras subfamily proteins, the least is known regarding M-Ras biological function. Like R-Ras and TC21, M-Ras controls cell proliferation and survival and may also regulate actin cytoskeletal dynamics. M-Ras is only weakly transforming in NIH3T3 fibroblasts [6] and also induced transformation of melan-a immortalized mouse melanocytes [4].

Activated M-Ras also caused IL-3-independent proliferation of RX-6 mast cell/megakaryocyte or BaF3 mouse myeloid cells and blocked serum withdrawal induction of C2 myoblast differentiation [3,7,8]. Like Ras, R-Ras, and TC21, activated M-Ras also promoted cell survival in PC12 cells, by a mechanism that requires PI3K function [60]. It should be noted that these activities of M-Ras were revealed by overexpression of mutated M-Ras proteins, and hence, it is not clear whether they accurately reflect functions of endogenous M-Ras activated by extracellular stimuli.

Conclusions

In summary, the three R-Ras subfamily members, R-Ras, TC21/R-Ras2, and M-Ras/R-Ras3 mediate cell growth, division, differentiation, and death by utilizing both novel pathways and those regulated by Ras and other Ras-related proteins. Further, R-Ras subfamily members, especially R-Ras itself, clearly contribute to cellular processes such as integrin-mediated cell adhesion in a manner distinct from that of Ras. Deciphering the full contribution of R-Ras, TC21, and M-Ras to cellular growth control clearly awaits further study.

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CHAPTER 235

Molecular and Structural Organization of Rab GTPase Trafficking Networks

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Introduction

Rab proteins are small GTPbinding proteins (20 to 25 kDa) that belong to the Ras superfamily. Rab proteins specifically direct membrane traffic in eukaryotic cells. Having been first discovered early in the 1980s in the budding yeast *Saccharomyces cerevisiae* and referred to as Ypt proteins, the name "Rab" comes from "the *Ra*s protein from *b*rain" as the first Rab (Rab3/Smg25) in mammalian cells was identified in bovine and rat brain [1]. The Rab family of GTPases is now the largest family of GTPbinding proteins in the cell [2]. They have been found in all eukaryotes studied and new members have been recently identified through the genome sequencing projects: *S. cerevisiae* contains 11 members, *Caenorhabditis elegans* has 29 members, *Drosophila melanogaster* has 26 members, and *Arabidopsis thaliana* has 57 members. *Homo sapiens* tops the list with 63 members. The fact that the number of Rab proteins is correlated with the complexity of the organism underlines their biological importance in membrane traffic. Rab proteins play a crucial role in regulating the intracellular protein and lipid trafficking between the different specialized organelles of a eukaryotic cell [3,4]. They are important in a myriad of vesicle-mediated transport pathways where they are found to be crucial in recruiting molecular motors, tethering factors, and SNAREs (SNAp REceptors) that guide, dock, and fuse the transport vesicle with target membrane compartments [5,6].

Rab Proteins are Recycling GTPases

Like all other GTPases, Rab proteins cycle between inactive GDP-bound and active GTP-bound forms. Because Rab

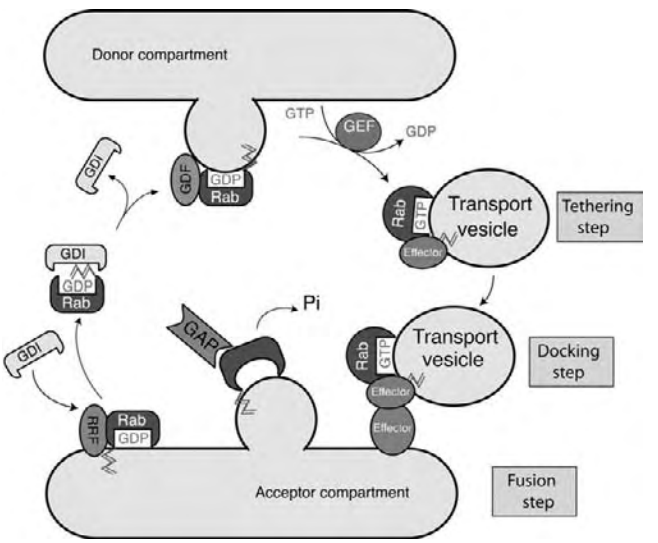


Figure 1 The general Rab GTPase cycle. Rab GTPases undergo a dynamic cycle of delivery and retrieval from the membrane. Delivery is initiated by the cytosolic form of Rab-GDP bound to GDI. Following recruitment, Rab is activated to the GTP-bound form by a Rab-specific GEF leading to recruitment of motors, tethers, and fusion factors. During or following fusion, Rab is converted back to the GDP-bound form and retrieved from the membrane by GDI through RRF.

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proteins are prenylated and insoluble, they are maintained in the cytosol in their inactive GDP-bound state in a complex with a chaperone-like protein, the guanine-nucleotide dissociation inhibitor (GDI) (Fig. 1) [7,8]. During Rab recruitment to membranes, GDI is released and exchange of GDP for GTP is catalyzed by a guanine nucleotide exchange factor (GEF), activating the Rab protein. In the GTP-bound state, the Rab protein can recruit various effectors that direct the vesicle to the target membrane compartment and promote fusion [3–5,9]. Following hydrolysis by a GTPase-activating protein (GAP), the GDP-bound Rab protein is then extracted from the membrane and recycled back into the cytosol by GDI [7,8].

Rab Proteins: An Evolutionarily Conserved Family

Rab proteins define a diverse, but conserved functional family. They share ~20–95% overall amino acid identity within the mammalian family. Although more divergent between species, higher eukaryote homologs of several yeast proteins can complement function. For example, it has been shown that mammalian Rab1, involved in endoplasmic reticulum (ER) to Golgi transport, can replace the function of the yeast counterpart Ypt1p [10]. Sequence alignment of Rab proteins shows the presence of 8 conserved and 11 non-conserved regions (Fig. 2). Conserved domains include the universal 4 nucleotide binding regions (G1, G3, G4, G5) that are signature motifs found in nearly all Rassuperfamily proteins, 3 phosphate/Mg^{2+} binding regions (PM1–PM3), and carboxyl-terminal cysteine motifs required for prenylation [11]. The variable regions are represented by the effector region G2 (also named switch I domain), the switch II domain, and the Rab family (RabF1–RabF5) and Rab subfamily (RabSF1–RabSF3) specific sequence motifs [2,12,13]. Based on the identification of these Rab-specific sequence motifs, Rab proteins have been clustered into 10 subfamilies (Rab1, 3–6, 8, 11, 22, 27, and 40). Based on the crystal structure of Rab proteins, Rab-specific motifs (RabF) are located in and around the switch domains and are thought to contribute to the binding of common regulatory molecules through such conserved regions. These have also been referred to as the complementarity determining regions (RabCDR) based on the structure of Rab3a bound to rabphilin3A (a Rab3A effector) [14]. Molecules that bind through RabF motifs may include GEFs, GAPs, GDIs, and Rab escort protein (REP) (also referred to as the choroideremia gene product; CHM [8]) involved in prenylation [8,15]. On the other hand, the RabSF regions, which show more variation, might allow specific binding of Rab proteins to a variety of divergent effector molecules involved in each of the different membrane trafficking pathways regulated by Rab [2,12].

Structural Organization of the Rab Proteins

Given their homology to other Ras superfamily GTPases, as early as the 1990s, the structural and biochemical information

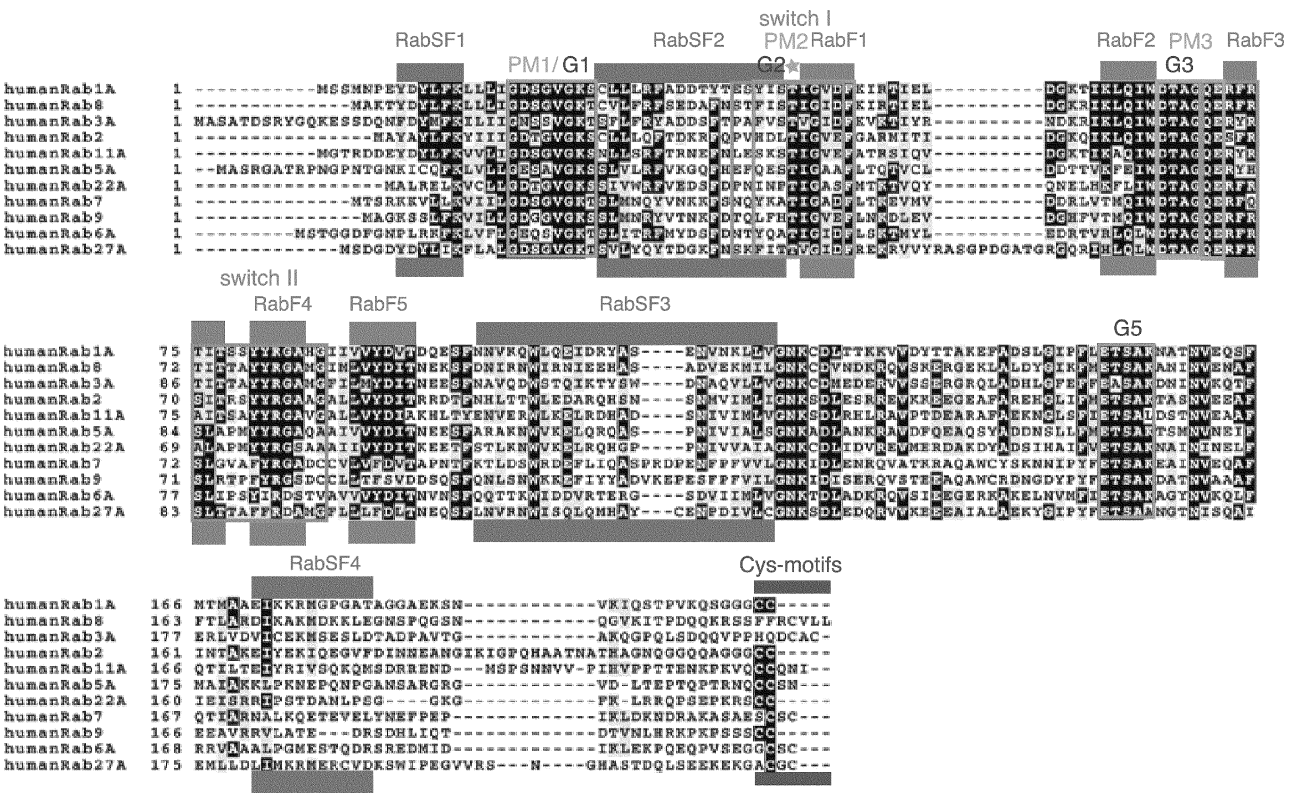


Figure 2 Rab-family and Rab subfamily domains direct Rab function. Illustrated are the location of the Rab family (RabF) and Rab subfamily (RabSF) domains in the context of nucleotide binding domains (G1–G5) and switch regions (switch I and II). These regions play an important role in defining the organization of the Rab gene family. (Adapted from Peirera-Leal and Seabra, *J. Mol. Biol*. **313**, 889–901. With permission.)

available from the three-dimensional structure of the *ras* oncogene protein $p21^{Ras}$ has been used to dissect Rab function [11]. This approach was confirmed by recent structures of Rab proteins. These now include Rab3A and Rab3A in complex with the effector Rabphilin-3A [13,14,16], Ypt51p (the Rab5 homolog) in its active GppNHp-bound conformation [17], and the yeast Sec4p (the Rab8 homolog) in both inactive GDP- and active GPPNHP-bound conformations [18]. Analysis of these structures reveals that the structure of Rab proteins is largely superimposable on the core structure *ras* oncogene despite its complete sequence divergence.

The structural core of the Rab proteins is comprised of six central β-strands in a relatively flat β-sheet that is sandwiched by three helices on either side, forming the guanine-nucleotide pocket. Comparison of the two Sec4 structures confirms that the protein undergoes a drastic conformational change in the regions called switch I and switch II which interact with effectors when Rab switches from the GDP- to the GTP-bound state [18]. Structurally, switch regions differ significantly from other Ras superfamily members. Consistent with this, mutagenesis studies have shown that the switch regions are crucial for the interaction of Rab proteins with regulatory proteins, such as GEF and GAP. Thus, Rab function recapitulates that classic *ras* oncogene GTPase paradigm.

Posttranslational Modification and Localization

To bind to membranes where they direct the assembly of protein complexes directing vesicle traffic, Rab GTPases need to be posttranslationally modified by the attachment of two 20-carbon geranylgeranyl moieties to cysteine residues located at their hypervariable carboxyl terminus [19,20]. Their consensus sequence for this posttranslational modification has been identified to be either -XXCC, -XCXC, or -CCXX where C is cysteine and X is any aliphatic amino acid [21]. The exceptions are Rab8 and Rab13, which contain only a single cysteine residue attached to a more traditional Ras-like -CAAX box. Deletion or mutation of the cysteine residues to serine eliminates the prenylation and inactivates all Rab species examined to date [22]. Prenylation requires the accessory component REP/CHM, and the enzyme complex geranylgeranyltransferase II (RabGGTase) composed of α and β subunits [23,24]. Unlike other members of the Ras superfamily that can be directly farnesylated by farnesyl transferase [19], newly synthesized Rab proteins are not recognized by the RabGGTase complex until they bind REP. Following prenylation, REP delivers the newly synthesized Rab proteins to the correct compartment through information found in their hypervariable 30–40 amino acid carboxyl terminus [25]. Indeed, each Rab protein has a very limited subcellular distribution diagnostic of its function [1,3,26]. For example, Rab1 is localized to the ER-Golgi interface in the exocytic pathway, Rab5 is found restricted to early endocytic compartments and clathrin-coated vesicles forming at the cell surface, Rab3A to synaptic vesicles, etc. The fact that 63 different Rab proteins have been identified to date suggests that there are at least this many different specialized trafficking pathways found in the differentiated cells of higher eukaryotes. As expected, many Rabs are associated with only a single tissue type, suggesting that the limited number of Rabs found in yeast (11 Rabs total) may define the rudimentary trafficking comprising the basic functions of the exocytic and endocytic pathways. In this regard, it is interesting to note that only 2 Rabs in yeast are essential proteins. Ypt1 is required for ER to Golgi transport, and Sec4 is required for Golgi to cell surface transport. We have proposed the "homing hypothesis" whereby each Rab dictates the formation of a effector complexes (the Rab interactome) that mediate a specific trafficking event in the cell, thereby ensuring high fidelity to membrane trafficking [6]. In addition, it was proposed that Rabs also control the mosaic organization of endocytic compartments by segregating cargo and transport factors into subdomains [27].

Effector Molecules: REP/CHM, GEF, Effectors (Motors/Tethers/Fusogens), GAP, and GDI

Rab proteins can interact with a diverse range of proteins [4,9]. GEFs are believed to play a critical role in the activation of Rab-GDP to Rab-GTP upon delivery to a specific membrane. Only a few exchange factors have been identified to date and they appear quite divergent indicating highly specialized function. However, it is likely that a conserved domain(s) promoting exchange will be a common feature of Rab GEFs. Once activated to the GTP-bound form and bound to membranes, Rab proteins recruit effector molecules [4,5,9]. While they are a highly heterogeneous group, they appear to fall into discrete categories in which they can be thought to function as (1) "tethers" that promote vesicle docking (such as p115, a Rab1 effector [28]; Rabphilin-3, RIM1 and 2, Rab3 effectors [29]; and EEA1, a Rab5 effector [5]), (2) SNAREs that direct vesicle fusion (SNAREs [30]), or molecular motors and their cognate receptor proteins that guide movement of membranes through the cell via the actin and microtubule cytoskeletons. A Rab6b effector is rabkinesin-6-involved microtubule binding during mitosis [31,32]. A Rab27a effector is the melanophilin/myosinVa complex involved in melanosome biogenesis and trafficking [33]. As many of the interactions between the Rab and various effector molecules could be indirect, this leads to the general principle that Rab GTPases serve as the focal point to regulate the assembly/disassembly of large macromolecular complexes to integrate the activity of these diverse components to move a vesicle from one cellular location to the next with speed and accuracy. While subfamily isoforms >80% homology (i.e., Rab3A–3D) may use evolutionarily related effectors; the high heterogeneity in effector function in more divergent Rabs emphasizes that they have become highly specialized in order to direct the specific function of a particular membrane transport step in the endocytic and exocytic pathways.

Following docking and fusion, conversion of Rab from the GTP- to the GDP-bound state is mediated by Rab-specific GAPs. Again, this appears to be a heterogeneous group,

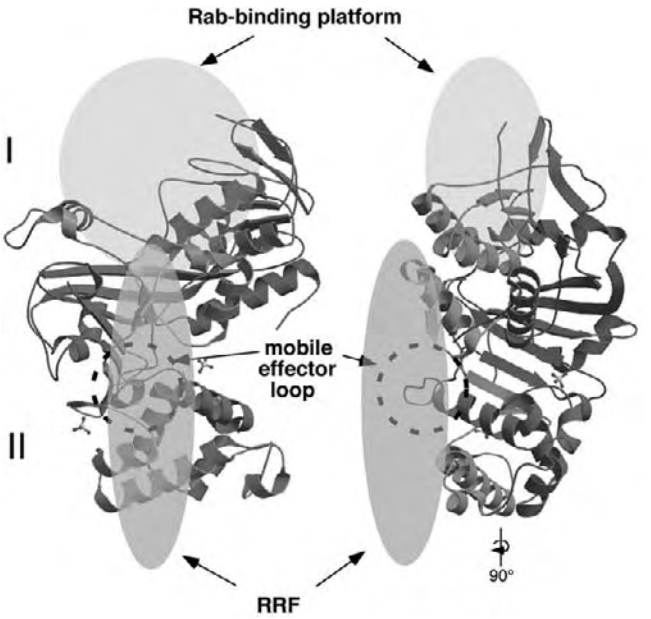


Figure 3 Structural organization of GDI, a Rab recycling factor. Shown is the two-domain organization of αGDI in which the upper domain (I) functions as a platform to bind Rab, whereas the lower domain (II) is involved in interaction with RRF, a membrane-associated recycling complex that assists GDI in removal of Rab from the membrane.

although only a few have been identified and their function in vesicle docking and fusion remains to be determined [9]. Following hydrolysis Rab-GDP must be recycled. This is the job of a common recycling protein, GDI, that interacts with all Rab GTPases studied to date [7,8]. The function of GDI has been extensively investigated using both structural and molecular approaches [34–38]. The structure of GDI shows that it is a two-domain protein—an upper domain I involved in Rab binding and a lower domain II involved in Rab extraction (Fig. 3). By binding of Rab through domain I in a reaction assisted by a membrane receptor (Rab recycling factor or RRF) which interacts with domain II [36], the prenyl groups of Rab are transferred from the lipid bilayer to the surface of GDI. Lipid binding triggers release of the Rab-GDI complex from the membrane. The cytosolic pool of the GDI-Rab complexes provides the principle source for re-recruitment of Rab to membrane. While the exact mechanism by which GDI releases Rab during delivery is unknown, it is believed to involve a guanine nucleotide dissociation factor (GDF) that displaces Rab from GDI in conjunction with interaction with the activity of Rab-specific GEFs found on the membrane [7,8].

Rab Dysfunction and Disease

Defects in intracellular trafficking can lead to a large variety of human diseases [39–41]. For example, mutation of the brain-specific guanine nucleotide dissociation inhibitor (αGDI), involved in Rab recycling, leads to X-linked mental retardation [42]. In contrast, mutation of the REP1/CHM involved in Rab prenylation leads to choroideremia, an X-linked recessive eye degeneration disease [43]. To date, only one Rab protein, Rab27, has been directly implicated in hereditary disease. Mutation of Rab27a results in Griscelli syndrome (GS) in humans and the *ashen* phenotype in mice [15]. Rab27a has been shown to play a critical role in the transport of secretory granules, such as melanosomes in melanocytes, lytic granules in cytotoxic T lymphocytes and platelet-dense granules [15]. Moreover, Rab27a might also be the trigger for the retinal degeneration in CHM, since it is present at high levels in the retinal pigment epithelium and choriocapillaris of the eye, and it is not prenylated in CHM patients where REP1/CHM is missing [44]. Because Rab proteins are the key regulators of all the membrane trafficking activity and dictate the function of highly specialized cellular trafficking pathways, we anticipate that a variety of other hereditary disorders are likely to be discovered that directly involve Rab dysfunction.

Perspective

Rab GTPases comprise a large gene family that dictates the organization of the exocytic and endocytic pathways of eukaryotic cells. Without Rabs, the subcellular organization typically found in eukaryotes would not exist. Thus, they provide a critical foundation for cell development and differentiation through their ability to organize divergent membrane trafficking complexes that dictate specificity to membrane interactions.

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CHAPTER 236

Cellular Roles of the Ran GTPase

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Introduction

Ran is an abundant nuclear GTPase that has been extensively characterized for its role in nuclear transport. The Ran pathway is involved in spindle assembly and postmitotic nuclear assembly, independent of its role in nuclear transport. The distribution of Ran's nucleotide exchange factor (RCC1) and GTPase activating protein (RanGAP1) leads to the formation of gradients of Ran-GTP within the cell. These gradients provide spatial cues that direct Ran-dependent processes. These cues are monitored through a common set of Ran-GTP binding effector proteins that were originally characterized as nuclear transport receptors. The past year has seen a considerable deepening in our understanding of the molecular features of this pathway and of how gradients of Ran-GTP are built and utilized. In this chapter, we relate these new developments to Ran's role in cellular processes.

Introduction to the Ran Pathway

Ran's intrinsic rates of nucleotide hydrolysis and exchange are slow. These reactions are accelerated *in vivo* by RCC1, a guanine nucleotide exchange factor (GEF) [1], and by RanGAP, a GTPase activating protein [2]. A family of proteins that share homologous Ran-GTP-binding domains are also important *in vivo* regulators of Ran [3]. RanBP1 is the best-understood member of this family. RanBP1 does not have intrinsic GAP activity, but it increases the rate of RanGAP1-mediated nucleotide hydrolysis by roughly an order of magnitude *in vitro* [4]. Moreover, it is essential *in vivo* for RanGAP1-mediated hydrolysis of Ran-GTP bound to nuclear transport receptors [5].

There are four notable aspects of the Ran pathway that distinguish it from many other members of the Ras superfamily. First, Ran is not isoprenylated, and is thus freely soluble. Second, Ran is an extremely abundant protein (roughly 0.4% of total cellular protein [6]), as are many of its interacting partners. Third, there is an asymmetric distribution of Ran's regulators throughout interphase [7]. RCC1 is chromatin bound and hence nuclear, whereas RanGAP1 and RanBP1 are predominantly cytoplasmic. This causes a steep gradient of RanGTP across the nuclear membrane [8,9]. Fourth, as mentioned above, Ran-GTP binds to a family of proteins that have a common structural motif (RanBD) first found in RanBP1. RanBP2, another Ran binding protein with four RanBD domains, is localized to the cytoplasmic side of the nuclear pore complex (NPC) [10]. The role of RanBP2 in promoting nucleotide hydrolysis has not been clearly demonstrated. A third member of this family, RanBP3, acts as a cofactor for the formation of complexes destined for nuclear export [11,12].

Structural Analysis of Ran Pathway Components

Several points emerging from structural studies on Ran and its regulators are notable. First, the structure of Ran itself is dramatically regulated by nucleotide binding, particularly in the switch I region [13]. The orientation of the switch I region precludes association between Ran-GDP and transport receptors [14], providing a molecular rationale for the specificity for transport receptors in binding to Ran-GTP only. Second, the Ran pathway shows an interesting divergence from other Ras-like GTPases with respect to its mechanism of GAP-mediated hydrolysis [15]. RanGAP consists of 11 leucine rich repeats of 28 to 37 residues each, forming a crescent [16]. While it was widely assumed that GAP-mediated hydrolysis of all ras family GTPases would require an arginine residue provided by the GAP protein, this is not the case for Ran [15]. Structures of a Ran-RanBP1-RanGAP ternary complex in the ground-and in the transition-state show that RanGAP does not provide a catalytic arginine. Rather, Ran alone provides the machinery for GTP hydrolysis, with tyrosine 39 of Ran replacing the arginine provided by other GAPs.

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Third, RanBP1 is essential *in vivo* because it dissociates Ran-GTP from transport receptors to permit access of RanGAP [5]. Transport receptor binding to Ran-GTP blocks RanGAP-mediated hydrolysis, because the interaction sites for RanGAP and receptors overlap [14,15]. However, binding of Ran to transport receptors does not inhibit association between Ran-GTP and RanBP1 family members because they bind on different surfaces of Ran [14]. RanBD domains make extensive contacts that have been described as a "molecular embrace" of Ran [14], causing a shift in the Ran's acidic C terminus, and promoting Ran's release from bound transport receptors. Although RanBP1 can activate GAP-mediated hydrolysis *in vitro* about an order of magnitude [4], RanBP1 does not directly contact RanGAP within the RanGAP-Ran-GTP-RanBP1 ternary complex [15]. It appears that RanBP1 exerts a positive influence on GAP-mediated hydrolysis by shifting the C terminus of Ran, thereby facilitating interactions between Ran and RanGAP.

Fourth, RCC1 is a seven-bladed propeller protein [17], similar in structure to the WD40 propeller structure of the GEF for the G-β heterotrimeric GTPase. RCC1's interaction interface with Ran resides in the loops between the propeller blades on one face of the protein [18]. RCC1 associates with chromatin through other proteins [19]. It has recently been demonstrated that the interaction of RCC1 with chromatin is mediated by histones [20]. RCC1 binds directly to mononucleosomes and to histones H2A and H2B [20], resulting in a modest stimulation of the catalytic activity of RCC1.

Ran's Role in Nuclear Transport

The requirement for Ran in nuclear transport has been extensively studied (Fig. 1) (reviewed in reference [7]). Ran-dependent nuclear transport is mediated by a family of transport receptors that are able to freely pass through the NPC in an energy-independent manner. Importin β was the first member of this family to be described, and all members of this family (both import and export receptors) bind to Ran-GTP. Import receptors bind to their cargo in the cytoplasm, where Ran-GTP is absent, and permit cargo translocation through the NPC. In the nucleus, RCC1-generated Ran-GTP binds to the import receptors and releases the cargo. Conversely, export receptors and their cargo associate within the nucleus as complexes containing Ran-GTP. After these complexes translocate to the cytoplasm, RanGAP1 induces GTP hydrolysis thereby causing the dissociation of the complexes. A small Ran-GDP-binding protein, NTF2, re-imports Ran-GDP back into the nucleus after each round of transport, where regeneration of Ran-GTP is achieved by RCC1.

Within this model, Ran-GTP concentration regulates receptor loading and unloading in a manner that is appropriate to the nucleus or cytosol. A number of interesting variations have been reported recently upon the way in which different receptors utilize the Ran-GTP gradient. First, while it is essential in this model that each transport receptor can only carry a particular cargo in one direction, there is no requirement that receptors cannot carry other cargos in the opposite direction. Indeed, bidirectional receptors have recently been reported in yeast [21] and mammals [22].

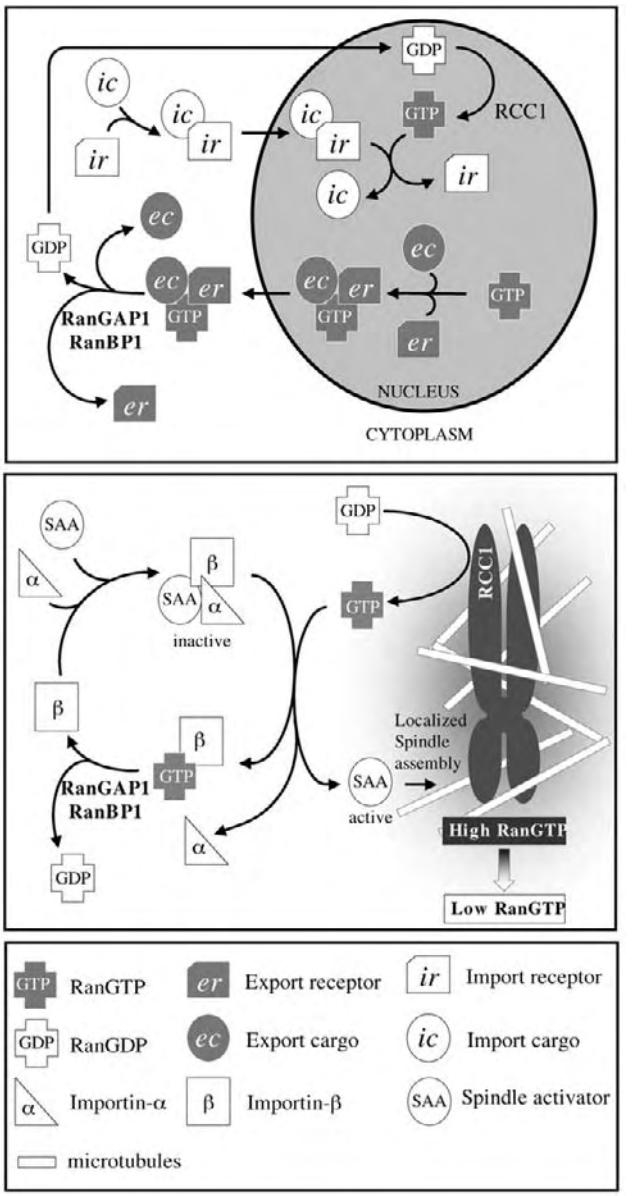


Figure 1 Ran regulates nuclear trafficking through the association and dissociation of transport complexes during interphase (upper panel). The compartmentalization of RanGAP1, RanBP1, and RCC1 cause an asymmetric distribution of Ran-GTP across the nuclear envelope. Import receptors and cargo form complexes in the cytosol and transit across the NPC. In the nucleus, Ran-GTP binds to the import receptors and causes cargo release. Import receptors return to the cytosol in association with Ran-GTP. RanGAP1, and RanBP1 hydrolyze Ran-GTP to Ran-GDP, promoting receptor recycling. Export receptors and Ran-GTP bind to export cargo in the nucleus. These complexes transit to the cytosol, where they dissociate after RanGAP1-mediated Ran-GTP hydrolysis. Elevated Ran-GTP levels near chromosomes promote mitotic spindle assembly (lower panel). Importin α and importin β inhibit spindle assembly activators (SAA) at low Ran-GTP concentrations in regions distant from chromosomes. Near chromosomes, Ran-GTP concentrations are higher. Ran-GTP binds to importin β, disrupting inhibitory complexes and allowing full SAA activity.

Second, many receptors interact with at least some of their cargo through adaptor proteins [7]. The best-characterized case of adaptor utilization is the import of substrates bearing classical nuclear localization signals (NLS) [7]. The Importin α adaptor protein binds to NLS sequences. Importin β associates with the importin α-NLS complex and promotes its transit through the NPC. Importin α is re-exported in association with Ran-GTP and CAS, an importin β-family export receptor. Two molecules of GTP are thus hydrolyzed during the import cycle of each NLS-bearing substrate, allowing substrates accumulation against a steeper nuclear-cytoplasmic concentration gradient through the expenditure of greater free energy.

Third, accessory proteins control the loading of cargo to receptors in many instances [7]. For example, the Crm1 export receptor binds RanBP3, a nuclear RanBD-containing protein [12,23]. RanBP3 does not directly bind to export substrates, but it increases the affinity of Crm1 for both Ran-GTP and export cargo. RanBP3 also associates with RCC1 in a manner that is stimulated by Ran, and activates RCC1s as Ran GEF [11]. Moreover, RanBP3 promotes the association of Crm1 and RCC1, perhaps acting as a scaffold to coordinate the loading of Ran-GTP onto Crm1. RanBP3 inhibits the association of unloaded Crm1 to the NPC in a manner that is relieved by Ran-GTP [12], suggesting that it permits Crm1 association to the NPC only after export complex assembly is complete.

Finally, import cargo unloading can be controlled by events in addition to Ran-GTP binding [24,25]. For example, DNA containing binding sites for the TBP transcription factor stimulates Ran-GTP-mediated dissociation of TBP from its import receptor (Kap114p) [24]. This stimulation suggests that TBP is released from Kap114p only when it finds its target DNA sequences within the nucleus. Import receptors may thus direct not only nuclear import but also intranuclear targeting or assembly of particular cargos into macromolecular complexes.

Ran's Function in Mitotic Progression

A number of observations have implicated Ran in regulating the onset of mitosis. When arrested in S phase and then shifted to the restrictive temperature, a mutant Hamster cell line with a temperature-sensitive allele of RCC1 (tsBN2 cells) progresses into mitosis prematurely, accompanied by nuclear envelope (NE) breakdown, precocious chromosome condensation, and activation of p34cdk1/cyclin B kinase [26]. These observations and others have shown that tsBN2 cells do not arrest appropriately in S phase in response to unreplicated DNA [27]. Moreover, mutant Ran proteins can block onset of mitosis in the presence or absence of nuclei in *Xenopus* cycling extracts, which would otherwise alternate between interphase and mitosis [28,29]. The molecular events whereby Ran regulates mitotic onset in metazoans remain unclear, and there is a lack of strong evidence from fission and budding yeast that Ran plays an analogous role for regulation of mitosis in either of those organisms [30].

Ran's Function in Spindle Assembly

Ran regulates spindle assembly in a manner that is independent of its nuclear transport function [31–35]. Spindle assembly is severely defective when Ran-GTP levels are lowered in *Xenopus* cytostatic factor (CSF) arrested egg extract, a mitotic system that is devoid of intact nuclei. Under these conditions, spindles are disorganized with low densities of microtubules (MTs) [33,35]. Conversely, increased levels of Ran-GTP in CSF extracts cause massive polymerization of MTs in a manner that does not require chromosomes or centrosomes [32,33,35]. It had been previously found that mitotic chromosomes can locally stabilize MTs, probably through the action of a diffusible MT-stabilizing factor produced by a chromatin-associated enzyme (reviewed in reference [36]). Since RCC1 binds to chromatin [37], it was natural to speculate from these results that Ran-GTP could play a role in the localized stabilization of mitotic MT by chromosomes. Consistent with this idea, it has recently been directly demonstrated through fluorescence resonance energy transfer (FRET) experiments that Ran-GTP concentrations are elevated in the vicinity of mitotic chromosomes [8].

The molecular mechanism by which Ran carries out its function in spindle assembly is closely related to its role in nuclear transport. Importin α/β bind and inhibit spindle assembly factors, such as the motor accessory proteins TPX2 and NuMA [38–40]. Ran-GTP near chromosomes destabilizes these inhibitory complexes, thereby allowing spindle assembly factors to function locally (Fig. 1). At a distance from chromosomes, Ran-GTP would presumably undergo nucleotide hydrolysis and inhibition would be restored. In reality, it is likely that the location of nucleotide hydrolysis may also be very important for spindle function, since vertebrate RanGAP1 is localized to the spindle in a highly regulated manner [41]. Another key aspect of this model is that importin α and β also promote the nuclear localization of spindle factors, ensuring that they are not inappropriately active on MT in interphase cytosol.

Since numerous other proteins involved in spindle assembly are nuclear during interphase, it is likely that additional targets are regulated in a similar manner. This notion is also supported by the fact that Ran regulates several different aspects of spindle assembly, such as the frequency of transition from shrinkage to growth of MTs (e.g., MT dynamics) [42,43], the capacity of centrosomes to nucleate MTs [42], and the behavior of other motor proteins or their accessory subunits [43,44]. Taken together, these data suggest that Ran may have acted in a multifaceted manner to coordinate assembly of spindles with respect to the mitotic chromosomes.

Ran's Role in Postmitotic Nuclear Assembly

Studies in *Xenopus* egg extracts have suggested that Ran is required for postmitotic NE assembly [45,46]. Nonhydrolyzable forms of Ran-GTP and loss of RCC1 activity or depletion of Ran all block early events of nuclear fusion

during NE assembly in egg extracts [45]. Addition of Ran-GTP reverses this inhibition, leading to the conclusion that Ran has an important role in re-formation of NE at the end of mitosis. Both nucleotide exchange and hydrolysis on Ran are required for this function to be fulfilled [45]. Remarkably, GST-Ran bound to beads can assemble structures resembling NE in egg extracts [46]. These structures contain NPC and are capable of nuclear transport. RCC1-bound beads do not assemble NE in this assay. Zhang and Clarke [46,47] have argued that Ran actually precedes RCC1 in binding to chromatin and can act as an RCC1-independent signal for NE assembly.

In an exciting set of new reports [48], it has been shown that importin β is required for NE assembly induced by Ran on beads. Concentration of importin β on beads is sufficient to induce NE assembly in *Xenopus* egg extracts in a manner similar to Ran-coated beads, while beads bound with other transport receptors did not have this property. Moreover, the function of importin β in NE assembly is disrupted by a mutation that decreases the affinity of importin β for nucleoporins, but not by a mutation that disrupts the interactions of importin β with importin α. In contrast to the mechanisms of nuclear transport and spindle assembly, it thus appears that importin β functions in NE assembly by recruiting NPC components rather than by importin α-dependent interactions with cargo protein.

Conclusions

Ran regulates nuclear transport, cell cycle progression, spindle assembly, and postmitotic NE assembly, suggesting a wide role for Ran in coordinating events during the cell cycle. These facets of Ran are mechanistically linked in two ways. First, in all cases where we have a rudimentary molecular understanding of Ran function, it appears that Ran-GTP gradients provide spatial cues that serve to indicate the localization of the chromatin. This notion is particularly well supported for nuclear transport and spindle assembly. Second, gradients of Ran are interpreted through a common set of Ran-GTP-binding effectors, with the importin β protein playing a particularly prominent role.

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CHAPTER 237

Rho Proteins and Their Effects on the Actin Cytoskeleton

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Introduction

Rho GTPases form a discrete family within the Ras superfamily of small GTPases, which currently comprises 23 members, in mammalian cells [1]. They play an important role in controlling many cellular activities including transcription, cell cycle progression, membrane traffic, transformation, and apoptosis, but they are best known for regulating the organization of the actin cytoskeleton [2,3]. The actin cytoskeleton is a highly dynamic network of actin polymers that controls many aspects of cell behavior, including morphology, adhesion, migration, and phagocytosis. This chapter summarizes our current knowledge on the control of the actin cytoskeleton by Rho GTPases.

Effects of Rho GTPases on the Actin Cytoskeleton

The role of Rho, Rac, and Cdc42 in controlling actin cytoskeleton organization was first discovered in quiescent Swiss3T3 fibroblasts, where it was found that the activation of each GTPase induces distinct filamentous actin structures in response to extracellular stimuli (Fig. 1). Activation of Rho by lysophosphatidic acid (LPA), for example, causes the formation of stress fibers (contractile bundles of actin:myosin filaments traversing the cell and ending in focal adhesions) [4], activation of Rac by platelet-derived growth factor (PDGF) or insulin results in the formation of lamellipodia (membrane protrusions driven by actin polymerization that can detach and fall backward onto the cell body creating membrane ruffles) [5], and activation of Cdc42 by bradykinin leads to the formation of filopodia (thin finger-like protrusions consisting of actin filament bundles) [6,7]. In addition, crosstalk can occur between the three GTPases: Cdc42 can activate Rac and stimulate membrane ruffling, and Rac can activate Rho to form stress fibers [5–7].

The effects of Rho, Rac, and Cdc42 on the actin cytoskeleton are not, however, restricted to fibroblasts, and over the last decade Rho GTPases have been implicated in many actin-dependent events in a wide variety of different cell types. Often, Rho GTPases function in a coordinated manner to promote complex changes to the actin cytoskeleton, while in other cases Rho GTPases can have opposing effects with Rac and Cdc42 causing membrane extension and Rho causing membrane retraction.

Rho, Rac, and Cdc42 also control the assembly of structures that are intimately associated with the actin cytoskeleton like cell-matrix and cell-cell contacts. Rho, for example, is required for the assembly of classical focal adhesions, while Rac and Cdc42 induce so-called focal contacts found at the leading edge of migrating cells [4,7,8]. Rho, Rac, and Cdc42 have also been reported to participate in the formation of cadherin-dependent cell-cell adhesion complexes [9], as well as tight junction complexes in epithelial cells [10]. Whether these effects on surface adhesion complexes are direct or mediated through the actin cytoskeleton is not entirely clear.

Cell Migration

Rho GTPases generate the protrusive forces needed for forward movement of migrating cells. Rac, in particular, is essential for the protrusion of lamellipodia at the leading

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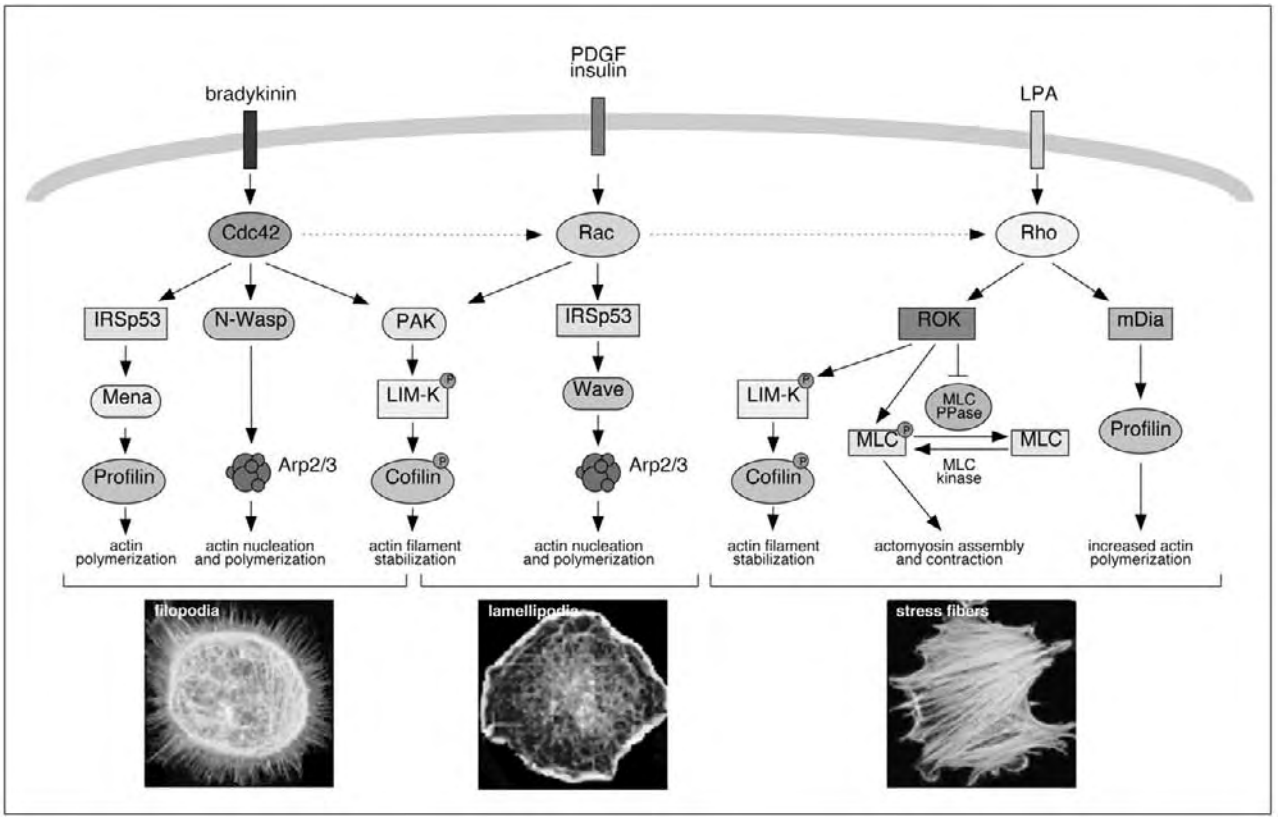


Figure 1 Schematic representation of Rho, Rac, and Cdc42 signaling pathways leading to organization of the actin cytoskeleton. Pictures of Swiss3T3 fibroblasts stained for filamentous actin structures are reprinted with permission from A. Hall (1998). *Science* **279**, 509–514, Copyright 1998 American Association for the Advancement of Science.

edge of a moving cell, while Cdc42-stimulated filopodia have been implicated in the sensing of chemotactic gradients and may be important for polarized movement [11,12]. By regulating focal adhesion sites, Rho, Rac, and Cdc42 can also control the transient loss and reactivation of substrate attachment required for traction during cell movement.

Neuronal Morphology and Guidance

Rho GTPases are important regulators of neurite outgrowth, guidance, and plasticity [13]. Rac and Cdc42 have been shown to stimulate neurite outgrowth and extension, dendritic branching and spine formation, and growth cone guidance toward attractive extracellular guidance cues. In contrast, Rho induces neurite retraction, prevents spine formation, and mediates growth cone collapse in response to repulsive cues.

Phagocytosis

Rho, Rac, and Cdc42 also drive cytoskeletal remodeling required for particle internalization during phagocytosis [14], and Rac and Cdc42 are required for uptake of fluids and particles during pinocytosis [15]. Interestingly, during bacterial invasion, pathogens have found ways to modulate the activity of Rho, Rac, and Cdc42 thereby eliciting changes in the actin cytoskeleton facilitating entry into the host cell [16].

Much less is known about the effects of other Rho GTPases on the actin cytoskeleton. The Cdc42-like protein TC10 induces filopodia [17], while two other Cdc42-like proteins, TCL and Chp, induce membrane ruffling [18,19]. Rnd1 and Rnd3 (RhoE), two apparently constitutively active Rho GTPases, cause disassembly of stress fibers and disruption of cortical actin filaments at least when overexpressed in cells [20]. RhoD stimulates the formation of actin-containing membrane protrusions and seems to antagonize Rho action by inducing stress fiber disassembly and blocking cytokinesis [21]. RhoG has been shown to induce Rac-dependent membrane ruffling and neurite outgrowth [22,23]. However, the physiological significance of these effects remains to be determined.

Signaling from Rho GTPases to the Actin Cytoskeleton

Signaling from Rho to the Actin Cytoskeleton

The pathways leading to Rho-induced actin:myosin filament assembly are relatively well understood and primarily involve two Rho-effectors, ROK and mDia (Fig. 1).

ROKα (Rho associated kinase, ROCKII) and its homolog ROKβ (ROCKI) are Ser/Thr kinases that bind activated Rho and are required for stress fiber formation and cytoskeletal contraction [24]. Constitutively active ROK stimulates the formation of stellate stress-fiber-like structures, whereas kinase-dead ROK or pharmacological inhibitors of ROK inhibit Rho-induced stress fibers. Several targets of ROK have been identified which are likely to be involved in stress fiber formation and contraction. ROK phosphorylates and thereby inhibits myosin-light chain (MLC) phosphatase and can also phosphorylate MLC directly. Together, this leads to an increase in phosphorylated MLC, activation of myosin motor function, and subsequent actin-myosin-based contraction. ROK can also phosphorylate and activate LIM kinase (LIM-K) which in turn phosphorylates and inhibits the actin-severing protein cofilin, leading to stabilization of actin-filaments.

The Rho effectors mDia1 and 2 are members of the formin-related protein family and contain three formin homology domains through which they interact with the actin monomer-binding protein profilin and stimulate actin polymerization [25]. Dominant-active mDia induces weak stress fiber formation and dominant-negative mDia inhibits Rho-induced stress fiber formation. Co-expression of active ROK and mDia induces stress fibers very reminiscent of those induced by activated Rho suggesting that the cooperative activity of these two target proteins is crucial for actin:myosin filament assembly [26].

Signaling Downstream of Cdc42 and Rac

Recently, considerable progress has been made in identifying downstream effectors of Cdc42 and Rac which may mediate filopodia and lamellipodia formation (Fig. 1). The Wiscott-Aldrich-syndrome protein (WASP) and its neuronal isoform N-Wasp have been implicated in filopodia formation downstream of Cdc42 [27]. Overexpression of N-WASP leads to the enhanced formation of filopodia, and Cdc42-induced filopodia can be blocked by anti-N-WASP antibodies or WASP-deletion mutants. Activated Cdc42 binds to WASP and synergizes with PI4,5P_2 to unfold the protein allowing it to interact with and activate the Arp2/3 complex. WASP also interacts with monomeric actin and profilin acting as a scaffold protein to bring together the machinery required for actin polymerization. The Arp2/3 complex is known to initiate actin nucleation and stimulate the formation of branched networks of actin as seen in lamellipodia [28]. However, it is not clear how Arp2/3 controls the formation and organization of the straight actin bundles seen in filopodia.

Another member of the WASP family, Wave/Scar, has been reported to mediate Rac-induced membrane ruffling by stimulating the Arp2/3 complex [27]. Rac activates Wave via the insulin receptor substrate p53 (IRSp53) which interacts both with GTP-bound Rac and Wave [29]. Interestingly, IRSp53 and its brain-enriched isoform IRSp58 also bind to activated Cdc42 and participate in filopodia formation and neurite outgrowth [30,31]. In this case, actin polymerization is mediated by binding of IRSp53 to Mena, a member of the Vasp/Ena family that interacts with profilin. Co-expression of Mena and IRSp53 strongly enhanced filopodia formation, though whether the interaction of Mena with profilin is needed in the formation of filopodia is unknown.

The PAK family of Ser/Thr kinases (PAK1, 2, and 3) also appears to promote actin assembly [32]. PAK binds to both Cdc42 and Rac and activated mutants of PAK1 have been reported to stimulate filopodia formation and membrane ruffling in fibroblasts, and induce neurite outgrowth in neuronal cells. Like ROK, PAK phosphorylates LIM-K leading to stabilization of actin filaments. PAK also phosphorylates and inactivates MLC kinase thereby reducing MLC phosphorylation and subsequent actomyosin assembly.

Conclusions

In the last decade it has emerged that Rho GTPases play a pivotal role in controlling actin filament assembly/disassembly during actin-based processes in all eukaryotic cells. A surprisingly large number of downstream effectors of Rho GTPases have been identified and some have been shown specifically to mediate signaling to the actin cytoskeleton. However, the detailed biochemical mechanisms by which this occurs are still unclear. The central role of Rho GTPases in regulating cell migration and bacterial invasion underscores their potential importance in the development of therapeutic agents to treat metastatic cancer or bacterial infections.

Acknowledgments

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CHAPTER 238

Regulation of the NADPH Oxidase by Rac GTPase

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Phagocytic leukocytes play critical roles in the innate immune response to pathogens. An important component of this response is the ability of leukocytes to generate reactive oxygen species (ROS) via a membrane-associated NADPH oxidase [1,2]. This multicomponent enzyme utilizes electrons derived from intracellular NADPH to generate superoxide anion, which subsequently dismutates to H_2O_2 and other ROS that are used for host defense. The NADPH oxidase of phagocytic leukocytes was the first identified, and remains one of the best-characterized, Rho GTPase-regulated systems. Historically, the involvement of a GTP binding protein in the NADPH oxidase regulation was suspected when guanine nucleotide analogs such as GTPγS and Gpp(NH)p were found to enhance superoxide production in cell-free assays [3–5]. A requirement for a prenylated cytosolic component suggested the involvement of a small GTPase [6]. Subsequently, it was simultaneously shown that either Rac1 or Rac2 GTPase was required for oxidase activity in the cell-free system [7,8], with Rac2 being the predominantly active isoform in human neutrophils [9]. Several additional lines of evidence have established that Rac is an integral and required component of the NADPH oxidase. Rac antisense oligonucleotide inhibited superoxide production in Epstein-Barr virus (EBV)-transformed B lymphocytes [10]. In neutrophils of bcr-null transgenic mice, Rac activity was increased due to the loss of a GTPase-activating protein, Bcr, and superoxide production was concomitantly increased, suggesting that not only is Rac activation required, but that it may be rate-limiting for oxidase activity [11]. Finally, the generation of Rac2-null mice led to the demonstration that *rac*2 –/– neutrophils had significantly reduced or absent superoxide production in response to various stimuli [12].

Components and Regulation of the NADPH Oxidase

The NADPH oxidase system of stimulated neutrophils catalyzes the one-electron reduction of oxygen to produce superoxide anion using NADPH as substrate (Fig. 1). In the unstimulated neutrophil, Rac exists as a complex with GDP dissociation inhibitor (GDI) in the cytosol [13]. When the phagocyte is activated through the action of soluble chemoattractants, chemokines, or phagocytic particles, Rac dissociates from GDI by an as yet undetermined mechanism. GDP is exchanged for GTP through the action of membrane-localized guanine nucleotide exchange factors (GEFs) [14] and Rac, now in its GTP-bound active form, becomes membrane associated [15]. Activation of the neutrophil also results in phosphorylation on multiple sites of cytosolic $p47^{phox}$, which exists in a complex with a third cytosolic component, $p67^{phox}$, followed by translocation of the $p47^{phox}$/$p67^{phox}$ complex to the membrane [16,17]. Phosphorylation of $p47^{phox}$ is thought to lead to the disruption of an inhibitory, intramolecular interaction within $p47^{phox}$, allowing exposed SH3 domains in $p47^{phox}$ to interact with proline-rich regions of other NADPH oxidase components [reviewed in references 1, 2, and 18]. The translocation of the $p47^{phox}$/$p67^{phox}$ complex occurs simultaneously and independently from the translocation of Rac GTPase [9,19]. $p47^{phox}$ is now known to be dispensable for NADPH oxidase activity under cell-free conditions, and appears to serve primarily as an adapter to facilitate membrane binding of $p67^{phox}$ [20]. The translocation of $p47^{phox}$, $p67^{phox}$, and Rac to the plasma membrane culminates in the formation of an active complex with integral flavocytochrome b_{558} (cyt *b*). Cyt *b* possesses an NADPH binding site, FAD, 2 hemes, and 2 subunits, $gp91^{phox}$ and $p22^{phox}$ [21]. The formation of this minimal

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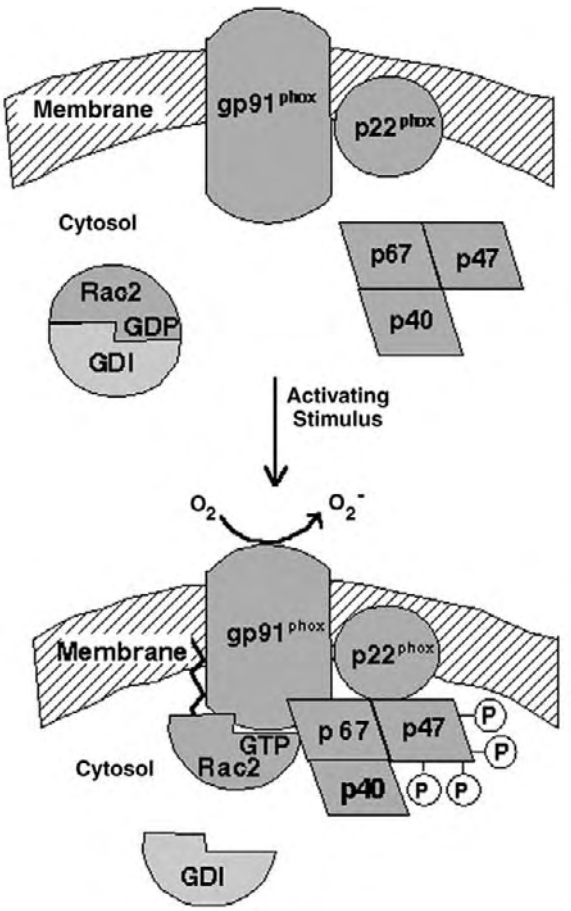


Figure 1 Formation of the NADPH oxidase complex. In the unstimulated neutrophil, cytosolic $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ are believed to exist as a complex in the cytosol. The other cytosolic component, Rac1/2 GTPase in its GDP-bound form exists in a separate complex with Rho GDP dissociation inhibitor (RhoGDI). Upon activation of the neutrophil, Rac is released from GDI and nucleotide exchange occurs. Rac in its GTP-bound form translocates to the membrane. Also during activation, $p47^{phox}$ is phosphorylated on multiple serines leading to the translocation of the p47/p67/(p40?) complex to the membrane; this event is separate from the translocation of Rac GTPase. At the membrane, the cytosolic components interact with cytochrome b_{558}, which is composed of two subunits, $gp91^{phox}$ and $p22^{phox}$. $gp91^{phox}$ contains the binding site for NADPH, FAD, and two heme groups. The interaction of the cytosolic components with cytochrome b_{558} allows electrons to flow from NADPH to FAD to the heme groups, and finally to the heme-bound oxygen to form superoxide anion.

complex allows electrons to flow from NADPH to FAD and from FAD to the heme of cyt *b*, and finally to the heme-bound oxygen whose reduction leads to the formation of superoxide anion. An additional cytosolic component, termed $p40^{phox}$ [22], may play a role in regulating the response of the system to phosphatidylinositol-3-phosphate *in vivo* [23], but is not required for NADPH oxidase activity in the cell-free system.

The Role of Rac in NADPH Oxidase Regulation

Understanding the precise nature of the role of Rac in the oxidase regulation is facilitated by an examination of the

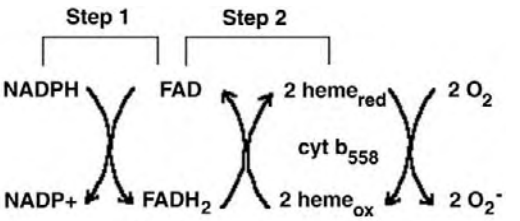


Figure 2 Electron transfer steps of the NADPH oxidase. Step 1 of electron transfer in the NADPH oxidase is defined as the pathway from the cytoplasmic electron donor NADPH to cytochrome b_{558}-bound FAD. Step 2 is defined as the pathway from FAD to the cytochrome b_{558}-associated heme groups.

specific domains of this GTPase. Most studies report that Rac is able to support oxidase activity only in its GTP-bound active form [24]. (There may be an exception to this—see reference [25]). This indicates that the switch I domain is important for Rac action on the oxidase (the switch II domain only undergoes minimal conformational changes upon GTP binding). Consistent with this, point mutations within the switch I or effector domain of Rac, including RacD38A, Y40K, A27K, and G30S, were unable to support oxidase activity [26–29]. The finding that Rac binds directly to $p67^{phox}$ (but not $p47^{phox}$) via the switch I domain provided important insight into Rac function in the oxidase [26,30]. It was shown that the tetratricopeptide repeat (TPR) in the N terminus of $p67^{phox}$ was the site of Rac binding [31], and this was confirmed upon determination of the crystal structure of the Rac-p67(TPR) complex [32]. The structure revealed specific stabilizing interactions between amino acids A27 and G30 of Rac and the TPR domain of $p67^{phox}$. At the same time, this structure revealed the availability of other Rac surface domains, particularly the insert domain present in members of the Rho GTPase subfamily, for possible protein interactions. Several prior investigations had suggested a requirement for the Rac insert domain in the activation of the NADPH oxidase. Peptide walking experiments indicated that blocking the insert domain of Rac abrogated NADPH oxidase activation [33]. Studies using insert domain deletion mutants of Rac have yielded conflicting results, however, concluding either that the insert domain was absolutely required [34,35] or unnecessary [36,37] for Rac oxidase activity.

The role of Rac in NADPH oxidase regulation had been generally supposed to be that of a docking protein, in which the prenylated C terminus of Rac bound to membrane phospholipids, while the switch I domain facilitated the binding of the $p67^{phox}$/$p47^{phox}$ complex to cyt *b*. Our laboratory recently investigated the role of Rac in the electron transfer reactions of the NADPH oxidase using a purified cell-free system [34]. We determined that Rac2 was required for both electron transfer steps (from NADPH to FAD and from FAD to cyt *b*) (Fig. 2). In order to assess whether Rac2 had a functional role that was distinct from its interaction with $p67^{phox}$, we used Rac2 and p67 constructs that were mutated in domains involved in Rac/$p67^{phox}$ interactions. We observed

that the non-Rac-binding mutant, p67 Δ178-184, could still support electron transfer from NADPH to FAD (step 1), but not from FAD to cyt *b* (step 2). In a reciprocal experiment, we observed that a Rac2 mutant (Rac2 D38A) that is not able to bind $p67^{phox}$ supported step 1, but not step 2 activity. These data suggested that Rac2 and $p67^{phox}$ did not have to functionally interact for electrons to be transferred from NADPH to FAD, but that a Rac/$p67^{phox}$ interaction was required for completion of electron transfer from $FADH_2$ to cyt *b*. Furthermore, since Rac2 was operative in the absence of $p47^{phox}$, this indicated that Rac2 must interact directly with cyt *b* to support the step 1 reaction. Based upon these observations, we proposed a two-step model for regulation of the NADPH oxidase by Rac2 GTPase (Fig. 3).

We hypothesized that the insert domain of Rac2 may play a role in binding to cyt *b* and regulation of electron transfer. Using an insert domain deletion mutant of Rac2 in our electron transfer assays, we found that the insert domain was critical for the step 1, and consequently, step 2 reactions. We furthermore demonstrated that the intensity of a fluorescent analog of mant-GppNHp bound to Rac2 increased in the presence of cyt *b*, indicating direct interaction between Rac2 and cyt *b*. In addition, using the insert domain deletion mutant of Rac2 in place of wild-type Rac2 eliminated this interaction, indicating that the insert domain of Rac2 is necessary for both the functional and physical interaction of Rac2 with cyt *b*. In support of the fluorescence data, we have recently demonstrated that glutathione-S-transferase (GST)-Rac2 can specifically bind cyt *b* purified from neutrophils in pulldown assays (Diebold and Bokoch, unpublished observations).

Current Models of Rac Function in NADPH Oxidase Regulation

Overall these data suggest a novel paradigm for NADPH oxidase regulation by Rac2 GTPase, showing that Rac2 can interact directly with cyt *b* and that Rac2 has a separate and required function in regulating catalytic activity of the NADPH oxidase (Fig. 4C). These two points differ from other models. The model of Lambeth *et al*. [2] (Fig. 4A) agrees with the first point that the insert domain of Rac may bind to cyt *b*. However, the only role of the insert domain of Rac according to this model is to facilitate binding of $p67^{phox}$. $p67^{phox}$, which has an activation domain and also binds cyt *b*, is proposed in the model of Lambeth *et al.* to be the only protein influencing the rate-limiting electron transfer step (step 1) of the NADPH oxidase (reviewed in [2]). Their model is based on the observation that a nonprenylated Rac1 mutant, which lacks the insert domain, decreased the affinity (EC_{50}) of Rac for the oxidase, but had no effect

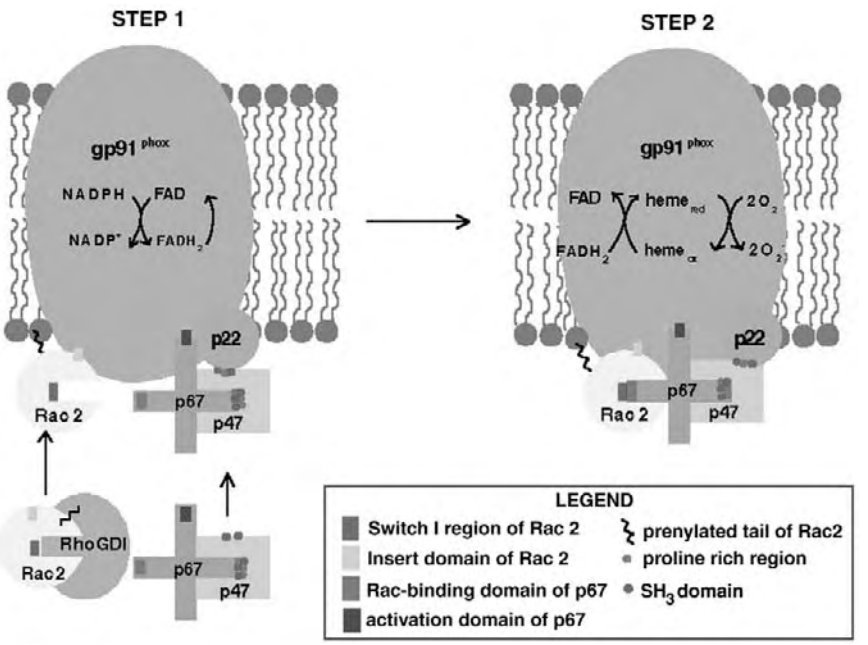


Figure 3 Two-step model for the regulation of the NADPH oxidase by Rac GTPase. Diebold and Bokoch [34] proposed a two-step model for the regulation of NADPH oxidase by Rac. In step 1, Rac translocates to the membrane and interacts with the phospholipid bilayer via its prenylated C terminus. In addition, Rac, via its insert domain, interacts with cytochrome b_{558} and contributes to the regulation of electron flow from NADPH to FAD without interacting with $p67^{phox}$. $p67^{phox}$ is still required for electron flow to occur in step 1 and regulates electron flow via its activation domain. The interaction of the insert domain of Rac with cytochrome b_{558} may induce a conformational change in cytochrome b_{558} that improves the interaction of $p67^{phox}$ and cytochrome b_{558}. In step 2, the interaction between the switch I domain of Rac and the Rac-binding domain of $p67^{phox}$ is required for electrons to continue to flow from FAD to the heme groups of cytochrome b_{558}.

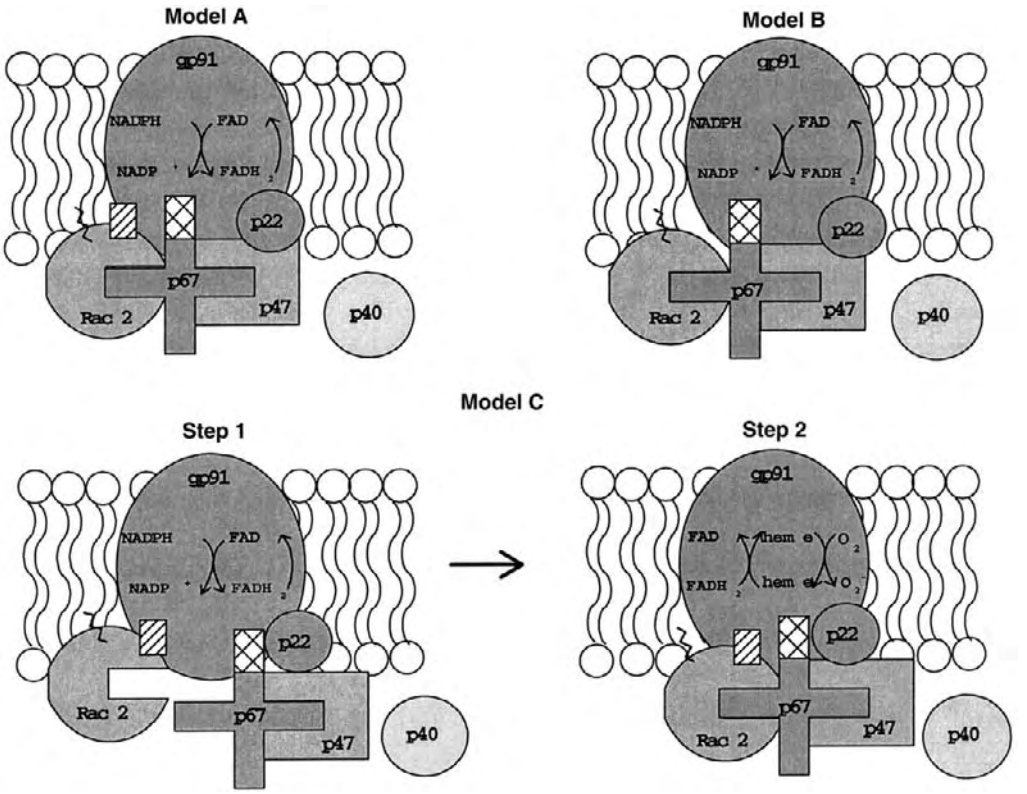


Figure 4 Comparison of proposed models of NADPH oxidase regulation by Rac. In each of the existing models, the switch I region of Rac (not indicated) interacts with $p67^{phox}$, the prenylated tail of Rac (shown as a zigzag line) interacts with the membrane, and the activation domain of $p67^{phox}$ (a crosshatched section) interacts with cytochrome b_{558}. In the model of Diebold and Bokoch (C) [34] and that of Lambeth *et al.* (A) [2], the insert domain of Rac (hatched section) has direct protein interactions with cytochrome b_{558}. The Diebold and Bokoch model differs in that Rac contributes to the regulation of electron flow from NADPH to FAD, while Lambeth proposes that only the activation domain of $p67^{phox}$ regulates this electron transfer step. In the latter model, Rac and $p47^{phox}$ serve as adapters aiding in the interaction of $p67^{phox}$ with cytochrome b_{558}. In model B proposed by Pick *et al* [38], Rac interacts only with the phospholipids of the membrane via its prenylated C terminus and does not interact with cytochrome b_{558}. In this model, the insert domain is not involved in protein interactions or regulation of the NADPH oxidase. As in the model of Lambeth *et al*., $p67^{phox}$ is the only regulatory protein, while Rac and $p47^{phox}$ serve as adapters for $p67^{phox}$.

on the maximal rate, V_{max}, of superoxide production [35]. Our laboratory, on the other hand, has observed a decrease in V_{max} when using a prenylated Rac2 version of this mutant [34].

The model of Pick *et al.* [38] (Fig. 4B) opposes the view that Rac interacts with cyt *b*. Instead, they propose that Rac interacts only with the phospholipids within the plasma membrane via its prenylated C terminus. This model is based upon observations that prenylated Rac can bind to phospholipid vesicles devoid of cyt *b*. (Phospholipids are used to re-lipidate cytochrome b_{558} purified from neutrophil membranes before it is used in cell-free assays). Like the previous model, this model portrays Rac and $p47^{phox}$ only as adapter molecules that aid $p67^{phox}$ in binding to cyt *b*. Interestingly, this group of investigators initially observed in a peptide walking study that peptides overlapping the insert domain of Rac inhibited superoxide production in the cell-free system [33]. Recently, however, this group used non-prenylated Rac1-$p67^{phox}$ chimeras in the cell-free system and showed that deletion of the insert domain of Rac1 did not affect the ability of this chimera to support superoxide production [37]. Consequently, based on these observation and observations by Lambeth's group that deletion of the insert domain does not affect V_{max} [35], the model of Pick *et al*. [38] does not support an interaction of the Rac insert domain with cyt *b* (Fig. 4B). At least part of the discrepancy between these models may be due to the use of prenylated Rac (Bokoch model) versus nonprenylated Rac (Lambeth and Pick models) in the cell-free system. The concentration of prenylated Rac required in the cell-free assay is at least 100-fold less than nonprenylated Rac. The use of such high concentrations of unprocessed GTPase may obscure relevant protein-protein interactions that normally occur at physiological concentration of reactants. Ultimately, a consensus model for the role of Rac in the oxidase will depend upon *in vivo* studies using neutrophils or cell lines bearing resemblance to neutrophils.

Rac GTPase—A More General Role in Regulating Oxidant-Based Signaling?

The finding that Rac can bind to cyt *b* and regulate electron transfer in the NADPH oxidase of neutrophils is relevant to other ROS-generating signaling pathways used by nonphagocytic cells. Recently, homologs of cyt *b*, called Nox, have been found in several tissues (reviewed in [39]). These new *N*ADPH *ox*idases produce low levels of superoxide anion that appear to be used as signals for the control of cell growth and transformation. It has been known for some time that Ras and Rac contribute to the control of signaling pathways that are critical for mitogenesis and oncogenesis. In stimulated NIH3T3 cells, transient expression of a constitutively activated form of Ras leads to a significant increase in intracellular ROS, and expression of a dominant negative allele of Ras or Rac1 inhibited this rise in ROS production [40,41]. ROS production in NIH3T3 cells was suppressed by treatment with a diphenylene iodonium (DPI), a flavoprotein inhibitor of NADPH oxidase of phagocytes, indicating that a Nox protein may be involved [41]. Interestingly, it has been shown that the insert region of Rac1 was essential for mitogenesis and superoxide production in fibroblasts [42]. Rac1 also appears to be involved in the signaling pathway leading to reperfusion injury caused by ROS production during reoxygenation of vascular smooth muscle [43,44]. Recombinant adenoviral expression of a dominant negative Rac1 suppressed the reperfusion-induced injury in an *in vivo* model of mouse hepatic ischemia/reperfusion injury. This was also observed in mice deficient for the $gp91^{phox}$ of phagocytic NADPH oxidase, suggesting that the Rac mutant inhibited ROS production by a Nox system rather than by one employing $gp91^{phox}$ [44]. Thus, it appears that ROS production in nonphagocytic cells involves both a Nox protein and Rac GTPase. The possibility exists that Rac may directly regulate superoxide production by Nox proteins in nonphagocytic cells by a mechanism similar to its binding to cyt *b* and regulating oxidant production by phagocytes. Determining if Rac has a function in superoxide production by Nox may lead to a better understanding of redox signaling pathways in other cell types and potentially provide a means to therapeutically intervene in ROS-related pathological disease states.

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CHAPTER 239

The Role of Rac and Rho in Cell Cycle Progression

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Introduction

The ability of cells to progress through the cell cycle depends on the concerted action of mitogen- and anchorage-stimulated signal transduction pathways. The regulation of the cell cycle machinery is often disrupted in tumor cells, with the most common targets being proteins involved in G1 progression. Identifying the signaling pathways responsible for G1 progression is therefore important for increasing the understanding of control mechanisms that operate during normal cell proliferation and their subversion in tumor cells. One class of signaling proteins that has recently been recognized to play a significant role in G1 phase progression is the small GTP binding proteins of the Rho GTPase family. This chapter will focus on the role of two members of the Rho GTPase family, Rac and Rho, in G1 progression.

Regulation of G1 Progression

Cell cycle progression through G1 is a complex and tightly controlled process. It is regulated by stimulatory and inhibitory signals, both of which are targets of the Rho GTPases. Three activities are recognized to be important for progression through the G1 phase of the cell cycle: early-G1 transcriptional activation of immediate early genes, mid-G1 activation of cyclin D/cdk4/6, and late-G1 activation of cyclin E/cdk2 [1,2]. Mitogenic stimulation results in the induction of many immediate early genes containing the serum response element (SRE) in their promoter [1]. The SRE is activated by binding to a ternary complex containing the transcription factors, serum response factor (SRF) and ternary complex factor (TCF) [1]. Although activation of immediate early genes is necessary for early G1 progression, it is not sufficient for progression to S phase, and progression through later phases of G1 requires the activity of cyclin-dependent kinases (cdks).

Cdks are a group of serine/threonine kinases that are activated by binding to their respective cyclin partners and by phosphorylation [2]. Two main cdk activities play a role in G1, cyclin D/cdk4/6 functions in mid G1 and cyclin E/cdk2 functions in late G1. The major substrate of the G1 kinase complexes is the retinoblastoma protein (Rb). In its unphosphorylated form, Rb functions as an inhibitor of E2F, a transcription factor that controls the expression of genes required for G1 progression [3]. The inhibitory effect of Rb on E2F transcriptional activity is exerted by two mechanisms, one involving the direct binding to E2F and the other involving the recruitment of histone deacetylase (HDAC) [4]. Both inhibitory effects are antagonized by the coordinated and sequential phosphorylation of Rb by cyclin D/cdk4/6 and cyclin E/cdk2 which in turn allows the ordered expression of E2Fdependent genes [4,5]. Phosphorylation of Rb by cyclin D/cdk4/6 initially releases HDAC thereby alleviating transcriptional repression, and phosphorylation of Rb by cyclin E/cdk2 dissociates the Rb-E2F complex [6,7].

An important mechanism for regulation of cyclin/cdk activity involves inhibition by cyclin-dependent kinase inhibitors. The main inhibitors of cyclin D/cdk4/6 complexes are $p16^{ink4a}$ and $p21^{Cip1}$, whereas inhibition of cyclin E/cdk2 occurs by $p21^{Cip1}$ and $p27^{Kip1}$[2]. The levels of these inhibitors are regulated by multiple mechanisms. $p21^{Cip1}$ is regulated predominantly at the level of transcription [8] and mRNA stability [9]. $p27^{Kip1}$ can be regulated at multiple

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levels including transcriptional [10], translational [11,12], and posttranslational [13,14] mechanisms dependent on the cell type and the extracellular signal. However, the predominant mechanism by which p27 ^{Kip1} levels are controlled is through cyclin E/cdk2-dependent phosphorylation [15,16], which targets p27^{Kip1} for ubiquitination and proteolytic degradation [13].

The Function of Rac and Rho in Cell Cycle Progression and Transformation

The Rho family of GTPases functions as molecular switches by oscillating between an active GTP-bound form and inactive GDP-bound form. Activation of the Rho GTPases can be induced by soluble growth factor stimulation and cell adhesion to the extracellular matrix (ECM). Their biological effects are exerted through the activation of multiple effector pathways that control transcription, cytoskeleton organization, and changes in the redox state [17].

The importance of the Rho GTPases in cell cycle progression was initially illustrated through studies demonstrating their involvement in both growth-factor-induced proliferation and oncogenic transformation. Rac1 and RhoA are each required for transformation by Ras and co-expression of a constitutively active form of Raf, a Ras effector, with either constitutively active RhoA or Rac1 synergistically enhances focus-forming activity [18,19].

Rac and Rho have been shown to be necessary and sufficient for cell cycle progression. In Swiss 3T3 cells, introduction of dominant interfering forms of Rac1 and RhoA inhibits progression of growth-factor-induced cell cycle progression, while a dominant active form of each is sufficient to induce cell cycle progression [20]. However, the capacity of Rac1 and RhoA to promote cell cycle progression is cell-type specific. For example, in rat embryo fibroblasts, G1 to S transition requires the synergistic activities of Rac and Raf [21]. Using partial loss of function mutants of Rac it has been shown that the contribution of Rac to cell cycle progression is dependent on two distinct effector functions, cytoskeleton rearrangements and superoxide production [21,22]. Rac-induced cytoskeleton rearrangements are mediated by the effector binding loop [21], a region spanning amino acids 26 to 40 that interacts with multiple downstream effector molecules. Rac-dependent superoxide generation is controlled by the insert region, a sequence of 11 amino acids common to all of the RhoGTPase family members, but not found in the Ras GTPase family members [23]. Significantly, superoxide generation has been shown to be essential for Ras-induced proliferation [24] indicating that Rac-mediated superoxide production might be functionally relevant to Ras-induced proliferation. It is noteworthy that the insert region of Rho also plays an important role with regard to cell cycle progression through the activation of the Rho effector, Rho kinase, which cooperates with activated Raf to promote transformation [25,26].

Cell Cycle Targets of Rac and Rho

Increasing evidence indicates that Rac and Rho influence cell cycle progression by targeting multiple regulatory steps throughout G1. A well-documented mechanism by which Rac and Rho affects early-G1 involves the activation of genes controlled by the SRE. Rac, through its effector PAK, promotes the phosphorylation of both Raf and MEK, two components of the signaling cascade leading to ERK activation [27,28]. Both PAK-mediated phosphorylation events act synergistically with the Ras pathway to promote full activation of ERK [27,29]. Subsequently, activated ERK phosphorylates and activates TCF thereby stimulating SRE-dependent transcription (Fig. 1). Rac activity has also been demonstrated

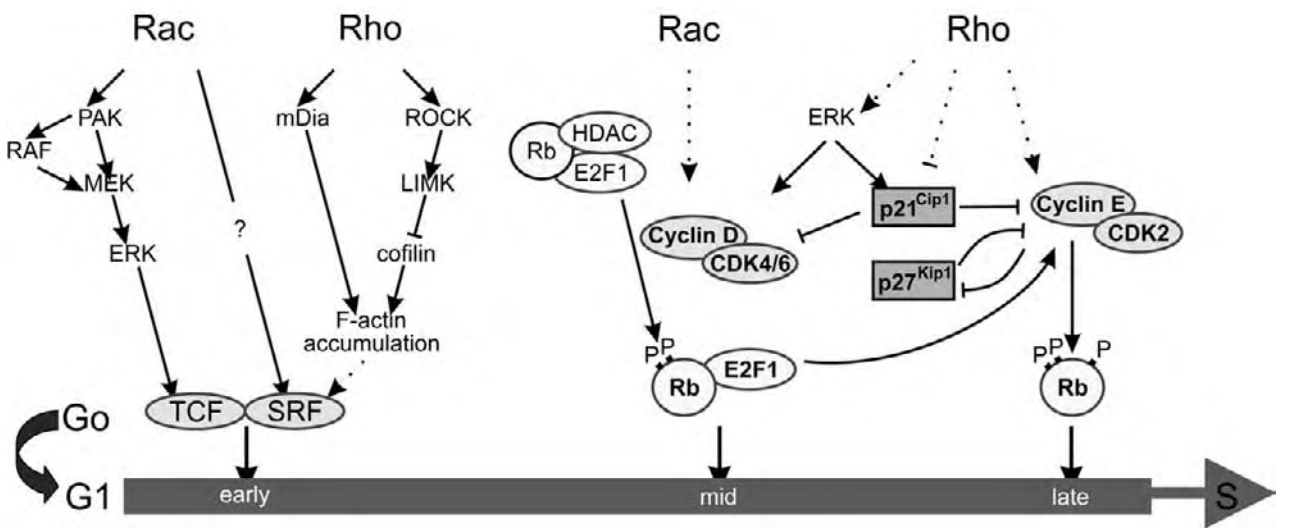


Figure 1 Integration of Rac and Rho signaling pathways with the cell cycle. Rac and Rho target multiple regulatory events during the G1 stage of the cell cycle. The contribution of Rac and Rho to TCF/SRF and cyclin/cdk complexes promotes G1-phase progression.

to potentiate SRF activity, but the signaling pathways regulating this response have not been identified [30,31].

RhoA activity is essential for mitogen-induced activation of SRF [30]. The ability of Rho to activate SRF is linked to its effect on actin cytoskeleton dynamics. This is indicated by studies showing that the Rho effectors LIMK and mDia potentiate SRF activity independently of extracellular signals [32–34]. Although the relative contribution of the Rho-dependent pathways to SRF activity seems to be cell-type dependent, both LIMK and mDia pathways contribute to F-actin accumulation, suggesting a causal role for F-actin levels in the activation of SRF (Fig. 1).

Progression through mid G1 of the cell cycle is dependent upon upregulation of cyclin D and formation of the cyclin D/cdk4/6 complex. Both Rac and Rho have been shown to contribute to the upregulation of cyclin D1 through ERK-dependent and -independent pathways (Fig. 1). Rac-mediated induction of cyclin D1 occurs in part through the Rac effector PAK [31], and is also dependent on NF-κB activation as evident from the findings that an intact NF-κB binding site in the cyclin D1 promoter is required for Rac-dependent cyclin D1 transcription [35].

Recent evidence by Welsh *et al.* demonstrates that Rho plays a central role in controlling adhesion- and mitogen-dependent cyclin D1 expression [36]. First, in early G1, Rho inhibits Rac-induced expression of cyclin D1 by antagonizing Rac through an unknown mechanism [36]. Second, in mid-G1 phase of the cell cycle, Rho promotes cyclin D1 induction by maintaining a sustained activation of ERK [36]. The mechanisms by which Rho might contribute to ERK activity are not well defined, but the Rho kinase pathway seems to be necessary for this effect [36]. Thus, Rho appears to have an important role in setting up the correct timing of cyclin D1 expression during cell cycle progression.

In addition to its role in the regulation of cyclin D1 expression, Rho regulates cyclin D/cdk4/6 activity by inhibiting the accumulation of the cdk inhibitor $p21^{Cip1}$ [37]. For example, in some cell types, high levels of Ras or Raf activities induce $p21^{Cip1}$ expression and cell cycle arrest [38–40], and this effect can be rescued by ectopic expression of activated Rho [37]. Furthermore, mouse embryo fibroblasts lacking $p21^{Cip1}$ do not require Rho for Ras-induced S-phase entry [37]. Rho can also be involved in the regulation of late-G1 progression by activating the cyclin E/cdk2 complex, which in turn promotes the degradation of the cdk inhibitor $p27^{Kip1}$ (Fig. 1) [41]. Together, the effects of Rho on the levels of cdk inhibitors are likely to contribute to the ability of cells to undergo G1 to S progression in response to proliferative signals.

Future Perspectives

Although, as outlined in this chapter, the involvement of Rac and Rho in regulating cell cycle progression is supported by many lines of evidence, the biochemical mechanisms that couple the signaling activities of these GTPases and the cell cycle machinery remain to be established. By virtue of their effects on the actin cytoskeleton, Rac and Rho play a key role in the regulation of cell shape changes that accompany adhesion and motility. It is well recognized that cell shape is an important determinant for the proliferative capacity of normal anchorage-dependent cells and loss of cell-shape-dependent growth control is a hallmark of oncogenically transformed cells. Thus, understanding the molecular basis for the involvement of Rac and Rho in cell cycle regulation should provide insights into the mechanisms by which alterations in cellular morphology can be sensed and converted to a growth response.

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CHAPTER 240

Cdc42 and Its Cellular Functions

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Cdc42 is a member of the Rho subfamily of small GTP-binding (G) proteins. A variety of biochemical and cellular studies have shown that Cdc42 plays important roles in the regulation of cell growth, differentiation, programmed cell death, and in the establishment of cell polarity. The molecular mechanisms underlying these biological activities have emerged from recent breakthrough studies of the interactions of Cdc42 with its various downstream targets including Wiscott-Aldrich Syndrome Protein (WASP), γ-coatomer (γ–COP), and the p21-activated kinase (PAK), activated Cdc42-associated kinase (ACK) and partitioning-defective (PAR) proteins. Through these interactions, Cdc42 impacts two major cellular activities: F-actin polymerization and membrane vesicle trafficking. These findings now lead to the suggestion that Cdc42 serves as a convergence point for pathways that influence actin cytoskeletal architecture and intracellular trafficking.

Introduction

Cdc42 is a member of the Rho subfamily of Ras-related (small) GTP-binding proteins. The CDC42 gene was initially identified in *Saccharomyces cerevisiae* as being essential for polarized growth and assembly of the bud-site [1]. The human homolog, originally designated Cdc42Hs, was independently cloned following its identification as a potential participant in epidermal growth factor (EGF) receptor-coupled signaling [2,3]. Early microinjection studies in Swiss3T3 cells indicated that Cdc42 caused filopodium or microspike formation, thus linking it to actin cytoskeletal organization [4,5]. Now after a decade of studies, a variety of upstream signaling activators, which catalyze the guanine nucleotide exchange activity of Cdc42 (thus designated guanine nucleotide exchange factors or GEFs) have been identified including the prototype GEF, Dbl (diffuse B-cell lymphoma, as well as Cdc24, Fgd1 (facial genital dysplasia, intersectin-l/Ese1 (EH domain and SH3 domain regulator of endocytosis), hPEM (human homolog of ascidian protein posterior end mark-2), Brx (breast cancer nuclear hormone receptor auxiliary factor), and Clg (common-site lymphoma/leukemia GEF) [1, 6–8]. It is now felt that a variety of receptor-coupled signaling pathways feed into these GEFs, including those mediated by receptor tyrosine kinases, trimeric G-protein-coupled receptors, neurotrophic receptors, integrins, and cytokine receptors [12–18]. A number of targets or effectors suspected to function downstream of Cdc42 have also been discovered and characterized, including PAK, ACK, myotonic related Cdc42-associated kinase (MRCK), WASP, IQGAP, γ-COP, partitioning-defective protein 6 (PAR6) and binder of Rho GTPases (BORG) [1,9,10]. These discoveries seem to position Cdc42 in signaling pathways that regulate a vast array of cellular activities, ranging from cell growth and differentiation to apoptosis, as well as a number of fundamentally important molecular events including gene transcription, actin cytoskeletal organization, membrane vesicle endocytosis and trafficking, cell adhesion and migration, and RNA processing and transport [11]. In this review, we will focus on the molecular mechanisms underlying the ability of Cdc42 to mediate its cellular functions.

Biological Effects of Cdc42

Cell Growth Regulation

Cdc42 is essential for cell growth. Early genetic studies in *S. cerevisiae* showed that yeast cells containing function-defective mutants of Cdc42 were not viable [1], and more recently, it was shown that Cdc42-knockout mice were embryonic-lethal [19]. Overexpression of a GTPase-defective Cdc42 mutant, Cdc42(G12V), as well as a mutant capable of constitutive GTP-GDP exchange, Cdc42(F28L), induced

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the transformation of fibroblasts [20,21]. The dominant-negative mutant, Cdc42(T17N) blocked transformation by the oncogenic Ras protein, Ras(G12V), indicating that the activation of Cdc42 is somehow required for transforming signals of Ras. Mutations or truncations of various GEFs for Cdc42, including Dbl, Vav, Brx, and Clg, have been shown to give rise to transformation [8,22,23], further suggesting a connection between the activation of Cdc42 and cell growth regulation.

Differentiation and Development

Cdc42 has been shown to serve important functions in cellular differentiation and embryonic development, through roles in myogenesis, neurite outgrowth, monocyte differentiation, and embryogenesis [24–27]. For example, overexpression of the GTPase-defective Cdc42(G12V) mutant in L6 rat myoblasts blocked myotube formation [24]. Ectopic expression of Cdc42(G12V) in chick spinal cord neurons stimulated neurite outgrowth and enhanced the size of the growth cones and their number of filopodia [25]. In *Drosophila*, Cdc42 negatively regulates notch signaling during wing development [28].

Apoptosis

Cdc42 has been shown to regulate the apoptosis of neuronal cells and the survival of epithelial cells [29,30]. Mixed lineage kinase 3 (MLK3), a downstream effector of Cdc42, was shown to induce the apoptosis of neuronal cells through the activation of the c-Jun kinase (JNK [31]). However, it has also been suggested that Cdc42 provides a survival (antiapoptotic) signal through its activation of PAK [32], which in turn phosphorylates Bad [33]. Recent studies have shown that Cdc42 contains a caspase-substrate motif and is sensitive to caspases 3 and 7 [34]. Caspase-insensitive mutants of activated Cdc42 exhibit protective effects against Fas-induced apoptosis [34]. These data led to the suggestion that Cdc42 may somehow influence the timing of apoptotic signals, protecting against a full-scale apoptotic response, until a caspase-catalyzed degradation of Cdc42 occurs.

Cell Adhesion and Migration

Cell adhesion and migration require active cytoskeletal organization and rearrangements. Many lines of evidence indicate that Cdc42 participates in cell adhesion signaling pathways and promotes cell migration through its effects on actin cytoskeletal organization [35–37]. However, the mechanism of activation of Cdc42 by cell adhesion is still not clear.

Cell Polarity

Cell polarity encompasses asymmetrical properties of cells that are manifested during cell division, differentiation, morphogenesis, and embryogenesis. To generate or sustain cell polarity, many processes, including actin cytoskeletal organization and membrane vesicle trafficking, are required. Cdc42 has been shown to play a key role in cell polarity [1,38]. Two such polarity-dependent processes that are controlled by Cdc42 and its downstream effectors are bud-site assembly in *S. cerevisiae* and apical/basolateral protein transport in epithelial cells [1,39].

Molecular Mechanisms Underlying the Biological Activities of Cdc42

The recent identification and biochemical characterization of the downstream effectors of Cdc42 have provided important insights into the molecular mechanisms that underlie its many biological effects. Below, we describe the molecular mechanisms that are thought to be responsible for the effects of Cdc42 on actin cytoskeletal organization, membrane vesicle trafficking, and the establishment of cell polarity.

Cdc42/WASP/Arp2/3 Complexes in F-Actin Polymerization

Although a complete understanding of how Cdc42 influences actin cytoskeletal organization has not yet been achieved, it is becoming clear how Cdc42 stimulates F-actin polymerization. WASP is a specific downstream effector for Cdc42. The carboxyl terminus of WASP contains a VCA (verprolin-homology, cofilin-homology, and acidic) domain that serves as a binding site for the Arp2/3 complex to organize the actin nucleation core and initiate F-actin polymerization [40,41]. In its inactive conformation, the VCA domain is masked through an intramolecular interaction with its Cdc42/Rac-interactive binding (CRIB) domain. When activated Cdc42 binds to the CRIB domain on WASP, the VCA region is released and interacts with the Arp2/3 complex to form an actin nucleation core and initiate F-actin polymerization [41]. Cdc42-induced filopodia formation appears to be mediated through its interaction with WASP, as the overexpression or microinjection of an Arp2/3-binding defective mutant of WASP inhibits the ability of EGF or bradykinin to stimulate filopodia [41].

Cdc42/γ–COP Complexes in Cdc42-Induced Cellular Transformation and Intracellular Transport

Cdc42 is required for cell-cycle progression and DNA synthesis. It appears that the ability of Cdc42 to stimulate actin polymerization or produce actin filopodia is not required for its effects on cell growth [42]. A Cdc42 mutant, Cdc42 (Y40C), which excludes the binding of all CRIB-domain-containing effectors, only interfered with Cdc42-mediated cytoskeletal organization and JNK activation, but did not influence cell-cycle progression or DNA synthesis [42]. This implied that the effector responsible for mediating

the effects of Cdc42 on cell-cycle progression is a non-CRIB-domain-containing protein. The γ subunit of the γ–COP, which lacks a CRIB domain, was found to be an essential effector for Cdc42-induced cellular transformation [10]. Because γ–COP is part of the COP1 complex, which is involved in vesicular trafficking, the interaction between Cdc42 and γ–COP suggests a direct link between Cdc42-mediated cell growth regulation and some type of a trafficking function.

Cdc42/WASP/Intersectin and Cdc42/ACK/Intersectin Complexes: Possible Roles in Receptor Endocytosis

Intersectin or Ese1 is a prototype for a family of endocytic proteins whose members contain two EH domains and five SH3 domains [43,44]. The long-form of Intersectin, designated Intersectin-l, contains a DH/PH domain and is a specific GEF for Cdc42 [6]. WASP interacts with the SH3 domains of Intesectin-l, activates its GEF activity, and influences receptor endocytosis [6,45]. It has been proposed that Cdc42/WASP/Intersectin complexes may play an important role in the regulation of endocytic vesicle transport by connecting endocytic vesicles to the actin cytoskeleton.

ACK is a nonreceptor tyrosine kinase that specifically interacts with activated forms of Cdc42 [46,47]. There are two isoforms of ACK that have very similar functional domains [46]. It has been shown that ACK contains a clathrin-binding motif and directly interacts with clathrin, an endocytic vesicle-coating protein [48,49]. Overexpression of ACK2 inhibits transferrin-receptor endocytosis via a competition with AP-2 for binding to clathrin [48]. Interestingly, both ACK2 and Intersectin-l are enriched in neuronal tissues and ACK2 interacts with the SH3 domains of Intersectin-l (Ese1) via its proline-rich domain 2 (PRD2) (Smith, W. *et al*., unpublished data). This raises the interesting possibility that a ternary Cdc42/ACK/Intersectin-l may form in neuronal tissues, containing both a specific upstream activator and downstream target for Cdc42. Recently, it has been reported that ACK2 phosphorylates SH3PX1 (sorting nexin 9) in both *Drosophila* and mammalian cells [50,51] and facilitates the degradation of the EGF receptor [51], suggesting that the Cdc42/ACK/Intersectin-l complex may form a functional unit that directly influences receptor degradation.

Cdc42/PAR6/PKCζ Complexes in Cell Polarity

The PAR protein family has 6 members (PAR1–6) and were originally identified in *Caenorhabditis elegans* embryos as being essential for the establishment of polarity [52]. The PAR6 protein directly interacts with activated Cdc42, thus serving as a putative target/effector [53–55]. It has been shown that PAR6 binds to PAR3 through its PDZ domain [53] and that this heterodimer recruits both activated Cdc42 and PKCζ. The resultant tetrameric complex appears to function as a determinant for cell polarity as well as regulates tight junction structures in mammalian epithelial cells [54]. The Cdc42/PAR6 complex also stimulates PKCζ kinase activity and this activation has been reported to induce cellular transformation [55].

The Cdc42/PAR6/PKCζ complex apparently is not the only functional unit involved in Cdc42-mediated cell polarity. The Cdc42/γ–COP complex may also play an important role in cell polarity through the regulation of vesicular trafficking. Moreover, a ternary complex consisting of activated Cdc42, the putative Cdc42-effector IQGAP, and β–catenin has been reported to regulate cell-cell contact adhesion and cortical F-actin polymerization and to play an important role in embryogenesis and the establishment of epithelial cell polarity [56]. Recent studies have shown that Cdc42 regulates polarized exocytosis by directly interacting with Sec3p, a component of the exocyst complex [57].

Cdc42/PAK/Cool(Pix) Complexes in Cell Growth, Adhesion, and Arf6-Mediated Membrane Vesicle Trafficking

The PAK family has been studied extensively and its members have been reported to exhibit very broad effects on cell growth, differentiation, apoptosis, and actin cytoskeletal organization. However, the specific role of PAK in cells is not clear. It has been reported that PAK can mediate the Cdc42/Rac-stimulated activation of JNK, which leads to increased gene expression [58,59]. PAK activity has also been shown to be crucial for Ras-induced transformation, and PAK-catalyzed phosphorylation of Raf has been reported to be required for Raf activation [60]. PAK also phosphorylates the BAD protein to prevent cellular apoptosis [33]. There is a subfamily of Dbl-related proteins named Pix (PAK-interactive exchange factor) and Cool (cloned-out of library), whose members specifically interact with PAK [61,62]. In cells, Cdc42, PAK, and Cool(Pix), together with a Cool(Pix)-binding partner called Cat (Cool-associated tyrosine phosphosubstrate; also very similar if not identical to the G-protein-coupled receptor kinase interactor or GIT, and the Paxillin-kinase linker or PKL), form a stable complex [63]. The Cat protein has an Arf-GAP domain and specifically stimulates Arf6 GTPase activity, thus connecting Cdc42/Rac signaling to Arf6 signaling [64]. The Cat protein also directly interacts with Paxillin, an important component in focal adhesion complexes, thereby providing a possible link between Cdc42/Rac signaling and cell adhesion [65].

Conclusions

Cdc42 influences a broad range of biological and cellular activities that are mediated by multiple downstream effectors. How Cdc42 is able to discriminately interact with its many upstream activators, GEFs, and downstream targets to give rise to specific signals will be an important issue for further understanding how this GTP-binding protein mediates its multiple functions. One intriguing possibility is that specific signaling complexes are constructed that contain both a specific GEF and a specific target/effector for Cdc42.

Two such examples are the Cdc42/PAK/Cool(Pix) complex and the Cdc42/WASP/Intersectin-l complex. At present, there appear to be two major cellular activities that are regulated by Cdc42: actin cytoskeletal organization and membrane vesicle trafficking. Whether these two functions are often intimately linked or more typically represent distinct activities under the control of Cdc42 remains to be established. There are some data implicating Cdc42 in the organization of F-actin cytoskeletal structures in vacuole membranes, as well as in T-cell receptor endocytic vesicles and in Golgi vesicles [66,67]. It is thus intriguing to hypothesize that Cdc42 organizes actin cytoskeleton-based vesicle "motors" that power and guide vesicle transport. Hopefully, in the not-too-distant future we will be able to determine the validity of this hypothesis.

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CHAPTER 241

Tissue Transglutaminase: A Unique GTP-Binding/GTPase

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Tissue transglutaminase (TGase) is capable of GTP-binding/GTPase activities like members of the large and small families of G proteins, as well as exhibiting an enzymatic activity that catalyzes the crosslinking of glutamine residues to primary amino groups or to the epsilon amino groups of protein lysine residues (transamidation). The binding of GTP to the TGase has a negative regulatory effect on transamidation, whereas Ca^{2+}, which is essential for transamidation activity, weakens the binding of guanine nucleotides. The recent determination of the three-dimensional structure for the guanosine diphosphate (GDP)-bound form of TGase sheds light on the molecular basis of the interplay between guanine binding and transamidation activity. Recent studies also point to an unexpected role for the GTP-binding activity, as well as transamidation activity, in the protection of cells against apoptosis.

Introduction

Tissue transglutaminase (TGase) is capable of multiple types of catalytic activity. Namely, it undergoes a GTP-binding/GTP hydrolytic cycle like members of the families of large heterotrimeric G proteins and small Ras-related G proteins [1–3], as well as it exhibits an ATP hydrolytic activity which appears to be noncompetitive with GTPase activity [4], and an acyl transferase activity [5,6] that results in the crosslinking of proteins (transamidation). Recent breakthroughs on the three-dimensional structure of TGase [7–10], and new insights into how TGase influences whether cells undergo differentiation versus apoptosis [11–13], provide interesting perspectives regarding the relationship between the GTP-binding/GTPase activities and the transamidation capability that resides on this single chain polypeptide. It is the relationship between these activities and how they might fit into new biological roles for the TGase that is the subject of this chapter.

General Overview

Transglutaminases are Ca^{2+}-dependent acyl transferases that catalyze the formation of an amide bond between the y-carboxamide groups of peptide-bound glutamine residues (which serve as acceptor residues in the reaction) and the primary amino groups in various (donor) compounds, and in particular, the e-amino group of lysine residues in proteins [5,6]. These enzymes contain an active site thiol group (6) within a Cys-His-Asp catalytic triad [7,10]. Six classes of transglutaminases have been identified and characterized in mammals. These are factor XIII-A and the keratinocyte (type I), tissue (type II or TGase), epidermal (type III), and prostate (type IV) transglutaminases, as well as TGX [6,14]. The erythrocyte membrane proteins Band 4.2 are also typically classified as a noncatalytic transglutaminase homologs [15]. It has been suggested that the different members of the transglutaminase family participate in diverse biological activities.

Tissue transglutaminase (TGase) is particularly interesting because of its ability to bind and hydrolyze GTP like traditional G proteins. There have been a number of suggestions that TGase functions as a signal transducer by mediating the stimulation of phospholipase C activity by α1-adrenergic

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receptors [16–21]. An important breakthrough came when it was appreciated that a GTP-binding protein, designated Gh, which was being studied for its ability to couple α adrenergic receptors to phosphoinositide lipid metabolism, was identical to TGase [18]. Other studies have shown that TGase binds most effectively to certain αl adrenergic receptor subtypes (α1B and α1D but not α1A) [21,22], and that the target/effector for TGase is not phospholipase C-β, which is regulated by the heterotrimeric Gq protein [23], but rather phospholipase C-δ [16,24]. It remains to be established just how analogous a role TGase plays in α adrenergic-coupled phospholipase C activity compared to the well-characterized role of the Gq protein. The expectation is that some insight will come from comparisons of the structures of the GDP- and GTP-bound forms of TGase (see the following section), and whether there are specific conformationally sensitive regions on the TGase that are analogous to the switch I and II regions of large and small G proteins.

Recent Implications

Tissue transglutaminase appears to be ubiquitously distributed in mammalian tissues and exists in a variety of cellular locations. It has been generally found in cytosol, although TGase has also been purified from the nucleus [3], and appears in the plasma membrane upon stimulating the cells with certain factors (e.g., retinoic acid [25]). It has been suggested that TGase participates in a variety of biological activities including wound healing [26]), cell-matrix interactions [27], differentiation [25,28–30], neuronal degeneration [14,31], and programmed cell death [32–34]. In particular, possible roles for TGase in both the induction of and protection against apoptosis have received a good deal of attention over the past few years. A number of correlative observations have been made regarding TGase expression and transamidation activity and cellular apoptosis, among the most interesting being the TGase-catalyzed crosslinking of the retinoblastoma protein (Rb) [34]. Given the potential for transamidation to disrupt protein function, conditions that generate hyperactive forms of TGase could well have deleterious effects on cells and likely account for the tight regulatory control over TGase expression and activation that has been observed [25]. However, it is becoming increasingly attractive to consider that TGase is expressed and/or activated in response to factors that would normally cause some insult or stress to cells, and that even in some cases, TGase-catalyzed transamidation is directed toward protecting against these perturbations rather than exacerbating them (see the following sections). There are now reasons to believe that the GTP-binding activity and potential signaling function of the TGase, as well as its enzymatic transamidation activity, contribute to its protective (anti-apoptotic) effects. This then makes it important to understand the molecular basis for the GTP-binding/GTP hydrolytic activity and the transamidation activity and their functional interplay.

TGase as a GTP-Binding/GTPase

Biochemical Characterizations

It has been realized for some time, starting with the initial characterizations of purified preparations of TGase, that there is a negative relationship between guanine-nucleotide-binding and transamidation activity [1–3]. In some studies, it has been shown that GTP is a much more potent inhibitor of transamidation activity, compared to GDP [1,2], thereby raising the provocative possibility of a direct coupling between the protein crosslinking activity of the TGase and its GTP-binding/GTPase cycle. However, in other studies, the differences in the abilities of GTP versus GDP to inhibit transamidation activity were minimal [4]. If true, this would then raise questions regarding the cellular conditions necessary for transamidation to occur, and in particular, whether those physiologically relevant transamidation substrates, upon binding to the TGase, weaken guanine nucleotide binding and stabilize a guanine-nucleotide-depleted state of the protein. Unlike the large and small G proteins, which show a high degree of preference for guanine nucleotides relative to adenine nucleotides, the TGase is able to bind and hydrolyze ATP. It is interesting that ATP binding and hydrolysis are not competitive with GTP binding and hydrolysis, suggesting that distinct binding sites exist on the TGase for these two nucleotides. At the present time, there is little known regarding the role of the ATP hydrolytic activity and further insight awaits the generation of TGase mutations that are highly specific for blocking ATP binding and hydrolysis without perturbing guanine nucleotide binding or GTP hydrolysis. There also have been questions regarding the affinity of guanine nucleotides for the TGase, as compared to guanine nucleotide binding to G proteins, as well as how rapidly GTP is hydrolyzed by the TGase. Some estimates have suggested that guanine nucleotides bind to the TGase with relatively weak affinity (dissociation constants in the micromolar range) and that GTP is hydrolyzed relatively slowly [4]. However, the turnover number for GTP hydrolysis catalyzed by the TGase purified from rabbit liver nuclei [3] was measured to be ~1 mol ^{32}Pi released per minute per mol protein, which is on the order of the intrinsic GTP hydrolytic activity for heterotrimeric G proteins. Moreover, the TGase has recently been crystallized with bound GDP (see the following sections), suggesting a high-affinity interaction similar to what has been demonstrated for members of both the large and small G-protein families.

Three-Dimensional Structure for the GDP-Bound TGase

The three-dimensional structure for the GDP-bound form of the human TGase has recently been determined to 2.8 Å resolution by x-ray crystallography [10]. The TGase organizes as three dimers within the unit cell. Each monomer contains a bound GDP molecule and is made up of four

distinct domains, an amino terminal β-sandwich domain (Met1 to Phel39), a transamidation catalytic core (Alal47 to Asn460), and two carboxyl-terminal β-barrel domains (Gly472 to Tyr583 and Ile591 to Ala687, respectively). Cysteine 277 lies within the middle of a groove within the transamidation active site [thus the TGase(C277A) mutant is transamidation-defective] and is part of a catalytic triad, Cys277-His-335-Asp358. The transamidation active site and the general domain structure of TGase are similar to those reported for factor XIIIa and for TGase III [7,8].

The guanine-nucleotide-binding site lies in a cleft formed by the catalytic core domain and the first β-barrel domain [10]. Most of the residues contacting GDP come from the end of the first β-strand of the first β-barrel domain and the loop that connects it to the second β-strand. There are also two residues contributed by the catalytic core domain that are involved in binding the guanine ring moiety. In this regard, the overall architecture for the guanine-nucleotide-binding site on TGase differs significantly from the guanine-nucleotide-binding domains of the α subunits of heterotrimeric (large) G proteins or the Ras-related (small) G proteins. For both the large and small G proteins, the guanine-nucleotide-binding site consists of five helices surrounding a six-stranded β-sheet [35]. The α subunits of the large G proteins also contain a helical domain that is adjacent to (and in effect closes over) the guanine-nucleotide-binding site and enables the α subunits to bind GDP with high affinity. Ras and other small G proteins lack this helical domain and bind GDP with high affinity through the coordination of Mg^{2+}. Both large and small G proteins require Mg^{2+} for the GTP hydrolytic reaction and thus share conserved serine and threonine residues that bind to the β- and γ-phosphates of the guanine nucleotide. The TGase also requires Mg^{2+} for its GTP hydrolytic activity, however, the location of the Mg^{2+}-binding site is not obvious as the TGase lacks amino acids with either hydroxyl or carboxyl side chains in the immediate vicinity of the nucleotide phosphate groups. Mutation of serine 171 does inhibit the GTP-binding activity of the TGase, however, the position of this serine is closer to the guanine ring rather than to the nucleotide phosphate groups and thus would seem to be an unlikely participant in the GTP hydrolytic reaction.

The importance or location of the normally essential arginine (i.e., arginine finger) for the GTP hydrolytic reaction of the TGase is also not known at the present time. There are various positively charged groups that surround the phosphate moieties of the bound GDP on the TGase and could conceivably play a similar role as the essential arginine residues provided by the helical domains of the large G protein and by the GTPase-activating proteins (GAPs) of small G proteins. These include Arg 580 from the last β-strand of the first β-barrel domain of the TGase, which forms two ion-pairs with the α- and β-phosphate groups of the guanine nucleotide, and Arg 478 from the first β-strand, which is located close to the β-phosphate of GDP. Both Arg 478 and Arg 580 are conserved in all TGases (i.e., from different species) but are missing in other members of the transglutaminase family that are incapable of binding GTP. Because the β-phosphate is pointed toward Arg 478, the γ-phosphate of GTP would likely need to rotate to avoid clashing with the side chain of Arg 478 and its hydrogen-bonding partner Val 479. This would bring the γ-phosphate into the vicinity of the positively charged side chains of Lys 173 and Arg 476. Because the TGase lacks the conserved glutamine residue found in all large and small GTP-binding proteins, which functions to position water for nucleophilic attack during GTP hydrolysis, a plausible mechanism for the GTP hydrolytic activity of the TGase would be that a water molecule hydrogen bonded to either the side chain of Lys 173 or Arg 476 would serve as the attacking group. Consistent with this idea, mutation of Lys 173 yields a GTPase-impaired TGase molecule [36].

Another notable difference between the guanine-nucleotide-binding pockets of large and small G proteins and that of the TGase is the absence of the NKXD motif that is conserved in virtually every large and small G protein. The asparagine residue within this motif always forms a hydrogen bond with the N7 atom of the guanine moiety, and the aspartic acid residue hydrogen bonds with the N1 and N2 atoms. In the TGase, a main chain oxygen from Tyr 583 from the last β-strand of the first β-barrel domain forms hydrogen bonds with the N1 and N2 atoms of the guanine ring, and Ser 482 from the first β-strand forms an additional hydrogen bond with N2.

There is one interesting similarity regarding the guanine-nucleotide-binding site in the TGase, compared to that for Ras or other small G proteins like Cdc42. In the TGase, the guanine ring lies in a hydrophobic pocket that includes a phenylalanine residue (Phe 174), which appears to be positioned to stack with the guanine moiety. The α subunits of heterotrimeric G proteins lack such a phenylalanine residue, however, in Ras and other small GTP-binding proteins, a conserved phenylalanine approaches one side of the guanine ring at an ~90° angle and participates in π-π stacking interactions. This stabilization has been noteworthy, as mutation of the phenylalanine (Phe 28) to leucine in Ras and Cdc42 yields proteins that show an accelerated dissociation of tightly bound GDP and are capable of constitutive GTP-GDP exchange in cells, thereby giving rise to malignant transformation [37,38]. It will be interesting to see if the corresponding mutation in TGase yields a molecule capable of constitutive GDP-GTP exchange activity.

An examination of the TGase structure also provides some possible insight into the nature of the interplay between guanine nucleotide binding and transamidation activity. In the GDP-bound form of TGase, it appears that access to the enzymatic (transamidation) active site is restricted by a loop that connects the third and fourth β-strands, and a loop that connects the fifth and sixth β-strands of the first β-barrel domain. Tyrosine 516, a residue conserved in different members of the transglutaminase family, is located within the first loop and hydrogen bonds with the essential cysteine residue (Cys 277). It is expected that Tyr 516 needs to move

to make the essential cysteine accessible to participate in catalysis, and it is easy to imagine how guanine nucleotide binding might stabilize the tyrosine residue and hinder the accessibility to the catalytic cysteine. The binding of GTP could further stabilize this catalytically compromised conformation thereby accounting for the enhanced inhibitory effects exhibited by GTP toward transamidation activity.

Based on comparisons of the structures of TGase, factor XIIIa, and type III transglutaminase [7–10], a major Ca^{2+}-binding site on the TGase is formed by the side chains of Asn 436, Asp 438, Glu 485, and Glu 490, as well as by the main chain oxygen from Ala 457. These are located near the end of the loop that connects the transamidation active site to the first β-barrel domain. In the TGase, Ile 416 and Ser 419 form a β-strand (anti-parallel) with Leu 577 and Glu 579 in a manner that maintains the first β-barrel domain and stabilizes the guanine-nucleotide-binding site [10]. It seems reasonable to expect that the binding of Ca^{2+} alters the position of Ile 416 and Ser 419 thus eliminating their stabilizing effects and weakening guanine nucleotide binding. Increasing Ca^{2+} would then potentially have a dual effect on transamidation activity by inducing a conformational change that is both essential for catalysis and weakens guanine nucleotide binding, which in turn reverses a negative regulatory effect normally imparted by bound guanine nucleotide.

New Links to Biological Function

It has been well documented that TGase activity is under the control of retinoic acid (RA) in a variety of cell types [11,25]. In some cases (e.g., the human leukemia cell line HL60), both the expression and activation of TGase are stimulated by RA treatment, whereas in HeLa cells, we have found that RA promotes the activation of TGase without having a significant effect on its expression. Because of its ability to trigger cell cycle arrest and cellular differentiation, RA has been examined as a possible therapeutic agent against human cancers. Whereas the natural retinoids have shown somewhat limited activity, the synthetic analog all-*trans*-*N*-(4-hydroxyphenyl) retinamide (HPR) has shown some promise in the treatment of breast and prostate cancers [39–041]. Interestingly, HPR has been consistently linked to apoptosis, while RA has been reported to give rise to cellular differentiation as well as apoptosis [42,43]. We have found that only HPR, and not RA, induces an apoptotic response in both NIH3T3 cells and HL60 cells. A major difference that has been detected in response to treatment with RA versus HPR is that RA stimulates the expression and/or activation of TGase, whereas HPR appears to inhibit its expression. It is interesting that pretreatment of cells with RA, prior to addition of HPR, induces TGase expression and activation and completely protects against HPR-induced apoptosis. The RA-mediated protection effect can be mimicked by the expression of wild-type TGase in NIH3T3 cells or by a TGase (C277S) mutant where the active site cysteine residue has been changed to serine. However, TGase mutants that are unable to bind GTP [e.g., TGase (S171E)] do not provide protection against HPR.

These results have led us to propose that the TGase may be functioning as a protection-factor that is activated by differentiation agents in order to ensure that cells remain viable and not susceptible to cell death programs during cellular differentiation. Apparently, the GTP-bound TGase can activate a signaling pathway that is essential for this protection effect. It will be interesting in the future to delineate this survival pathway and to identify the target that binds to GTP-bound TGase and mediates these effects. It should be noted that addition of the TGase competitive inhibitor, monodansylcadaverine (MDA), to RA-treated cells results in converting the RA response to one that mimics HPR treatment (i.e., apoptosis). This is interesting in light of our finding that mutation of the active site cysteine on the TGase did not abrogate its protection capability or give rise to apoptosis. Thus, the effects of MDA may be directed at altering the ability of TGase to bind GTP or preventing GTP-bound TGase from initiating signaling activities that can protect against apoptosis. However, recently, we have found that in Rb knock-out cells, which are sensitive to HPR, co-expression of wild-type TGase and Rb can overcome the apoptotic effects of HPR, whereas expression of TGase (C277A) together with Rb cannot [13]. Thus, there may be cell types where both the GTP-binding activity of the TGase and its transamidation activity are critical to trigger pathways that protect against programmed cell death.

Future Directions

A number of questions regarding the role of TGase, and in particular its GTP-binding/GTPase cycle, in various cellular functions await future studies. It will be important to know whether in fact the TGase undergoes significant conformational changes as an outcome of GDP-GTP exchange, analogous to large and small G proteins, and where those changes occur. How are such changes translated into the binding of target/effector proteins and what are the identities of these proteins? At the present time, phospholipase C appears to be a leading candidate, however, similar to the cases for other G proteins, there are likely to be additional cellular targets for the TGase. We also know very little regarding how the GDP-GTP exchange reaction for the TGase is regulated, and it will be important to determine whether guanine nucleotide exchange factors (GEFs) exist with functions analogous to the serpentine receptors (which stimulate the activation of heterotrimeric G proteins) and the GEFs for small G proteins. Likewise, nothing is known regarding the regulation of the GTP hydrolytic activity of the TGase. Are there specific GTPase-activating proteins? If so, how are they regulated, as GTP hydrolysis may be necessary to promote optimal transamidation activity? Can the TGase bind aluminum fluoride and will this induce a conformation that mimics the transition state for GTP hydrolysis similar to what has been observed in large and small G proteins?

Finally, what other binding partners exist for the TGase and how do these interactions give rise to survival or anti-apoptotic effects? We are at a much earlier stage in appreciating the functional importance of the GTP-binding/GTPase cycle of the TGase, compared to what we know about other more traditional G proteins, but there is every reason to believe that the TGase will prove to be an important, albeit somewhat unusual, type of G-protein switch.

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CHAPTER 242

The Role of ARF in Vesicular Membrane Traffic

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The ARF Family of Regulatory GTPases

*A*DP-*r*ibosylation *f*actors (ARFs) are a family of highly conserved, ~20-kDa GTPases with a number of cellular activities; most notably the regulation of vesicular membrane traffic. ARFs are ubiquitous in eukaryotes with as many as 23 members of the ARF family expressed in mammalian cells. The ARF family includes both ARF and ARF-like (ARL) proteins, based upon the extent of sequence and functional identities; e.g., the 6 mammalian ARFs share > 60% identity to one another, while most ARLs share 40–60% identity to each other or to any ARF [1]. With this high level of structural conservation we expect that ARLs perform mechanistically and functionally homologous roles to those of ARFs, but these are much less well defined and will not be covered here. The ARF subfamily can be further divided into three classes. In mammals, class I includes ARF1–3, class II includes ARF4 and ARF5, and class III is ARF6 [2]. In contrast, *Caenorhabditis elegans* and *Drosophila melanogaster* each have single representatives of each ARF class. The results discussed in the following sections were obtained primarily from studies of ARF1. The extent to which ARF4 and ARF5 act as described below is unknown, while ARF6 clearly has some differences in location and presumed actions [3,4].

Like other regulatory GTPases, ARFs cycle between GDP- and GTP-bound conformations in a cycle whose rate is determined by binding to guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and effectors. In addition to these functionally critical protein interactions, ARFs are unique among regulatory GTPases in that their affinity for biological membranes is affected by the nucleotide bound [5]. Specifically, ARFs are co-translationally modified by the addition of myristic acid to the N terminal glycine [6]. This hydrophobic N-terminal anchor functions coordinately with the amphipathic N terminus to orient the protein on the surface of the membrane when GTP is bound. ARFs are predominantly soluble when GDP is bound. Thus, activation of ARF (GTP binding) alters both its location and its affinity for effectors.

All of the currently identified actions of ARFs take place on membrane surfaces. These different functions can be divided into four different groups, (1) regulation of lipid metabolism, (2) regulation of vesicle transport, (3) cofactor for bacterial toxins [7,8], and (4) GTP-dependent binding to other miscellaneous proteins [9–11] with less well understood functions. Because of the likelihood of functional interplay, the rest of this review will focus only on the first two.

ARF as a Regulator of Membrane Traffic

The primary cellular roles for ARFs are the regulation of membrane traffic and lipid metabolism. Whether these represent one integrated or two distinct aspects of ARF biology is controversial. Most likely, ARF-dependent changes in lipid metabolism occur both during vesicle biogenesis and at other times. The observations that (1) activation of ARF is sensitive to its lipid environment, (2) specific lipids serve as cofactors in some ARF-dependent activities, (3) ARF can alter lipid metabolism, and (4) ARF is required for the biogenesis of a broad array of vesicles in cells (see Fig. 1),

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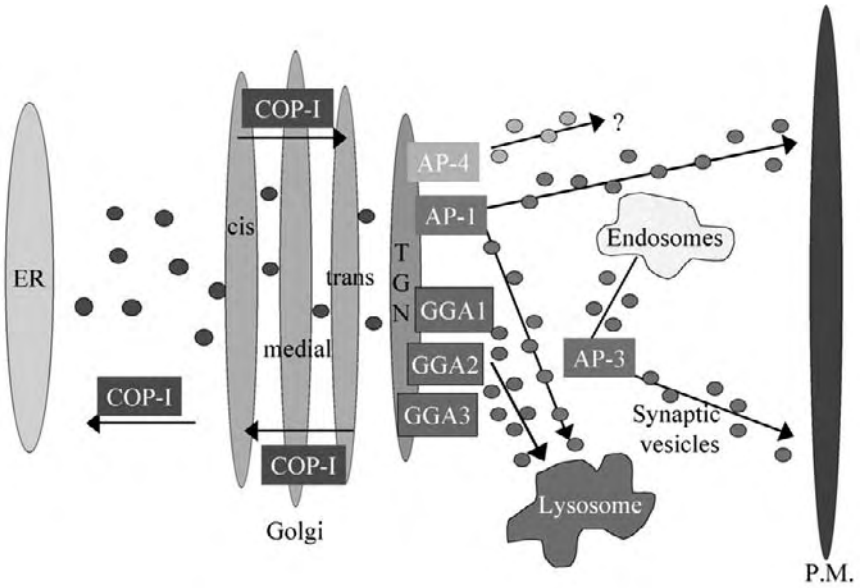


Figure 1 ARFs act to regulate the recruitment of coat proteins to a wide variety of intracellular membranes. The different compartments implicated as a source or destination of vesicles regulated by ARF proteins are shown. Coat/adaptor proteins or complexes are represented by shaded rectangles at the membranes at which they are thought to initiate vesicle budding. The direction and destination of the vesicles are indicated by arrows. Arrows emanating from AP-1 and GGA2 are intended only to indicate traffic from TGN to endosomes/lysosomes, and P.M. routing between different endosomal compartments is not shown. Note that the destination and possibly directionality of transport of each of the coated vesicles are still under investigation (particularly, e.g., AP-4). P.M. = Plasma Membrane.

implicate ARFs as both sensors and modulators of different lipids and membranes. The role of ARF as regulator of membrane traffic is very likely to be intimately linked to roles in lipid metabolism and signaling.

ARF is Activated on Membranes

The dependence on a hydrophobic environment for GTP binding was first described when ARF was purified and shown to bind guanine nucleotides [12]. The ability of a broad list of different lipids and detergents to satisfy this requirement reveals a nonspecific requirement for lipids in the activation process. This is likely an *in vitro* correlate of the GTP-stimulated translocation of ARF to membranes. ARFs are sufficiently hydrophobic to interact weakly with membranes in the GDP-bound state [13]. Once at the membrane they encounter ARF GEFs, which promote GTP binding and a more stable association with the membrane. In this way, specificity in membrane binding is likely tied to the localization of ARF GEFs, though we cannot exclude the lipid composition of the membrane from playing a role.

Specific Lipids are Cofactors for ARF-Stimulated Activities

The search for an ARF GEF led to the observation that acid phospholipids can stimulate the release of GDP from ARFs, and can stabilize the protein in the nucleotide-free state [14]. Phosphoinositides, specifically phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), were the most active lipids in these studies. At about the same time, ARF was found to be a potent, direct, GTP-dependent activator of phospholipase D (PLD), which catalyzes the conversion of phosphatidylcholine to phosphatidic acid and choline [15,16]. Surprisingly, ARF-stimulated PLD activity was dependent on the addition of $PI(4,5)P_2$ [15]. Similarly, some ARF GAPs were found to be potently stimulated by $PI(4,5)P_2$ [17,18]. The product of PLD, phosphatidic acid, can also increase ARF GAP activity and alter affinity of ARF GAP for $PI(4,5)P_2$ [18]. Thus, there exist both nonspecific effects of lipids to stabilize GTP binding and specific effects of lipids to bind and modulate ARF, ARF effectors (PLD), or ARF GAPs. As seen in the following section, the interplay between ARF actions and $PI(4,5)P_2$ gets even more complicated.

ARF as Modifier of Lipid Metabolism

A role for ARF in lipid metabolism was first revealed by its role as co-activator of PLD [16,19]. This led to speculation that effects of ARF on vesicle traffic may result from changes in membrane lipid composition. This theory was supported by the observation that PLD was localized to Golgi membranes [20], a major site of ARF actions, and that a persistently active form of PLD can mimic many of the actions of activated ARF on coated vesicle formation *in vitro* [21]. More recently, ARF has been shown to recruit both PI 4-kinase and PI(4)P 5-kinase to membranes, [22] and is a

direct activator of the latter [23]. The sequential actions of these two enzymes produce PI(4,5)P_2, the allosteric activator of PLD and some ARF GAPs and a molecule with widespread effects on cell signaling and morphology. Thus, ARF can increase PLD activity and PA levels both through direct activation of PLD and indirectly, through increased production of PI(4,5)P_2 via activation of PIP kinases. ARF activity can produce changes in local and perhaps cellular levels of phosphatidylcholine, phosphatidic acid, PI, PI(4)P, and PI(4,5)P_2.

Together these data reveal extensive interplay between ARF and phospholipids that may have profound effects on signal transduction, through the activation of specific enzyme effectors and generation of PI(4,5)P_2 and on changes in the lipid composition in microdomains that could impact the rate or ability to form transport vesicles.

The Role of ARF in Vesicle Biogenesis

Cell biological, genetic, and pharmacological data all pointed to intracellular membranes, and particularly the Golgi and *trans*-Golgi network (TGN), as important sites of ARF action. For example, electron microscopy and indirect immunofluorescence of cultured mammalian cells localized ARF to cis-Golgi [24] and TGN membranes [25]. Further, deletion of yeast *ARF1* resulted in defects in the processing of secreted proteins as they pass through the Golgi and genetic interactions with other Golgi proteins [24]. Later, brefeldin A was shown to progressively cause the release of proteins from the cytosolic surface of Golgi and TGN membranes [26], loss of Golgi integrity with fusion of Golgi elements with the endoplasmic reticulum (ER) [27,28], inhibition of protein secretion [29], and death of some cells [30]. All the cellular actions of brefeldin A result from inhibition of ARF GEFs [31], suggesting a role (though perhaps an indirect one) for ARFs in each.

A breakthrough occurred with the observation that the inhibition of intra-Golgi transport by GTPγS led to the accumulation of vesicles of uniform size and possessing an electron-dense coat. Purification of these vesicles allowed the identification of the coat proteins; consisting of the heptameric COP-I complex and ARF [32]. The brefeldin A sensitivity of both ARF and COP-I localization at Golgi membranes led to the proposal that binding to activated ARF is required for COP-I to bind Golgi membranes and promote the budding process [33]. Since that time the number of different coat proteins recruited by ARF has increased markedly, leading to the need for mechanistic details that can explain specificity in recruitment of cargo and coats into the nascent vesicle.

Thus far, seven coat complexes have been identified whose binding to ARF is GTP-dependent and to membranes is brefeldin A-sensitive. These include COP-I, the tetrameric adaptin complexes AP-1 [34], AP-3 [35], and AP-4 [36], and the three monomeric GGAs, GGA1-3 [37–39]. COP-I is required for retrograde transport from the Golgi to the ER and for transport between the Golgi stacks (see Fig. 1). The adaptin complexes are involved in vesicle traffic from the TGN (AP-1 and AP-4) and from endosomes and synaptic vesicles (AP-3). GGAs regulate vesicle traffic from the TGN to endosomes/lysosomes. Thus, ARF regulates the recruitment of coat complexes from a variety of membranes within a cell, including multiple coats at a single membrane, the TGN.

The initiation of vesicle budding requires a minimum of three components: ARF, the coat/adaptor[1] protein or complex, and a transmembrane anchor protein (see Fig. 2). For COP-I, these roles are filled by ARF, COP-I, and the p24 family of transmembrane proteins (Fig. 2A) [40]. The transmembrane proteins have cytosolic "tails" that carry sorting motifs, such as the acidic dileucine motif that bind GGAs (Fig. 2B) [41,42] or the tyrosine-based signals that bind adaptins (Fig. 2C) [43]. Once the initial three components are in place, the adaptor proteins recruit other components necessary for vesicle formation, transport, uncoating, and fusion. In only some cases does this include clathrin. It is not clear when ARF dissociates from the bud or vesicle but it is likely to be soon after initiation of the coating process. Thus, activation of ARF promotes vesicle budding through the recruitment of vesicle coat proteins which can act as scaffolding in the assembly of the components necessary for vesicular transport.

The correct matching of cargo with adaptor is required for appropriate targeting of the vesicle. An attractive model for how the three components act to "proofread" the coating process was provided by Goldberg [44] and was based on the observation that ARF GAP activity was increased by COP-I. When cargo with an incorrect sorting signal is present, GAP activity is stimulated by COP-I; leading to GTP hydrolysis on ARF, release of COP-I, and prevention of coat assembly and bud maturation. If the sorting motif on the cargo is appropriate to the adaptor, GTP hydrolysis is not stimulated, and vesicle coating proceeds. Though data supporting this model have been generated only with COP-I, we present a more general model for ARF-dependent vesicle biogenesis, showing the different sorting motifs and adaptors that result in the production of different coated vesicles (Fig. 2). Further experimentation is needed to support this three-component model for ARF action. An even more challenging goal is the integration of this protein-based model with the changes in membrane lipids that are known to occur in response to ARF activity. Such a holistic model will be required before detailed understanding of membrane traffic and signal transduction by ARFs is achieved.

[1]The terms coat protein and adaptor are used interchangeably here. The former name originates from the electron-dense coat first seen on the non-clathrin coated, COP-I vesicles. Such a coat can be seen in AP-1 bearing clathrin-coated vesicles but has not been described for other ARF coats. The term adaptor is more generic and intended to refer to the fact that whether monomeric or oligomeric, a key component in their biological functions is the ability to bind multiple proteins, including ARFs, transmembrane cargo, clathrin, and accessory proteins.

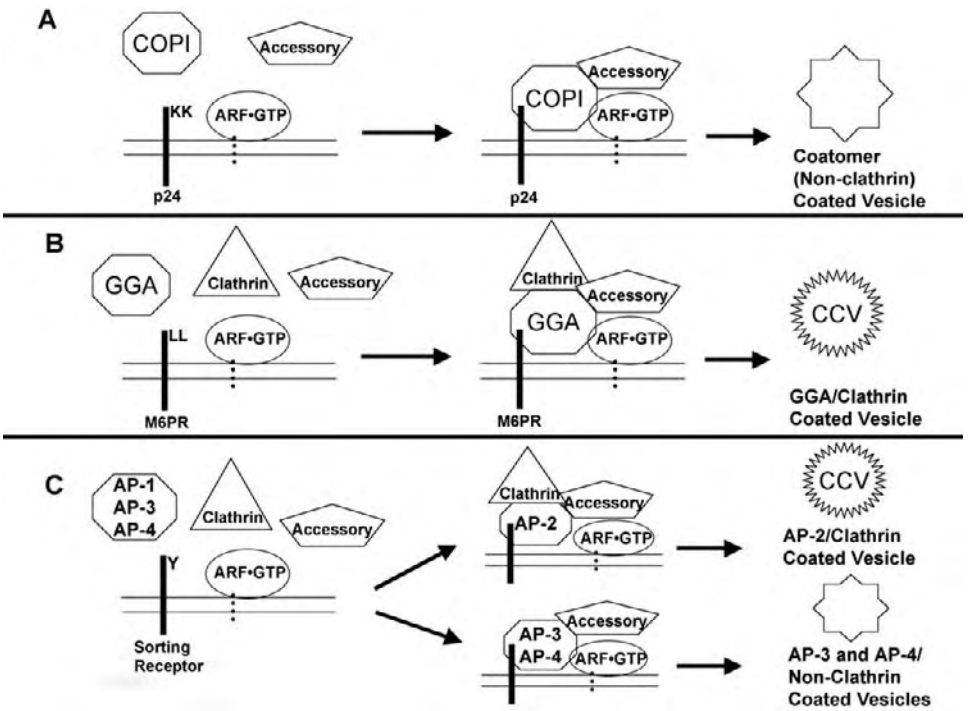


Figure 2 Three-component model for the initiation of ARF-mediated coated vesicle assembly. A minimum of three components are proposed as being critical to the assembly of coat proteins and initiation of vesicle budding; ARF-GTP, a coat/adaptor protein or complex, and a transmembrane anchor or sorting receptor. The latter carries sorting motifs which help match cargo and coat. The p24 family has a dilysine (KK) sorting motif, the mannose 6-phosphate receptors (M6PR) carry an acidic dileucine motif, and sorting receptors have a tyrosine-based signal. Once assembled the adaptor proteins are proposed to act as scaffolds to recruit clathrin (in some cases) and (poorly defined) accessory proteins. The minimal components required for coating of COP-I (panel A), GGA (panel B), and adaptin (panel C) vesicles are shown. Note that GGAs and AP-1 form clathrin-coated vesicles (CCV), while clathrin is not found on vesicles carrying COP-I, AP-3, or AP-4.

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CHAPTER 243

Yeast Small G Protein Function: Molecular Basis of Cell Polarity in Yeast

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Introduction

Cell polarity is critical for the function of the many cell types involved in vectored processes such as nutrient transport, neuronal signaling, and motility. Development of a polarized cell, regardless of cell type, follows a common plan [1]. First, a spatial cue (landmark) marks the site of polarized growth. Signal transduction molecules then interpret the identity of the landmark and signal the establishment of polarity by an asymmetric organization of the cytoskeleton. Polarity is then reinforced with the secretory transport and deposition of molecules needed for growth at the site chosen for polarized growth.

The budding yeast *Saccharomyces cerevisiae* is an outstanding experimental organism with which to decipher the molecular mechanisms of polarity development by small G proteins (hereafter GTPases). GTPases make numerous molecular interactions with effectors and regulators and can be involved in multiple distinct signaling pathways in response to the internal and external cues. The facile experimental genetics of yeast affords the opportunity to study GTPases in individual signaling pathways under different physiological conditions [2]. Herein, the role of GTPases in linking spatial landmarks on the cell cortex to the reorganization of the cytoskeleton will be presented in the context of yeast budding.

The Rsr1/Bud1 Ras-Like GTPase Module Interprets Spatial Landmarks

Yeast cells undergo oriented cell division by selecting a specific site for polarized growth, the bud site, on their cell cortex. Haploid **a** and α cells bud in an axial pattern in which both mother and daughter cells select a bud site immediately adjacent to their previous division site. Diploid **a**/α cells bud in a bipolar pattern: mother cells select a bud site adjacent to their daughter or on the opposite end of the cell, whereas daughter cells always choose a bud site directed away from their mother [3–5] (Fig. 1). Thus, the choice of a bud site determines the axis for cell polarity and ultimately the cell division plane.

A GTPase module consisting of the Ras-like GTPase Rsr1/Bud1, its GDP-GTP exchange factor (GEF) Bud5, and its GTPase activating protein (GAP) Bud2, is essential for selecting the proper site for polarized growth in both haploid and diploid yeast cells [6–10]. Deletion of *RSR1/BUD1, BUD2,* or *BUD5* leads to random budding [6,7,10] as does the expression of $rsr1^{G12V}$ (predicted to be constitutively GTP-bound) or $rsr1^{K16N}$ (predicted to be constitutively GDP-bound or nucleotide-empty) [11], suggesting that Rsr1/Bud1 needs to be cycled between GTP- and GDP-bound states for proper bud-site selection. Rsr1/Bud1 localizes to the plasma membrane and then becomes

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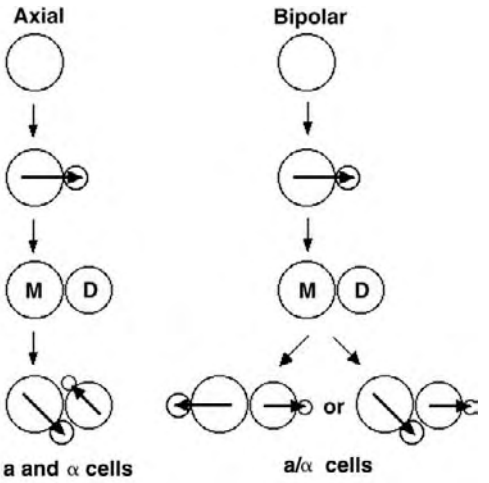


Figure 1 Wild-type patterns of bud site selection in yeast. In the axial pattern (exhibited by an **a** or α cell), the mother cell (M) buds immediately adjacent to its last daughter; the daughter cell (D) buds toward its mother. In the bipolar pattern (exhibited by an **a**/α cell), the mother cell can bud at or near either of its poles; the daughter cell buds away from its mother. The arrows within the cells indicate the axis of polarity. (Reprinted, with permission, from Chant, J. and Herskowitz, I. *Cell,* **65**, 1203–1212, 1991. Copyright Cell Press).

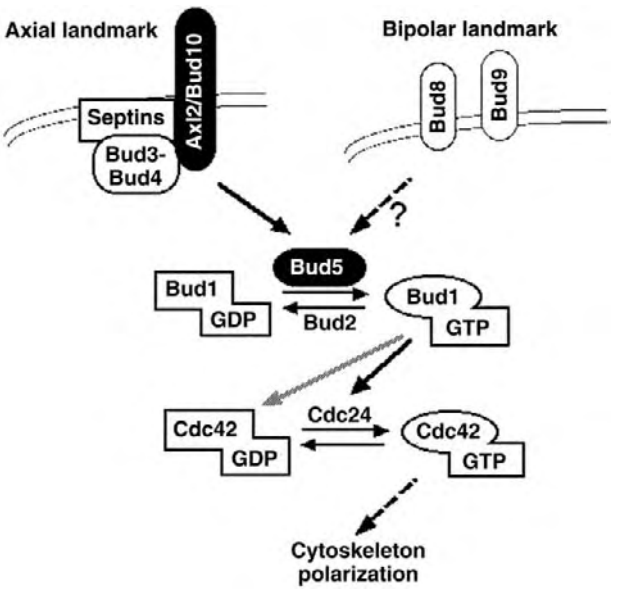


Figure 2 A model for spatial control of cell polarity during yeast budding. Bud5 is likely to be localized to the presumptive bud site in haploid **a** and α cells through the interaction with Axl2, a component of the axial landmark. Bud5 locally activates Bud1 to the GTP-bound state, which then associates with proteins necessary for bud-site assembly, such as Cdc24 and Cdc42. Bud5 may interact with Bud8 or Bud9, putative landmarks of diploid **a**/α cells, thus directing polarity establishment for bipolar budding pattern. (Adapted from Kang, P. J., Sanson, A., Lee, B., and Park, H.-O., *Science,* **292**, 1376–1378, 2001. With permission.)

concentrated at the presumptive bud site in G1 of the cell cycle [11a]. Bud2 and Bud5 proteins are also found at the presumptive bud site in unbudded cells [12,13]. Although each protein of the Rsr1/Bud1 GTPase module can localize to the presumptive bud site independently of each other, localization of each protein to the proper bud site cannot be maintained in the absence of any other protein in the module. Thus the localized action of the Rsr1/Bud1 GTPase module is required for proper bud site selection [12,13].

Isolation of cell-type specific alleles of *BUD2* and *BUD5* suggests that each of the proteins encoded by these genes localizes to the presumptive bud site through interactions with cortical landmarks unique to haploid or diploid cells [13,14]. Recent studies on Bud5 have established that Bud5 interacts physically with a transmembrane protein, Axl2/Bud10 [15,16], a potential landmark for axial budding in haploid cells [13]. Bud5 mislocalizes in cells lacking Axl2/Bud10 [13,17]. Thus, the interpretation of a cortical landmark by a GTPase module occurs by the interaction of the landmark protein with the GEF of the module, which may result in the localized activation of module at the site of polarized growth. It is not known whether Bud5 interacts with potential bipolar landmarks such as Bud8 or Bud9 [14,18] to establish the bipolar budding pattern in diploid cells (Fig. 2).

The Rsr1/Bud1 module does not complete the link between cortical landmarks and polarity establishment; rather, it links the spatial signals from both axial and bipolar landmarks to the Cdc42 GTPase module (discussed in the following section), which is essential for the establishment of cell polarity.

The Cdc42p Rho-Like GTPase Module Regulates the Establishment of Cell Polarity

A GTPase module consisting of the Rho-like GTPase Cdc42, its GDP-GTP exchange factor (GEF) Cdc24, its GTPase-activating proteins (GAPs) Bem3, Rga1, and Rga2, and guanine nucleotide dissociation inhibitor (GDI) Rdi1, is essential for the establishment of cell polarity prior to budding (reviewed in [19]). Mutation of *CDC42* or *CDC24* prevents an asymmetric organization of the actin cytoskeleton at the bud site as well as bud formation [20,21]. Association of Cdc42 with the plasma membrane via posttranslational geranylgeranylation of its C terminus is essential for Cdc42 function [22]. Deletion of each of the GAP-encoding genes does not result in a *cdc42* phenotype, due perhaps to a redundancy of function among this class of proteins [23,24]. How the Cdc42 module effects the establishment of cell polarity at the site chosen for polarized growth is a question of expanding complexity because the proteins of the Cdc42 module make numerous physical and genetic interactions [19,25,26] that result not just in the polarization of the actin cytoskeleton (see the following section), but of other proteins involved in polarized growth as well (e.g., Sec3 of the exocyst complex) [27].

Coupling the Rsr1/Bud1 GTPase Module to the Cdc42 GTPase Module

A web of genetic interactions among the *BUD* genes and *CDC24* hinted at a linkage between the Rsr1/Bud1 and

Cdc42 GTPase modules (reviewed in [28]). Subsequent studies showed that Rsr1/Bud1 interacts with Cdc24 (as well as Cdc42 and the scaffold Bem1) in a guanine nucleotide-dependent manner [29,30] (Fig. 2). The scaffold protein Far1 sequesters Cdc24 in the nucleus [31–33]. In late G1, Cdc24 is exported into the cytoplasm upon cyclin activation of Cdc28 kinase [32,33]. In the absence of Rsr1/Bud1, Cdc24 localizes in a patch randomly located on the plasma membrane [11a], consistent with the proposed role of Rsr1/Bud1 GTPase module guiding bud-site assembly proteins to the proper bud site.

The Cdc42 GTPase Module Spatially Restricts Actin Assembly to the Site of Polarized Growth

Localized activation of Cdc42 at the bud site by Cdc24 leads to an asymmetric distribution of filamentous actin structures within the cell. In late G1, actin cables align along the cortex of the cell and cortical actin patches concentrate at the bud site (reviewed in [34]). Recent studies suggest that new Cdc42-dependent actin assembly and stabilization at the bud rather than the movement of existing actin structures to the bud causes the asymmetric distribution of these cytoskeletal structures within the cell [35–38].

A permeabilized cell assay provided the first evidence for Cdc42-dependent actin assembly at the site of polarized growth. Permeabilized cells with impaired Cdc42 function did not assemble exogenously added actin at the site of polarized growth, except with the prior addition of recombinant Cdc42 or an activated form of the Cdc42 effector Ste20, a PAK family kinase [39,40]. This latter result is consistent with the ability of GTP-bound Cdc42 to relieve PAK autoinhibition (reviewed in [41]), although a role for Ste20 in actin assembly remains undefined. Cla4, a PAK kinase that is partially redundant with Ste20, may, however, have a bona fide role in cortical actin assembly. Phosphorylation of class I myosins by Cla4 may activate the actin nucleating complex, Arp2/3, directly or indirectly through interaction with the Arp2/3 activator Las17/Bee1 [38]. To what extent this activity depends upon the kinase activity of Cla4 is unclear [42]. Interestingly, overexpression of *CLA4*, but not *STE20*, is lethal [2]. Phosphorylation of Cdc24 by Cla4 may release Cdc24 from a Bem1 scaffold that also contains Cdc42, ending polarized bud growth [43, 43a]. Thus, activation of Cla4 by Cdc42 may initiate actin assembly at the site of polarized growth and may result in the negative regulation of Cdc42 by acting on its GEF Cdc24.

Polarization of the actin cytoskeleton also depends upon two other Cdc42 effectors, the redundant formin family proteins Bni1 and Bnr1. Bni1 localization at the site of polarized growth depends upon Cdc42 activity and is enhanced by interactions with proteins of the polarisome and the Cdc42 effectors Gic1 and Gic2 [44–46]. In the absence of formin function, the actin cytoskeleton becomes depolarized. Upon recovery from formin inactivation, actin cables assemble from the site of polarized growth in an Arp2/3-independent manner [36,37]. Formins are also required for maintaining a polarized distribution of Las17 [38]. It is unclear whether recruitment of this activator of Arp2/3-dependent actin assembly depends upon Cdc42.

Furcation of Cdc42-dependent actin assembly pathways may allow for greater regulation of polarized actin assembly among growth conditions and cell types. For example, the Cdc42 effectors Gic1 and Gic2 may be more important in haploids and at higher temperatures in contrast to the putative Cdc42 effectors Msb3 and Msb4, which are suggested to be more important in diploids and at low temperatures [47].

Conclusion

Substantial progress has been made in deciphering the molecular basis of cell polarity in yeast. One important aspect, but still poorly understood, is the regulation of each component, in particular GEFs and GAPs, involved in polarity development. Some of the machinery is specific to yeast, but the general principles underlying cell polarity and the components in the signaling pathways appear highly conserved throughout evolution. Thus understanding the spatial and temporal control of the GTPases and their regulators in yeast is undoubtedly relevant to other eukaryotes. This article aimed to be a brief guide to the field. For in-depth discussion, we recommend recent reviews [25,34,48–51]. A guide to yeast genes can also be found in the *Saccharomyces* Genome Database (SGD) [52].

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CHAPTER 244

Farnesyltransferase Inhibitors

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Introduction

Cell signaling requires proper localization of all the players involved. Many proteins are targeted to the appropriate subcellular location, and are directed to interact with their regulators and effectors, by lipid modification. Prenylation, the modification by a C15 farnesyl isoprenoid or a C20 geranylgeranyl isoprenoid, is required for the biological activity of small GTPases and other proteins containing specific C-terminal CAAX motifs. Inhibitors of farnesyltransferase (FTase), FTIs, have been designed for use as anti-Ras and anticancer drugs, but in fact they are selective for FTase, not for Ras. This distinction has important implications for their use as pharmacological tools to dissect signaling pathways. For example, FTIs can completely suppress the prenylation of H-Ras but not of the other Ras isoforms, so grouping all the Ras proteins together as FTI targets is an oversimplification that adversely affects the interpretation of results using FTIs to block Ras-mediated signaling events. Efforts are underway to identify the biological consequences of disrupting the farnesylation and geranylgeranylation of specific prenylated proteins, and it now seems clear that multiple proteins are involved in cellular responses to FTIs. For example, genetic evidence indicates that RhoB is required for the apoptotic response to FTIs, but not for the suppression of anchorage-independent growth. Understanding the contribution of specific targets to distinct consequences of FTI disruption will be important in identifying the roles of prenylated proteins in specific aspects of cell signaling.

Farnesylation and Protein Function

Like many other things in life, successful cell signaling requires accurate and specific localization of all the players. For many proteins, the correct subcellular localization and biological activity requires the attachment of specific lipids that mediate both positioning within the appropriate cellular compartment and interaction with upstream regulators and downstream effectors. It has become apparent in the last decade or so that the modification of proteins by prenylation, that is, the attachment of isoprenoid lipids, plays a critical role in the signaling activity of G proteins both large and small. In particular, the finding that farnesylation, the attachment of a 15-carbon farnesyl isoprenoid group, to the small GTPase Ras is required for its biological activity, provoked massive efforts to develop inhibitors of (FTase), the enzyme responsible for this attachment. The resulting (FTI) compounds have helped to shed considerable light on the role of such modifications in cell signaling events, but have also introduced a few mysteries of their own.

Ras—the Prototype of Farnesylated Proteins

Ras is an important hub in the complex network of intracellular signaling pathways. Ras proteins receive signals from a wide range of cell surface receptors including receptor tyrosine kinases, G-protein-coupled receptors, and integrins [1]. In turn Ras transmits these signals through an ever-increasing list of effector proteins, the most well characterized of which are the serine/threonine kinase Raf, phosphatidylinositol-3-kinase (PI3-K) and Ral-guanine nucleotide dissociation stimulator (Ral-GDS). Ras signaling ultimately influences gene transcription to alter properties including cell survival, proliferation, differentiation, adhesion, motility, and morphology. Normally GDP-bound and inactive, Ras proteins are converted to the active, GTP-bound conformation by guanine nucleotide exchange factors (GEFs), and back again to the resting state by GTPase-activating proteins (GAPs) that stimulate the

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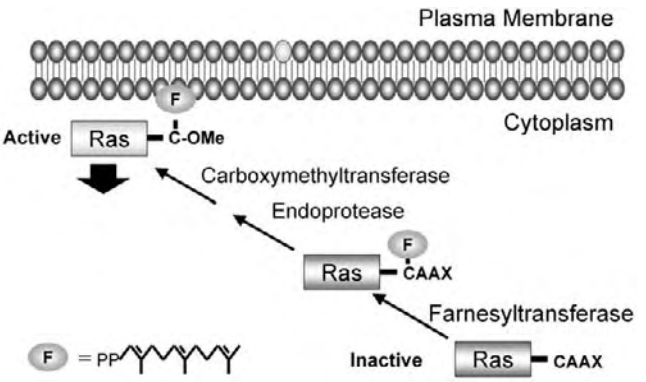


Figure 1 Posttranslational modifications are required for membrane localization and biological activity of prenylated proteins.

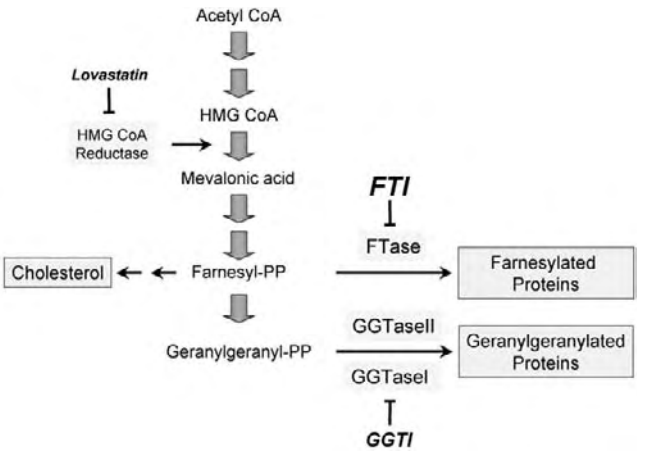


Figure 2 Farnesyl isoprenoids, obligate intermediates in cholesterol biosynthesis, are key elements of the mevalonate pathway.

intrinsic GTPase activity of Ras. When Ras is rendered constitutively GTP-bound and active by oncogenic mutations (typically at positions 12, 13, or 61), the resulting uncontrolled signaling leads to increased cell proliferation, survival in the absence of substratum, loss of contact inhibition, changes in adhesion, motility, and morphology, and the development of other aspects of the transformed phenotype. Alternative splicing results in the three *ras* genes encoding four Ras proteins, H-Ras, N-Ras, K-Ras4A, and K-Ras4B. Recent work suggests that these proteins, while highly similar, are not completely functionally equivalent. In keeping with this possibility, although activating Ras mutations in general are associated with approximately 30% of human cancers, the distribution of mutations of each Ras isoform differs among tumor types [2]. The high prevalence of mutated Ras in cancers has led to many attempts to develop compounds that block Ras signaling for cancer treatment.

Localization of Ras to the inner leaflet of the plasma membrane is an absolute requirement for both its normal and oncogenic functions, and is accomplished through a series of posttranslational modifications (Fig. 1) (reviewed in reference [3]). The first is the addition of a 15-carbon farnesyl isoprenoid to the cysteine of the C terminal four amino acid sequence called the "CAAX motif," where C = cysteine, A = aliphatic, and X = any amino acid. This prenylation step is then followed by proteolysis of the -AAX sequence, carboxymethylation of the resulting farnesylated C-terminal cysteine, and (in H-, N- and K-Ras4A) palmitoylation of either one or two cysteines upstream of the CAAX motif. The first and obligate step of all these posttranslational modifications is catalyzed by FTase. Mutants of Ras that lack a farnesylatable cysteine neither localize to membranes nor signal to downstream effectors. Thus, FTase has been an attractive target for anti-cancer drug development efforts directed toward inhibiting aspects of cellular transformation influenced by Ras. Farnesyl isoprenoids are obligate intermediates in the cholesterol biosynthetic pathway, but blocking FTase is far more selective than disrupting the entire pathway via statins, which are inhibitors of the mevalonic acid precursor (Fig.2). However, although FTIs were originally developed as anti-Ras drugs, the consequences of inhibiting FTase are more complicated than simply inhibiting the farnesylation of Ras. In this chapter we will describe the activities of FTIs, the current confusion surrounding the identity of the biologically relevant downstream targets of FTase, and the use of FTIs as pharmacological tools to disrupt cellular signaling events.

Identification and Development of FTIs

The first FTI was a simple synthetic tetrapeptide corresponding to the CAAX motif of K-Ras4B (CVIM), which was designed to act as a competitive inhibitor of full-length Ras for binding to FTase [4]. However, the instability and poor membrane permeability of simple peptides demanded that different chemical entities be developed. Thus, in rationally designed peptidomimetic FTIs, labile peptide bonds were replaced with more stable chemical moieties [5–7] to increase biological half-life, and modification of the negatively charged carboxyl terminus greatly improved cell permeability. Current FTIs are generally nonpeptidomimetics that have been identified by screening small molecule libraries generated by combinatorial chemistry [8]. Naturally occurring FTIs have also been discovered in screens of natural products from bacteria [9], fungi [10], and plants [11], although only the fungal FTI manumycin has been used extensively for research purposes. The mechanism and kinetics of Ras prenylation by the zinc-containing metalloenzyme FTase, and its inhibition by FTIs, have been studied extensively (reviewed in reference [12]). These studies have been facilitated by the determination of the crystal structure of FTase alone or in complex with Ras and/or farnesylpyrophosphate [13,14], and by the use of NMR to define the interactions between FTase and FTIs in solution. This work has further contributed to refining the structures of subsequent FTIs. Most FTIs developed to date have been competitive for the protein substrate of FTase, but prenyl-competitive [15,16]

and dual-competitive [17] FTIs have also been developed successfully. Peptide-competitive FTIs with IC_{50}s in the micromolar to nanomolar range are commercially available for research purposes. Two of the best of these compounds for use in cell-based assays are FTI-277 [18] (Calbiochem, San Diego, CA) and L744,832 [19] (BioMol, Plymouth Meeting, PA). Other peptide-competitive FTIs with favorable pharmacokinetic and pharmacodynamic properties are currently in clinical trials. These include R115777 (Zarnestra, Johnson & Johnson), SCH66336 (Sarosar, Schering-Plough), BMS-214662 (Bristol Myers-Squibb), and CP-609754 (Pfizer). The status of clinical trials using FTIs is the subject of recent reviews [20,21].

FTI Activity in Cell Culture and Animal Models

Early FTIs showed great promise *in vitro* against purified FTase, with many FTIs inhibiting H-Ras farnesylation in the low nanomolar range [5,22], and demonstrating excellent specificity for FTase compared to the highly related enzyme geranylgeranyltransferase I (GGTase I) [23]. Further, FTIs inhibited the prenylation of H-Ras and other farnesylated proteins in a variety of cell culture systems, as demonstrated most often by gel shift analysis wherein the unprenylated protein migrates slower than the prenylated form. FTIs selectively inhibited transcriptional transactivation of reporters driven by Ras compared to Raf, and also caused reversion of many aspects of the transformed cell phenotype (reviewed in references [3,24,25]). FTIs inhibited the proliferation of H-Ras-transformed cell lines and human tumor-derived cell lines, reverted Ras-transformed morphology, re-established actin stress fibers lost upon Ras transformation, inhibited Ras-dependent anchorage-independent cell growth and migration, inhibited DNA synthesis, and affected the transcription of a variety of genes downstream of Ras-responsive promoters. Oddly, FTIs showed remarkably little toxicity toward normal cells, which is a highly desirable feature for potential drugs, but a surprise at the time given the inability of cells to cope with dominant negative Ras. Except for the relative lack of toxicity, these studies suggested that Ras was an important target of FTIs, and encouraged further studies in animal models.

In both mouse tumor xenograft models and transgenic mouse models, FTIs also produced impressive effects with little overt toxicity (reviewed in reference [26]). In nude mice, FTIs inhibited the growth of a wide variety of human tumor-derived cell lines and human tumor explants from the pancreas, lung, colon, blood, brain, prostate, and bladder. Inhibition of the growth of tumors derived from rodent epithelial and fibroblast cell lines transformed with specific oncogenes including Ras was also observed. Regression of tumors was rare in nude mouse models, consistent with a cytostatic mode of action. Transgenic mice told a different story. In transgenic animals harboring mutations in H-Ras [27], N-Ras [28], or K-Ras [29] giving rise to stochastic mammary, salivary, or lymphoid tumors, FTI treatment inhibited the growth of existing tumors and prevented the growth of new ones. But in H-Ras-driven transgenic tumors, rapid regression of existing tumors was also observed [27]. The basis for the different responses of xenografted and transgenic tumors to FTIs is still not understood.

Although FTIs were originally intended to be used against solid tumors harboring Ras mutations, there is increasing preclinical and clinical evidence that they are actually more effective against various forms of leukemia (reviewed in reference [30]), as well as gliomas and breast cancer (reviewed in reference [31]). These tumors are not typically associated with Ras mutations, but instead may have upregulated Ras signaling due to elevations in epidermal growth factor receptor (EGFR) family activity via overexpression or mutation [1]. Thus, inhibition of endogenous Ras function may play a role in this sensitivity. Whether such inhibition is direct or indirect is a matter of debate.

Studies in human tumor cell lines have also shown that FTIs are additive or synergistic in combination with a wide variety of other anti-cancer agents including [32], taxanes [33,34], aminobisphosphonates [35], cyclophosphamide [36], inhibitors of the MEK pathway [37], and ionizing radiation [38]. It is not clear why FTIs work particularly well with taxanes, but interactions with farnesylated microtubule binding proteins including CENP-E and K-Ras have been proposed. Clearly, many questions regarding the consequences of FTI inhibition still exist.

Alternative Prenylation in the Presence of FTIs

Early in the development of FTIs it was observed that, while prenylation of H-Ras was effectively blocked by FTIs, prenylation of N- and K-Ras was not [39,40]. Instead, when FTase is inhibited, N- and K-Ras were shown to be alternatively prenylated by GGTase I [41,42]. Sharing a common α subunit with FTase, GGTase I catalyzes the addition of a 20-carbon geranylgeranyl isoprenoid to proteins terminating in appropriate CAAX motifs. The canonical CAAX motif specifying geranylgeranylation terminates in leucine (L) [23]. However, in the absence of FTase activity, the C-terminal methionine (M) of N-Ras (CVVM) and K-Ras (CVIM) permits alternative prenylation while the serine (S) of H-Ras (CVLS) does not. Because of this, N- and K-Ras, but not H-Ras, were shown to bypass functional FTI inhibition. Unless farnesylated and geranylgeranylated N- and K-Ras proteins were functionally distinct, which does not appear to be the case, this end-run around FTI suggested that functional compensation by N- and K-Ras for FTI-inhibited H-Ras might explain the lack of toxic side effects of FTIs [40]. However, it also suggested that FTIs might not be effective against the majority of human tumors that harbor N- or K-Ras mutations, an assertion that is inconsistent with the observation that such tumors were sensitive to FTIs in mouse models. Alternative prenylation of N- and K-Ras, as well as of other farnesylated proteins with a C-terminal methionine, further complicates attempts to define clearly the appropriate

downstream targets and mechanism of FTI action. Importantly, a critical role for non-Ras FTI targets is implied by these observations.

FTIs as Pharmacological Tools to Study Signaling and Biology

FTIs have been used to implicate Ras in numerous cellular processes including cell transformation, mitogenesis, neurite outgrowth, motility/migration, response to oxidative stress, transcriptional regulation, and protein secretion. Further, contributions of specific downstream Ras effectors, such as Raf/MEK/Erk, PI3-K/Akt, and upstream Ras activators such as receptor tyrosine kinases have also been implicated by using FTIs. However, the interpretation of such data can also be problematic. Despite their differing responses to FTI, H-, N-, and K-Ras proteins are often wrongly lumped together, and "Ras" is often wrongly presumed to be implicated in any process that is affected by FTI treatment. Thus, FTIs have been used as putative Ras inhibitors in ways analogous to inhibitors of other signaling intermediates such as the MEK inhibitors U0126 and PD98059, the PI3-K inhibitors LY294002 and wortmannin, and the p38 MAPK inhibitor SB203580. This is potentially problematic for two reasons. First, as was discussed in the above section, not all Ras proteins respond equally to FTIs (N- and K-Ras are resistant), and second, Ras proteins are neither the only farnesylated proteins affected by FTIs, nor necessarily the most sensitive. The mechanism of FTI action is still poorly understood because there are many farnesylated proteins other than Ras whose functions are affected by FTIs, making it difficult to conclude that a particular FTI-induced phenotype is Ras dependent. Other possible FTI targets will be discussed in the section about inhibition of signaling by FTIs.

In short, FTIs are literally FTase inhibitors, not Ras inhibitors. As will become apparent, only when the phenotype of interest is known to be H-Ras-dependent, is it really safe to use FTIs as Ras inhibitors. Instead, FTIs should be used as inhibitors of farnesylation, with the understanding that the processing, localization, and function of multiple farnesylated proteins are inhibited upon FTI treatment.

Targets of FTIs

During the early years of FTI research initial success was tempered by the increasing realization that the efficacy of FTIs was not simply the result of functional Ras inhibition, and that the original straightforward model of FTI-as-Ras-inhibitor was not the full story. FTI sensitivity does not correlate with the presence of activating Ras mutations in a series of human tumor cell lines [19], and FTIs inhibit N- and K-Ras-induced transformation, even though these isoforms are alternatively prenylated and therefore not rendered unprocessed and inactive by FTI. Together these observations support the notion that farnesylated proteins other than Ras proteins, whose prenylation and activity are also affected by FTIs, are also important targets of FTI action (reviewed in references [43] and [44]).

Our own search of the SwissProt database revealed approximately 250 human proteins containing a C terminus that resembles a CAAX motif. Some of these are shown in Table I. *In vitro* studies using synthetic or mutant CAAX sequences [4,45,46] demonstrated that many of these proteins are poor substrates for FTase and are not likely normally to be farnesylated. Of those that are potential FTase substrates, many, including numerous members of the Ras superfamily, are known to be farnesylated while others have not yet been evaluated. At present, several CAAX-containing, farnesylated human proteins are currently under investigation as mediators of FTI action.

The most intensively studied putative FTI target is RhoB [47], an immediate-early, inducible small GTPase that is highly related to RhoA and RhoC. Uniquely, RhoB exists in both a farnesylated (F) and a geranylgeranylated (GG) form [48]. The geranylgeranylated form of RhoB is growth inhibitory in both rodent fibroblasts and human carcinomas [49,50], and an increase in RhoB-GG is proposed to mediate the antineoplastic effects of FTIs [51]. It remains to be demonstrated whether a shift of RhoB-F to RhoB-GG or an induction of RhoB-GG correlates with FTI sensitivity in human tumor cells. Interestingly, RhoB may be upregulated as a consequence of FTI treatment, in keeping with its role as a stress detector. Genetic evidence from RhoB null MEFs shows that loss of RhoB impairs the apoptotic response to FTIs [51], suggesting that RhoB is required for apoptosis. By contrast, loss of RhoB does not impair the inhibition of anchorage-independent growth by FTIs [51], suggesting that RhoB is not the mediator of that response.

Other potentially critical FTI targets include the centromere binding protein CENP-E [52,53] which is involved in spindle formation and chromosome alignment during mitosis, the phosphatases PRL-1, -2, and -3, nuclear lamins, inositol 3,5-diphosphate phosphatases I and IV, kinases such as S/T kinase 11, and numerous small GTPases of the Ras (Rheb, Rap2) and Rho (RhoD, RhoE, Rho6, RhoN, and TC-10) families (reviewed in reference [43]). Proteins that have specialized expression patterns, such as the α and β subunits of skeletal muscle phosphorylase B kinase, may play important roles in specialized circumstances. CAAX-containing proteins whose prenylation status has not yet been explicitly determined, but whose CAAX sequences and homology to other farnesylated proteins imply that they too will be farnesylated, include the small GTPases RhoI, Ras-related protein 22 (RRP22), Rab28, and the putative tumor suppressor NOEY2/ARHI, as well as AGS1, a GEF for heterotrimeric G proteins. In addition, there are approximately 40 other human proteins unrelated to GTPases that contain CAAX motifs that could support farnesylation. Further complicating the issue is the observation that several potentially farnesylated proteins have a methionine-containing CAAX motif similar to N- and K-Ras, suggesting that they also might be resistant to FTI inhibition via alternative prenylation.

Table I CAAX-Containing Proteins as Potential FTI Targets

FTI targets	Accession no.	CAAX	Prenyl group
H-Ras	RASH_HUMAN	CVLS	F
hPRL-3	NP_116000	CCVM	F(=>GG?)
Lamin A/C	LAMA_HUMAN	CSIM	F(=>GG?)
Lamin B1	LAM1_HUMAN	CAIM	F(=>GG?)
Lamin B2	LAM2_HUMAN	CYVM	F(=>GG?)
CENP-E	CENE_HUMAN	CKTQ	F
hPRL-1 (PTPCAAX1)	NP_003454	CCIQ	F
hPRL-2 (PTPCAAX2/OV-1)	NP_003470	CCVQ	F
RhoB	RHOB_HUMAN	CKVL	F/GG
Known farnesylated			
DnaJ	DJA1_HUMAN	CQTS	F
gamma-Gt (Transducin)	GBG1_HUMAN	CVIS	F
gamma-T2	GBGU_HUMAN	CLIS	F
gamma11	GBGB_HUMAN	CVIS	F
G-protein-coupled recept.	GP41_HUMAN	CAES	F
InsP3 5-phosph.IV	AF187891	CSVS	F
Phosphorylase B kinase β	KPBB_HUMAN	CLIS	F
Rhodopsin kinase	RK_HUMAN	CLVS	F
K-Ras2A	RASK_HUMAN	CIIM	F=>GG
K-Ras2B	RASL_HUMAN	CVIM	F=>GG
N-Ras	RASN_HUMAN	CVVM	F=>GG
Peroxisomal protein (PxF)	PXF_HUMAN	CLIM	F(=>GG?)
Rheb	RHEB_HUMAN	CSVM	F(=>GG?)
Rho6(Rnd1)	RHO6_HUMAN	CSIM	F(=>GG?)
RhoE(Rho8/Rnd3)	RHOE_HUMAN	CTVM	F(=>GG?)
RhoN(Rho7/Rnd2)	RHON_HUMAN	CNLM	F(=>GG?)
RhoD	RHOD_HUMAN	CVVT	F
TC-10	NP_036381	CLIT	F
Hepatitis delta antigen	AANT_HDVAM	CRPQ	F
InsP3 5-phosph.I	I5P1_HUMAN	CVVQ	F
Phosphorylase B kinase α	KPB1_HUMAN	CAMQ	F
Rap2A	RAP2_HUMAN	CNIQ	F
Likely farnesylated			
AGS1	NP_057168	CVIS	F?
NOEY2/ARHI	U96750	CIIM	F(=>GG?)
RRP22	RR22_HUMAN	CSLM	F(=>GG?)
Rab28	RB28_HUMAN	CAVQ	F?
Serine/threonine kinase 11	ST11_HUMAN	CKQQ	F?

Also, the fact that RhoB (CKVL) is both farnesylated and geranylgeranylated, even though it ends with a leucine (L) that normally signals exclusively for geranylgeranylation, suggests that other proteins might be exceptions to the basic rules governing prenylation. Given the current confusion surrounding the identity of relevant FTI targets, it will be important to determine which of these proteins is farnesylated, affected by FTIs, and can account for the observed biological effects of FTIs. In any case, it is likely that FTIs will affect numerous farnesylated proteins to produce complex, cell-type-specific effects.

Inhibition of Signaling by FTIs

Why must farnesylated proteins be farnesylated? First, this lipid modification has been shown to be necessary for membrane localization. Both pharmacological inhibition with

lovastatin and similar compounds and with FTIs prevent farnesylation and cause normally membrane-localized farnesylated proteins to remain cytosolic. Mutagenesis of the CAAX motif to a SAAX motif, which cannot be prenylated, also renders normally farnesylated proteins cytosolic. These results imply the existence of specific protein:lipid interaction sites at membranes, whether simply to increase hydrophobicity or for specific docking interactions. Second, farnesylation is important for protein:protein interactions. Farnesylated Ras proteins have long been known to be better substrates for GEFs such as SOS, to interact better with downstream effectors such as PI3-K p110δ and adenylyl cyclase [54] and to stimulate downstream signaling to kinases such as ERK2 as compared to their nonfarnesylated counterparts, even in cell-free systems [55]. The assembly of β/γ dimers of heterotrimeric G proteins depends upon appropriate prenylation of the γ subunit [56,57], as does cell-free activation of downstream effectors including PLC-β and adenylyl cyclase [57]. The enhancement of protein:protein interactions by lipid modification even in cell-free systems suggests that this is not simply a secondary consequence of necessary subcellular localization conferred by the isoprenoid.

If the critical consequences of FTI activity are due to cellular stress responses, then the most critical FTI targets may not necessarily even be farnesylated (viz. RhoB). But if the consequences of FTI activity are primarily due to blocking FTase-mediated farnesylation of target proteins, then the challenge is to determine the identities and relative affinity for FTase of all farnesylated proteins. FTIs can demonstrate the existence of farnesylated proteins in a particular pathway. Growth inhibition by FTIs is associated primarily with their induction of cell cycle arrest in either G0/G1 or G2/M, depending on p53 status. FTIs do not generally kill transformed cells except in conjunction with a second apoptotic signal such as serum starvation [58] or the absence of substratum [59], but exceptions have been observed. In some human tumor cell lines, sensitivity to FTI-induced apoptosis is dependent on the inhibition of the PI3-K/Akt/BAD pathway and a concomitant reduction in the level of phospho-Akt in those cells [60]. What farnesylated protein is critical to regulate this pathway is unknown at present, although it could be endogenous H-Ras; it has also been suggested that FTI does not inhibit Akt via blocking the processing and activity of a farnesylated protein, but rather by upregulating the expression of RhoB [61]. However, even the correlation between FTI-induced apoptosis and phospho-Akt suppression is not universal across all cell types, so additional mechanisms are likely. FTIs may also have an indirect effect on tumor growth by inhibiting angiogenesis [62,63], and this is known to be the consequence of a decrease in vascular endothelial growth factor (VEGF). However, it is unclear whether the farnesylated protein controlling VEGF that is inhibited by FTI to produce the anti-angiogenic effect is a Ras protein, which is known to regulate VEGF in a PI3-K-dependent manner, or is another protein altogether. Therefore, at this stage it is not possible to draw definitive causal connections between most of the observed cellular effects of FTIs and particular biochemical pathways that may mediate those effects. Making such connections is now a major goal for understanding what FTIs are doing in the context of both whole cells and animals.

Summary and Prospects

The development of FTIs during the past ten years constitutes a convoluted story. Originally designed to treat Ras-related cancers by inhibiting oncogenic Ras function, FTIs are now thought to be ineffective against N- and K-Ras due to alternative prenylation, and to exert their effects primarily through inhibition of the farnesylation of other substrate proteins or the induction of proteins such as RhoB. With so many potential FTI targets, each with a different cellular function and sensitivity to FTIs, the task of unraveling the mechanism of FTI action is complex. But even in the absence of an understanding of which specific substrates mediate which specific consequences, it is clear that FTIs do have utility as anti-cancer agents, and can be useful tools to demonstrate the contribution of farnesylated proteins to the control of a particular signaling pathway.

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CHAPTER 245

Structure of Rho Family Targets

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The variety of cellular processes controlled by the Rho family proteins, of which Cdc42, Rac, and Rho are the most widely studied members, are mediated through a number of downstream effector proteins, many of which are still being characterized. The members of the Rho subfamily have a far greater number of effector proteins than other G-protein subfamilies. The effectors can be loosely divided into those that bind to Cdc42 and Rac and those that bind to Rho. The effectors are both functionally and structurally diverse, as is the nature of their interactions with the small G proteins.

Structurally, the Rho family proteins are distinguished from the Ras family by the presence of an extra pair of helices (the insert region) [1]. It has been reported that removal of this insert region in Rac expressed *in vivo* prevented both the formation of membrane ruffles [2] and transformation [3]. Thus it is expected that at least one target protein of the Rho family will interact with this region. The structures of six Rho family effectors and their complexes have been studied so far and will be described here.

CRIB Proteins

Many of the downstream effectors for the Rho family proteins Cdc42 and Rac contain a small (16 amino acid) consensus sequence known as the CRIB (Cdc42/Rac interactive binding), which is essential for mediating interactions with the G proteins [4]. In several studies it has been shown that additional residues, C terminal to the CRIB, are also necessary for tight binding, making the full G protein binding domain (GBD) 40–45 amino acids. All the CRIB proteins bind to Cdc42·GTP and some of them bind to Rac·GTP. Structural studies of the CRIB family proteins have addressed two fundamental questions: How do some CRIB proteins discriminate between the closely related Cdc42 and Rac, and how does binding a Rho family protein activate downstream events?

The solution structures of three different Cdc42/CRIB complexes have been solved: activated Cdc42 kinase (ACK), a tyrosine kinase, which has been implicated in integrin signaling and endocytosis [5]; Wiscott-Aldrich syndrome protein (WASP), which is thought to mediate interactions with the cytoskeleton [6], and p21 activated kinase (PAK), a serine/threonine kinase involved in JNK signaling and cytoskeletal rearrangements [7]. Comparison of these structures reveals interesting differences in the way that the effectors contact the G proteins (Fig. 1). In addition, since ACK and WASP are specific for Cdc42 while PAK binds to both Rac and Cdc42, the structures shed light on how the CRIB proteins may discriminate between two such similar molecules. In each structure, the CRIB consensus region binds in a similar manner, forming an intermolecular β-sheet with the β2-strand of Cdc42 and then interacting with switch I. This is reminiscent of the structure of Ras with its effectors Raf-1 [8] and Ral-GEF [9], both of which form a similar intermolecular β-sheet, although all the other features of these structures differ. The regions outside the CRIB consensus all interact with the same regions of Cdc42, helix α5, and switch II, although the details of the interactions are all different. ACK does not form any more secondary structure but wraps around the G protein, forming an irregular hairpin at the top of switch I. WASP and PAK both form a regular β-hairpin that interacts with switch I and switch II, followed by a short piece of α-helix which interacts with switch II. In WASP and PAK the relative orientation of this hairpin and α-helix are different, as is their orientation with respect to the switch II helix.

NMR studies on the free GBDs of these three proteins revealed no significant tertiary structure. In both PAK and WASP there was some evidence for the formation of the short section of α-helix that is seen in the complex with Cdc42 [7,10]. In ACK, where there is no secondary structure in the complex, none could be discerned in the free GBD [5].

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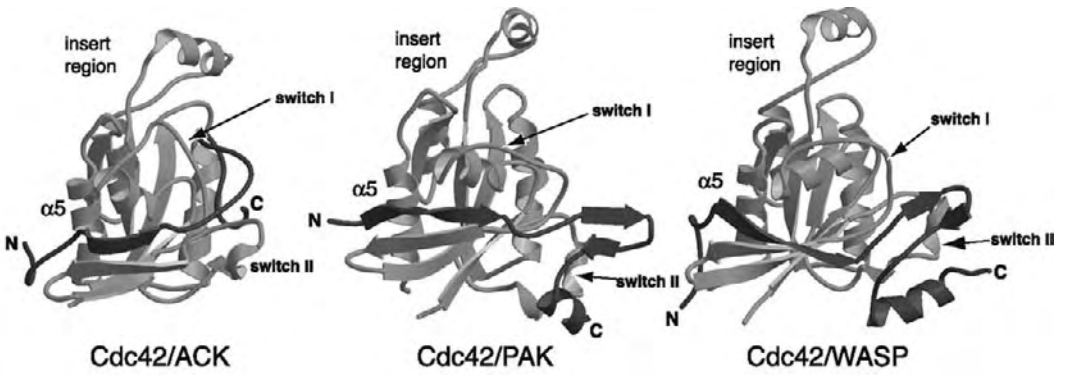


Figure 1 Comparison of CRIB/Cdc42 structures. In each case the G protein is shown in pale gray and the CRIB fragment in dark gray. The insert region that defines the Rho family is marked. The positions of the two switch regions that become fixed on effector binding are also shown, as is the position of the α5-helix, which interacts with the CRIB effector in all three cases and is the location of the mutation that specifically disrupts Cdc42/ACK and Cdc42/WASP binding.

Sequence alignments of Cdc42 and Rac reveal that switches I and II are almost completely conserved, the only difference being a single (conservative) substitution. It was therefore clear that the basis of the selectivity of CRIB effector proteins such as ACK and WASP for Cdc42 would lie outside these switches. Mutagenesis studies combined with measurements of K_d suggested that interactions with Leu-174 in helix α5 of Cdc42 contribute to binding of WASP and ACK to Cdc42 but do not contribute to PAK binding [11]. Position 174 is an Arg residue in Rac and thus it may represent one of the points of discrimination between G proteins. Analysis of single residue mutations may not lead us to a complete understanding of the discrimination between Cdc42 and Rac, because in all the Cdc42/effector complexes solved the buried surface area is large (2500–4000 Å^2). Also, since there is no Rac/CRIB fragment complex solved so far, the details of Rac/CRIB interactions are still unknown.

Insight into the activation mechanisms of the CRIB proteins came when the structures of both PAK and WASP in autoinhibited forms were solved [12,13]. WASP has no enzyme activity but it has a region at the C terminus that binds both the Arp2/3 complex and actin. It was found that a small region of the C terminus that is homologous to cofilin (CHR) interacts with residues within and C terminal to the GBD. The structure of a molecule comprising the CHR tethered by a flexible linker to the GBD extended at the C terminus by approximately 20 residues has been solved (Fig. 2). This structure showed that the β-hairpin and α-helix seen in the Cdc42 complex are still present but that they now interact with three extra α-helices C terminal to the GBD, mainly through hydrophobic contacts. These four helices form a hydrophobic surface, against which a helix from the CHR is packed. It is clear that in this form the protein cannot bind either to the Arp2/3 complex (via the CHR) or to Cdc42 (via the GBD). To bind Cdc42 the protein would have to undergo a conformational change that results in release of the CHR, allowing it to bind to other partners. The thermodynamic cost of this conformational change would be paid for by a lower binding affinity between the autoinhibited form of the protein and Cdc42 than between GBD fragments

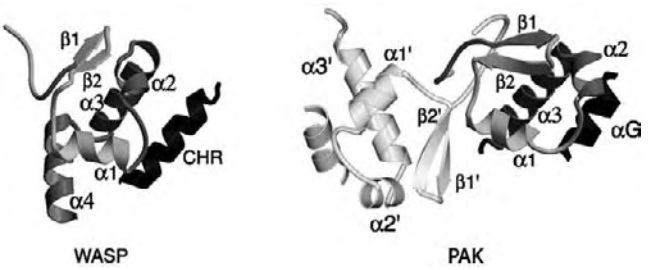


Figure 2 Structure of autoinhibited WASP and PAK fragments. The layers of the structures are shown in different shades. In both cases the β1/β2 and α1 elements are part of the GBD and are involved in the interaction with Cdc42; α2 and α3 are in the region immediately C terminal to the GBD that is involved in negative regulation of the C terminus of the molecule. In WASP, helix α4 follows α3; this helix has no counterpart in PAK. The CHR and αG are the cofilin homology region (WASP) and helix G from the kinase domain (PAK), respectively. The second PAK monomer is shown in pale gray and labeled β1′, α1′ etc.

and Cdc42 as is seen, with K_ds approximately 100-fold higher for the tethered construct [13].

It had been shown by yeast-two hybrid experiments with PAK that a region C terminal to the GBD, known as the kinase inhibitory (KI) domain could bind directly to its kinase domain, thus inhibiting its catalytic activity. An x-ray-derived structure has been solved of the GBD/KI bound to the kinase domain and reveals that the GBD/KI domain is strikingly similar to the equivalent region of WASP, although in PAK there are only three helices to WASP's four (Fig. 2). One of the helices from the kinase domain packs against the three helices of the KI domain in a manner closely resembling the CHR helix of WASP packing against its autoinhibitory domain. There is one striking difference in the PAK structure: it is a dimer that is held together by an interaction between the CRIB/KI regions. Given this structure it would seem to be impossible to form a Cdc42/PAK complex without breaking the dimer. This was shown in a later paper by the same authors [14]. However others have shown that PAK forms a stable dimer, even in the presence of Cdc42 [15].

The activation mechanism of ACK is not known at present but it is likely to involve a similar, intramolecular inhibition.

This is implied by the discovery that mutation of Leu-543 of ACK causes constitutive activation of the kinase [16]. This Leu interacts with switch II in the Cdc42/ACK complex [5] and mutation of the equivalent Leu residue in PAK (Leu-107) or WASP (Leu-270) also disrupts their autoinhibitory interactions [17,18]. The activation of ACK may, however, be more complicated since it appears that Cdc42 also disrupts an intramolecular interaction between the SH3 domain and a Pro-rich region [19]. An intramolecular SH3/Pro interaction is also thought to exist in another CRIB-containing protein, mixed lineage kinase 3 (MLK3) [20]. In both ACK and MLK3 the role of Cdc42 binding in activation is still unclear.

Non-CRIB Rac Effectors

p67^{phox}

p67^{phox} is one component of the multiprotein enzyme complex, NADPH oxidase. This complex, found in phagocytes, forms the principal defense mechanism against microbial infection in humans. Binding of Rac to p67^{phox} is a critical step in the activation of the latent NADPH oxidase complex. The Rac binding region of p67^{phox} had been localized to the N terminal 200 amino acids, which contains four copies of the tetratricopeptide repeat (TPR) motif. Structures of other TPR-containing proteins had shown that each TPR motif is composed of a pair of anti-parallel α-helices (A and B), and that these repeated units pack together to form an extended structure with an amphipathic groove on the A helix face of the domain (the TPR groove), which mediates interactions with other proteins [21,22]. The structure of Rac·GTP complexed with the N terminal 200 amino acids of p67^{phox} revealed both an effector GBD distinct from the CRIB family of effectors and also a different use of the TPR repeats as a binding motif [23]. The N terminus of p67^{phox} contains 9 α-helices: the first eight of these form four TPR motifs, while the ninth helix packs against the B helix of TPR4 (Fig. 3). The TPR groove is filled by a stretch of residues C terminal to the ninth helix, which binds in an extended conformation and thus it is not available for intermolecular interactions. Rather, contacts are made between Rac and one face of the TPR domain, which consist of a β–hairpin insertion between TPR3 and 4 and the loops connecting TPR1 with TRP2 and TPR2 with TPR3. Contacts on the G-protein side are also unusual and include residues from helix α1 and the following loop, residues from the N terminal end of switch I, and the loop between strand β5 and helix α5. The TPR domain does not contact all of switch I and no contacts are seen with switch II or the insert region. This is in contrast to the complexes between Cdc42 and the CRIB effectors, where extensive contacts are made with both switch I and II. The TPR/Rac complex also differs from the CRIB effector complexes in that no intramolecular β–sheet is formed. Still, 1170 Å^2 is buried in the TPR/Rac complex, less than that in the CRIB/Cdc42 complexes, possibly resulting in the lower affinity observed.

p67^{phox} binds specifically to Rac rather than Cdc42. Analysis of the residues involved in the interface showed that all were conserved between Rac and Cdc42, except Gly-30. Ala-27 and Gly-30 have been defined as critical residues for the specificity of the interaction between Rac and p67^{phox}: Mutation of these residues in Cdc42 to the corresponding residues in Rac results in a Cdc42 protein that binds p67^{phox} with a relatively high affinity [23]. Ala-27 does not directly contact the TPR domain but in Cdc42 this residue is a Lys, which would cause a steric clash, preventing binding.

Arfaptin

Arfaptin (or POR, partner of *R*ac) was identified independently as an effector for both the Rac and Arf small G proteins and, consequently, has been proposed to be a facilitator of crosstalk between signaling pathways. Arfaptin (residues 118–341) has been crystalized alone and in complex with both Rac·GDP and Rac·GMPPNP [24]. The Arfaptin domain consists of 3 α–helices (A–C) that form an anti-parallel α–helical bundle; 2 of these self-associate to give an elongated crescent-shaped dimer, with an overall length of 140Å, which binds to one Rac molecule. Rac sits on the concave surface of the Arfaptin crescent close to the dimer interface, and contacts are seen predominantly between switch I and II of Rac and monomer A of Arfaptin (Fig. 3). Switch I packs against helix αA while switch II interacts with helix αB. A single contact is seen to monomer B, at His 57 in helix αA′. This interaction is sufficient to preclude the binding of another Rac molecule to Arfaptin, thus accounting for the observed stoichiometry of 1 Rac:1 Arfaptin dimer. 1600Å^2 of solvent-accessible surface area is buried in the complex. Similar of Arfaptin affinities for both the GDP- and GTP-bound forms of Rac argues against its being a conventional effector for the G protein. In contrast, Arfaptin binds to both Arf1 and Arf6 in a GTP dependent manner. Examination of the Rac·GDP and Rac·GMPPNP molecules reveals crucial similarities between the two in complex with Arfaptin. Critically, Thr-35, which coordinates to the Mg^{2+} in G protein·GTP forms is instead in contact with Arfaptin, giving rise to a more GDP-like structure. It is predicted that a canonical Rac·GTP conformation could not be accommodated by Arfaptin [24]. Presumably, in the Arf/Arfaptin complex the G protein can take up its usual GTP-like conformation, thus allowing discrimination in that case. It is possible that Arfaptin's ability to bind both forms of Rac allows it to sequester Rac until Arf is activated, whereupon Arf displaces Rac freeing it to signal appropriately. In this model, the function of Arf is to allow coordinated activation of Rac and Arf.

Rho Effectors

Effectors for Rho include at least eleven proteins: DAG Kinase, PLD, PIP5-kinase, Kinectin, Rhotekin, Rhophilin, p140 Diaphanous, MBS, Citron, ROK and PRK. Within this

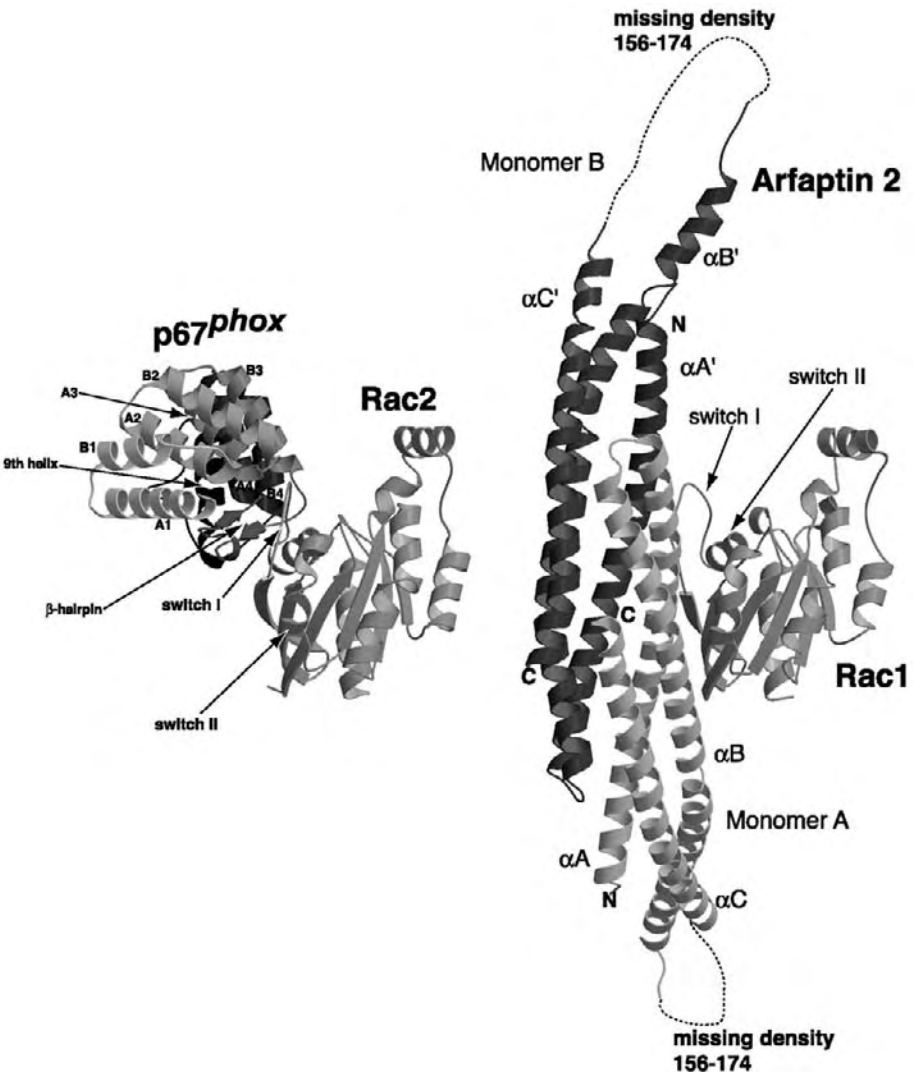


Figure 3 The structures of Rac2/p67^{phox} and Rac1/Arfaptin 2. The layers of the TPR repeats of the p67^{phox} become darker going from the N to the C terminus. The switch regions of Rac are shown in both structures. The two monomers in the Arfaptin 2 dimer are shown in different shades. In the Arfaptin structure the two regions where no electron density was observed are shown as dotted lines.

group of effector proteins there are at least two Rho binding motifs defined by sequence homology: REM proteins (or Class 1 Rho binding motif) include the PRKs, Rhophilin and Rhotekin, while RKH proteins (REM2 or Class 2 Rho binding motif) include the ROKs and Kinectin (reviewed in [25]). Structural information is limited at present to one of the REM proteins, PRK1, in complex with RhoA.

Protein Kinase C Related Kinases

PRK1 (PKN) and 2 are highly related serine/threonine kinases with a catalytic domain homologous to that of the protein kinase C family in their C termini and a unique regulatory domain in their N termini [26,27]. The N terminus of the PRK1 contains three HR1 repeats, one of which, HR1a, incorporates an inhibitory pseudosubstrate site [28]. Kinase activity is enhanced by binding of GTP-bound Rho or Rac [29–32].

The x-ray structure of RhoA in complex with the HR1a repeat of PRK1 describes the fold of the HR1a domain as an **a**nti-parallel **c**oiled-**c**oil (ACC) finger domain [33] (Fig. 4). The ACC finger domain is quite distinct from other G protein binding domains and known Rho family effectors. It does show limited similarity with the Rab binding domain of Rabphilin and the Rac/Arf effector Arfaptin, but the contacts that these effectors make with their G proteins are quite different [24,34]. The structure of the complex between HR1a and RhoA indicated two possible contacts sites on RhoA for HR1a. The major site, contact 1, has a buried surface area of 2080Å^2 and mainly involves hydrophilic interactions. This contact involves residues in the β2- and β3-strands of RhoA, the N-terminal part of helix α5 and residues at the ends of switch I. Contact 2, which involves more hydrophobic residues, buries a total surface area of 1640Å^2 and involves residues in switch I (Val38–Asn41),

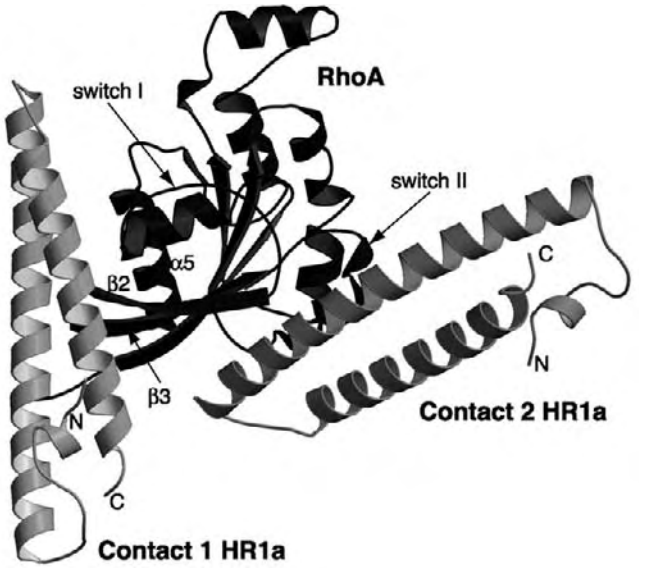


Figure 4 The structure of the RhoA/PRK1 HR1a complex. The two potential HR1a binding sites are shown as contact 1 and contact 2. Contact 1 was defined as the primary contact site on the basis of the buried surface area, although the HR1a in contact 2 makes more interactions with the switches.

strand β3, and switch II (Trp58 and Asp65–Asp76). The size of contact 2 means that it is unlikely to be an artifact of crystal packing. It is also noteworthy that it involves more residues in the switch regions of RhoA, which are likely to be involved in effector binding.

Concluding Remarks

We have attempted to summarize here the pertinent details of all the Rho family/effector structures determined. Several points emerge from this discussion. First, it is clear that the structural diversity in the Rho effectors is extensive. It is likely, for example, that the CRIB proteins, although they will all have some similarities in the way that they interact, will also differ in their details, particularly outside the short CRIB consensus sequence. The other three effector structures are also completely different both to each other and to the CRIBs, although it is likely that families will emerge whose members interact with the G proteins in a similar, but not identical manner. There are also, however, several other effector proteins with no sequence homology to Arfaptin, PRK, TPR domains, or CRIBs. It is likely that these proteins will adopt different structures and will interact with the Rho family proteins in novel ways.

The manner in which the effectors contact the Rho family G proteins is multifarious. They utilize β-strands (the CRIBs), β-hairpins (the CRIBs), α-helices (Arfaptin, PRK1, and the CRIBs), interhelical loops ($p67^{phox}$), and even a dimer interface (Arfaptin) to interact with the G protein. In some cases, such as the CRIB proteins, the effectors make an extensive set of contacts with the Rho family proteins, burying a large surface area (2500–4000 $Å^2$) while in others, such as $p67^{phox}$, which binds with a lower affinity, the buried surface is only ~1200$Å^2$.

The region of the G proteins that interact with the downstream targets is also not conserved. In most cases, switch I is involved in the interactions, which is perhaps not a surprise given that effectors bind preferentially to the GTP-bound form of the G protein. It is usually the case that other regions of the G protein are also involved in binding to effectors; this may be in part to bring about specificity. The switches are relatively well conserved within the family while the diversity in the other regions is higher. In most of the structures discussed here switch II is also involved in the interaction; the exceptions being $p67^{phox}$ and PRK1, contact I. The other regions of the Rho family that are involved in effector binding are helix α1, the β2- and β3-strands, the β5-α5 loop, and the C-terminal helix, α5. In no case so far is the insert region involved in effector binding.

Structural information on the Rho family targets has moved at an exciting pace over the last three years. The next stage must be to determine how the binding of the Rho family protein then causes the downstream effects that are seen. This point has begun to be addressed with the elegant work on PAK and WASP activation. We can look forward to many more such breakthroughs in the future, where a combination of structural and mechanistic studies will help us to understand fully these complex systems.

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CHAPTER 246

Structural Features of RhoGEFs

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Introduction

Effective cellular signaling relies upon the tight control of the various proteins within a signal transduction pathway. For GTPases (guanosine triphosphatases), common mechanisms of regulating the activation and attenuation of these critical molecules have sustained throughout evolution. All GTPases cycle between two discrete states, an inactive guanosine diphosphate (GDP)-bound form, and an active guanosine triphosphate (GTP)-bound form. The removal of GDP nucleotide from an inactive GTPase allows subsequent loading of GTP, thereby triggering these "binary switches" to recognize downstream effectors. This critical process of GTPase activation is rigidly controlled by guanine nucleotide exchange factors (GEFs).

Dbl family proteins are the major recognized class of GEFs for the Rho family of small GTPases [1–3]. Rho GTPases have risen to prominence since a large body of work over the last ten years has implicated these ~25-kDa members of the Ras superfamily in controlling vital cellular functions, including organization of the actin cytoskeleton, progression through the cell cycle, and regulation of transcriptional activities [4–9]. Given that Rho GTPases manage various critical cellular processes, it is not surprising that these small GTPases, as well as their activators (RhoGEFs), promote oncogenesis when constitutively activated [10–16]. Membership within the Dbl family of RhoGEFs is solely dependent upon the possession of an ~300 amino acid segment containing a Dbl homology (DH) domain directly adjacent to a pleckstrin homology (PH) domain [17]. While PH domains exist in a multitude of signaling proteins, the DH domain is unique to these RhoGEFs, and accordingly constitutes the primary catalytic portion of a Dbl protein by supporting nucleotide exchange activity within a substrate Rho GTPase *in vitro* and *in vivo* [18,19].

Recent biophysical investigations into the function of Dbl-family proteins have revealed substantial insight into the means by which these RhoGEFs catalyze the removal of bound nucleotide from Rho proteins. Specifically, an understanding at atomic resolution of the roles of the conserved DH and PH domains found within all Dbl-related proteins is now available. Here we survey the structural features of RhoGEFs and highlight the key determinants responsible for dictating the activation of Rho GTPases.

Structural Accomplishments

The founding member of the Dbl family, Dbl, was first identified as a transforming factor from a human *d*iffuse *B*-cell *l*ymphoma [20,21]. Further analysis delimited the DH/PH domain segment as the minimal transforming portion of Dbl-related RhoGEFs [22]. While most Dbl proteins possess a plethora of protein modules, which most likely regulate GEF activity, including Src homology 2 (SH2) domains, Src homology 3 (SH3) domains, Ras-binding domains (RBD), and regulator of G-protein signaling (RGS) domains, the conserved tandem DH and PH domains have been clearly characterized as the region possessing biochemical guanine nucleotide exchange activity for Rho GTPase targets. This combined information has set the stage for an in-depth structural examination of the catalytic mechanism of Dbl proteins.

NMR structures of the isolated DH domains from the exchange factors Trio [23], βPIX [24], and Vav [25], as well

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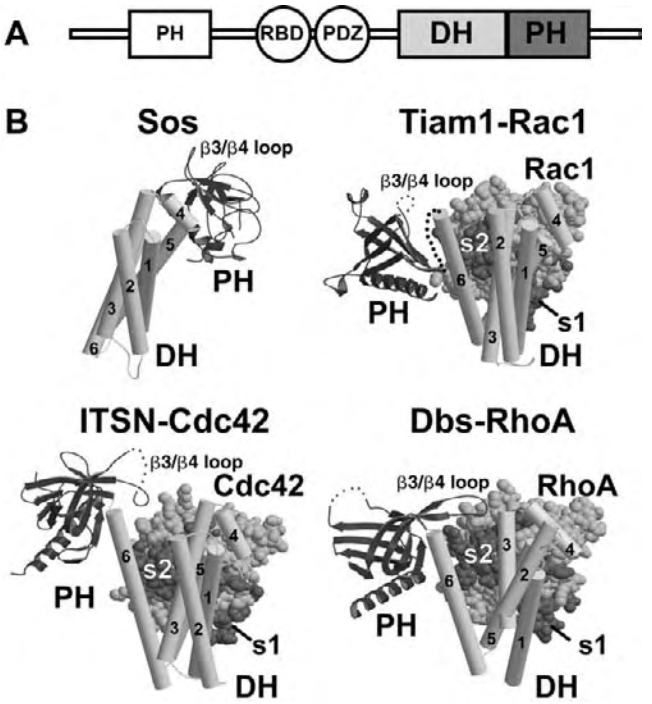


Figure 1 Structural comparison of the DH/PH portion of several Dbl exchange factors. (A) Domain architecture of Tiam1, a representative Dbl family protein, highlighting the conserved DH/PH domain cassette. Tiam1 also possesses an N-terminal PH domain, a Ras binding domain (RBD), and a PDZ domain. (B) The crystal structures of Sos DH/PH (PDB accession code #1DBH), Tiam1-Rac1 (PDB accession code #1FOE), ITSN-Cdc42 (PDB accession code #1KI1), and Dbs-RhoA (PDB accession code #1LB1) were aligned using the CR1 and CR3 regions of the DH domains. The identified helical segments of the DH domains (yellow) are depicted as cylinders, while the PH domains (blue) are presented as ribbon representations. Bound Rho GTPase substrates are portrayed as green CPK spheres, with the exception of the noted switch regions (s1, s2), highlighted in red. Black circles depict the linker region between the DH and PH domains of Tiam1, while blue circles illustrate PH domain loops that are disordered in the crystal structures. (A color representation of this figure is available on the CD version of the *Handbook of Cell Signaling*.)

as the crystal structure of the DH/PH portion of son of sevenless (Sos) [26], first presented the three-dimensional architecture of a DH domain (Fig. 1). In addition, significant insight has been gained by crystal structures of the DH/PH fragments of several RhoGEFs in complex with their cognate Rho GTPase substrates. Specifically, the initial structure of the DH/PH segment of Tiam1 bound to Rac1 (Tiam1-Rac1) [27], followed by subsequent similar structures, (Dbs-Cdc42, Dbs-RhoA, intersectin (ITSN)-Cdc42) [28,29] have explained several facets of the conserved mechanism of Dbl protein catalyzed nucleotide exchange.

DH Domain Features

DH domains form an elongated α-helical bundle composed of six major helical segments, with a unique fold unrelated to other nucleotide exchange factors (RCC1, Sec7 domain, EF-Ts, and the Cdc25 domain of Sos) for different G-protein families [30,31]. DH domains possess three evolutionarily *c*onserved *r*egions: CRs 1–3. Together CR1

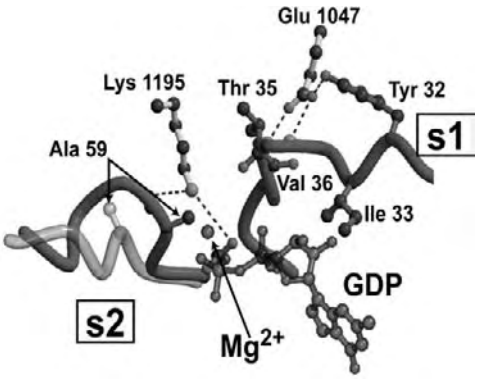


Figure 2 Mechanism of RhoGEF-induced release of GDP from a Rho GTPase. Close-up view of the switch regions of Rac1 (red) binding important Tiam1 residues (yellow) in the Tiam1-Rac1 complex. (Adapted from Worthylake, D. *et al*., *Nature*, **408**, 682–688.With permission). GEF-GTPase interactions are depicted with dotted blue lines. GDP and magnesium ion (in semi-transparent blue) have been superimposed in their natural binding sites of a Rho GTPase based on the Cdc42-GDP structure (PDB accession code #1AN0). A portion of switch II is shown both before (pink), and after (red) engaging Tiam1, to highlight the dramatic rearrangement of Ala 59, which occludes magnesium binding, and therefore promotes removal of nucleotide. (A color representation of this figure is available on the CD version of the *Handbook of Cell Signaling*.)

(within helix 1), and CR3 (within helix 5), along with a small region of helix 6, create the primary binding surface for a substrate Rho protein (Fig. 1). In sharp contrast, CR2 (within helix 2), maps to the opposite face from the active site, and most likely functions to stabilize the helical bundle.

The Tiam1-Rac1 structure first revealed the critical DH domain positions that engage the nucleotide-binding "switch" regions of a Rho GTPase [27]. Specifically, well-conserved, solvent-exposed DH domain residues equivalent to Glu 1047 and Lys 1195 in Tiam1 make important contacts with Thr 35 and Ala 59 of Rac1, respectively (Fig. 2). Subsequent RhoGEF/Rho GTPase structures [28,29], and mutagenesis within the DH domains of Trio [23], and Dbl [32] support the fundamental importance of these positions in catalyzing the removal of bound GDP from a Rho GTPase. In all four RhoGEF/Rho GTPase structures [27–29], the DH domains bury significant solvent exposed surface area to stabilize the GTPase in a nucleotide-free transition between the inactive and active states.

DH-Associated PH Domains

Extensively characterized as lipid-binding membrane tethers, PH domains from many different proteins display a wide assortment of affinities and specificities for phosphoinositide components of the cell membrane [33–35]. The invariant organization of the DH and PH domains in RhoGEFs suggests a vital role of the PH domain in Dbl-catalyzed nucleotide exchange. Specifically, the PH domain has been implicated in anchoring a Dbl protein to the cellular membrane [36], allosterically regulating GEF activity via phosphoinositide interactions [37,38], and directly engaging the substrate GTPase [28].

Structurally, within Sos, the PH domain is connected to the DH domain by a flexible interdomain linker, while for Tiam1, Dbs and ITSN, the PH domain begins immediately after the terminal helix (α6) of the DH domain. Overall, Dbl PH domains resemble the classical ~100 residue anti-parallel β-sandwich PH domain fold, composed of seven strands capped by a terminal α-helix. However, unlike PH domains from other proteins, DH-associated PH domains usually possess an additional short β-strand and a 3_{10} helix at the N terminus of the fold, that pack against the α6 helix of the DH domain using extensive hydrophobic interactions.

PH Domain Configurations

Comparison of the DH/PH structure of Sos with the Tiam1-Rac1, Dbs-Cdc42, Dbs-RhoA, and ITSN-Cdc42 structures provides significant insight into the dramatic conformational heterogeneity of the PH domain. Where the PH domain of Sos partially occludes the GTPase binding site of the DH domain, within the RhoGEF/Rho GTPase complexes, the PH domains are positioned to allow the Rho proteins access to the CR1/CR3 surface of the DH domain (Fig. 1). In addition, where the PH domains of Tiam1 and ITSN reside distant from Rac1 and Cdc42, respectively, the PH domain of Dbs is rotated about the α6 helix of the DH domain such that the β3/β4 loop of the PH domain now contacts the substrate GTPases, (Cdc42 and RhoA), with interactions shown to be functionally important for exchange activity [28]. Together, this structural data potentially reflect several of the configurations that DH and PH domains sample when engaging their target Rho GTPases. However, complicating this model is the fact that, *in vitro*, Dbs requires its PH domain for full catalytic activity when comparing DH to DH/PH elements [28], while the PH domain of ITSN is dispensable for robust exchange activity (our unpublished observations). Thus, it is quite possible that these structural snapshots depict two distinct classes of RhoGEFs, where Dbs and closely related family members can utilize their PH domains to assist in activating a Rho GTPase, while others cannot.

With regard to the functional role of the conserved PH domain, several reports document allosteric modulation of RhoGEF activity in response to specific phosphoinositides binding the PH domain [37–39]. In contrast, other studies find conflicting or no modulation of DH domain-catalyzed nucleotide exchange activity in response to phosphoinositides [40,41]. A large body of structural studies has revealed how PH domains specifically engage the head groups (inositol phosphates) of phosphoinositides, using a positively charged patch composed of variable length loops [33]. It is quite possible that at a cellular membrane, specific phosphoinositide-PH domain interactions could restrict the conformation of the PH domain leading to novel PH domain/GTPase, or PH domain/DH domain contacts that modulate exchange activity. However, studies involving prenylated Rho proteins, and using lipid bilayers will be necessary to dissect the complete functional roles of the DH-associated PH domain in activating Rho GTPases.

Mechanism of Nucleotide Exchange

An important feature revealed by the RhoGEF/Rho GTPase structures is the mechanism of release of bound GDP from the GTPase. As mentioned, all GTPases possess flexible switch regions that adopt distinct conformations depending upon the state of bound nucleotide [42]. Similar to other characterized GEFs [43], DH domains engage switches I and II of the Rho protein, and induce these regions to physically impede upon both magnesium and GDP binding sites. For example, significant rearrangement of switch II (residues 57–75) of Rac1 occurs in the Tiam1-Rac1 structure relative to the architecture of an inactive GTPase [27]. Importantly, Ala 59 of Rac1, stabilized by Lys 1195 of Tiam1 dramatically swings into the binding site of the Mg^{2+} ion normally associated with bound nucleotides (Fig. 2). The crystal structure of the Mg^{2+}-free form of RhoA-GDP documents the paramount consequence of the removal of magnesium in encouraging an open conformation of the switch regions of a Rho GTPase [44].

Additionally, other RhoGEF-induced rearrangements within the switch regions of a Rho GTPase help stabilize the nucleotide-free state of the protein. For instance, Glu 1047 in CR1 of Tiam1 binds several Rac1 residues of switch I (Tyr 32, Thr 35, Val 36) to effectively shift Ile 33 of Rac1 into the binding site of the bound GDP (Fig. 2). This reorientation of switch I destroys the positive interaction that Cys 18 of Rac1 normally makes with bound nucleotide in the Rho GTPase. Moreover, binding of the DH domain of Tiam1 is further secured by Tyr 64 of Rac1, which contacts a set of conserved Tiam1 residues [27]. In addition, Tiam1 binding prevents Phe 28 of Rac1 from making a positive contact with bound nucleotide. This observation is consistent with the finding that Rac1(F28L) exhibits a "fast-cycling" phenotype, showing an increased spontaneous nucleotide exchange rate, thereby mimicking a Dbl-induced Rho GTPase [16]. The combined network of DH domain-Rho GTPase interactions encourages release of nucleotide, and thus traps a Rho GTPase in a nucleotide-free state, where the large excess of GTP *in vivo* begets GTP loading and concomitant downstream effector activation. While the Tiam1-Rac1 structure, as well as the other RhoGEF/Rho GTPase complexes, serve as models to interpret the determinants responsible for nucleotide exchange in Rho proteins, a mechanistic study of the effect of these proposed critical residues is sorely lacking.

Molecular Recognition of Rho GTPase Substrates

The combined structural wealth of data has allowed a framework to interpret the determinants within RhoGEFs and Rho GTPases that dictate the proper pairing between

these oncoproteins. To date, the Rho GTPase family possesses 21 members [45], while greater than 70 distinct mammalian Dbl proteins exist. Discrimination by RhoGEFs for the highly similar Rho GTPase substrates remains a paramount issue, given that individual Rho proteins stimulate diverse cellular events. For example, three well-studied Rho GTPases provoke dramatically different cytoskeletal morphologies, where Cdc42 stimulates filopodia production, Rac1 induces lamellipodia formation, and RhoA initiates actin-myosin filament assembly [5].

DH domains selectively recognize a nonconserved patch of positions between the switch regions of Rho GTPases, and utilize these residues for selecting the appropriate target. Structure-based mutagenesis of complementary residues within DH domains and Rho GTPases has demonstrated the basis for this superb specificity. For instance, mutation of Trp 56 in Rac1 to mimic the equivalent Cdc42 side chain (Phe 56) prohibits exchange activity catalyzed by the Rac1-specific GEF Tiam1, while simultaneously removing steric constraint to allow the Cdc42-specific GEF ITSN, to engage and exchange nucleotides within W56F Rac1 [46,47]. In addition, recent work using subtle point mutations within DH domains has remodeled the exchange profiles of ITSN and Dbs to alter their specificity for Rho GTPase substrates [29]. These studies have focused entirely upon Cdc42, Rac1, and RhoA, and must be extended to the entire Rho family to truly appreciate the basis for this selective molecular recognition.

External Regulation of the DH and PH Domains

While the tandem DH and PH domains effectively activate members of the Rho family, outside portions of Dbl proteins surely contribute to the regulation of nucleotide exchange activity. For example, several RhoGEFs (LARG, p115, and PDZ-RhoGEF) possess an N-terminal RGS domain, which binds and hydrolyzes GTP within Gα subunits. Accordingly, emerging studies suggest that modulation of RhoGEF activity is induced by Gα subunit binding to the RGS domain of a Dbl protein [48–50], however, further investigation must decipher this complex mode of regulation. In addition, the upregulation of ITSN-catalyzed exchange activity within Cdc42 by N-WASP binding to an SH3 domain of ITSN has been reported [51].

An exceptional example of external regulation of RhoGEF exchange activity was presented by Rosen and colleagues [25]. Using NMR spectroscopy, the authors determined the structure of the DH domain of Vav along with an N-terminal helical extension that folds back and occludes the conserved DH domain residue (equivalent to the previously mentioned Glu 1047 of Tiam1) at the GTPase binding surface. Interestingly, this amino terminal sequence adjacent to the DH domain contains an Src phosphorylation site (Tyr 174), and accordingly, phosphorylation of Tyr 174 disrupts the inhibitory contacts made on the catalytic surface of the DH domain. These structural data coincide well with previous findings reporting that phosphorylation stimulates the exchange activity of Vav homologs [52]. Thus, outside of the conserved DH/PH cassette, external autoregulation of exchange activity may prove a common theme in all Dbl proteins. Although, given the diverse protein modules present in these RhoGEFs, numerous means of regulating the capacity of the DH and PH domains are anticipated.

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CHAPTER 247

Structural Considerations of Small GTP-Binding Proteins

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Introduction

The basic switching apparatus of small GTP-binding proteins is the G domain. The G domain itself is a conserved structural unit with a canonical switch mechanism, with minor modifications in different members of the family. Their regulators and effectors show a large diversity in their structures and modes of interaction. Here we will try to define some underlying principles.

The G Domain Functional Unit

The basic structural unit of small GTP-binding proteins consists of about 160–170 residues. It is α,β fold typical for nucleotide binding proteins and is called the G domain (for earlier reviews and references, see references [1–3]). This G domain consists of a mixed six-stranded β-sheet and five helices on both sides. It contains four to five conserved sequence elements, which are lined up along the nucleotide binding site and mediate binding, GTP hydrolysis, and the switching apparatus (Fig. 1). The most important contributions to binding are first due to the interactions of the nucleotide base with the N/TKxD motif and an invariant Ala and second of the phosphates with the conserved P-loop GxxxxGKS/T motif [4]. Specificity is due to an Asp side chain forming a bifurcated H bond with the guanine ring, but also due to the Ala main chain interaction with the guanine oxygen, which for steric reasons does not allow replacement by the adenine amino group [5]. Other conserved sequence elements are a conserved Thr and a DxxG motif, both of which are involved in the conformational change.

Structures of several Ras-related proteins like Ras/Rap/Ral, Rac/Rho/Cdc42, Arf/Arl, Ran, and Rab have been solved. The easiest way to compare these structures is to consider the 166–171 residue long G domain of the Ras protein as the minimal switch unit, and to describe the others as variations of this canonical structure. In addition to the G domain, small GTP-binding proteins contain a C- and N-terminal extension of varying length and various insertions. Rho proteins Rho/Rac/Cdc42 contain a α-helical insertion of approximately 13 amino acids, Ran has an elongated C-terminal element very crucial for its function in nuclear transport, whereas Arf/Sar1 proteins contain a myristoylated N-terminal extension that is important for nucleotide-dependent insertion into the plasma membrane. The G domain with its conserved features is also found in multidomain GTP-binding proteins such as the heterotrimeric G proteins and the protein biosynthesis factors eIF5B, EF-Tu, and EF-G.

Whereas the small G proteins are structurally highly homologous, and the structural features of the switch mechanism are conserved, the regulators actually show a variety of shapes and the effectors have, in addition, a variety of function. Here we will concentrate on the major regulators GEF and GAP and the effectors.

The Conformational Switch

Requirements of the molecular switch can be defined from comparing GDP- and GTP-bound structures. Structural differences are mostly subtle and are primarily confined to two regions, which have first been observed in Ras and are called the switch regions [6]. These regions usually show an increased flexibility in X-ray structures and in magnetic

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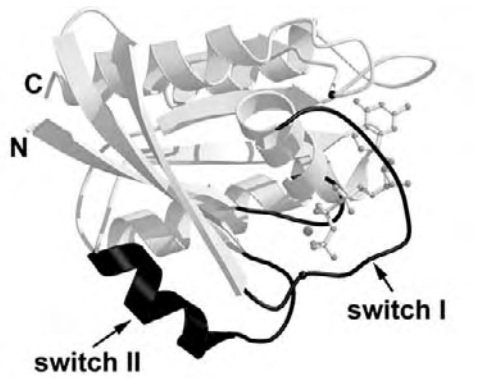


Figure 1 Structure of the G domain. Ribbon plot of the minimal G domain in gray, containing six β-strands and five α-helices, with the conserved sequence elements and the switch regions in black. GTP and Mg^{2+} ion are shown in ball and stick representation.

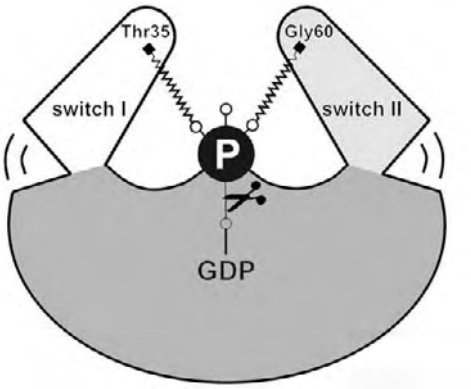


Figure 2 Schematic diagram of the universal switch mechanism where the switch I and II domains are bound to the γ-phosphate via the main chain NH groups of the invariant Thr and Gly residues, in what might be called a loaded spring mechanism. Release of the γ-phosphate after GTP hydrolysis allows the switch regions to relax into a different conformation.

resonance studies using NMR and electron paramagnetic resonance (EPR) [7,8]. Furthermore, whereas the GDP-bound proteins show a large variation of structural details, the GTP-bound forms of the G domain are remarkably similar [3]. Most important, the trigger for the conformational change is rather universal.

In the triphosphate form there are two hydrogen bonds from γ-phosphate oxygens to the main chain NH groups of the invariant Thr and Gly residues (Thr35/Gly60 in Ras) in switch I and II, respectively. The conformational change can best be described as a loaded-spring mechanism where release of the γ-phosphate after GTP hydrolysis allows the two switch regions to relax into the GDP-specific conformation (Fig. 2). The extent of the conformational change is different between the different proteins. In Ras the switch regions involve residues 32–38 for switch I and 59–57 for switch II.

The canonical switch mechanism is modified in many ways. Whereas Ras/Rap/Rho/Rac/Rab show minor changes involving only switch I and II, Ran experiences a large conformational change in switch I with an unfolding of an extra β-strand which in turn induces the long C-terminal extension, the so-called C-terminal switch, to drastically alter its location [9–11]. An even more dramatic change in switch I of Arf and Arl involves the change in register of two β-strands relative to the rest of the sheet and the detachment and subsequent membrane insertion of the N-terminal helix. This has been called the N-terminal switch [12,13].

Guanine Nucleotide Exchange Factors

Guanine nucleotide release from small GTP-binding proteins is slow and is stimulated by several orders of magnitude by guanine nucleotide exchange factors (GEFs). The mechanism of GEF action involves a series of fast kinetic steps, which lead from a binary protein-nucleotide complex via a trimeric complex to a binary nucleotide-free complex, which is stable in the absence of nucleotide. This series of reactions is reversed by rebinding of nucleotide, which is predominantly GTP due to its higher concentration in the cell. In principle, these reactions are fast and fully reversible such that GEF merely acts as a catalyst, which increases the rates at which equilibrium between the GDP- and GTP-bound form of the protein is reached.

Structures of GEFs are conserved within a given subfamily. GEFs for the Ras subfamily have a Cdc25 catalytic domain, whereas the GEFs for Rho-type proteins contain a DH (dibble-homology) domain (see chapter by John Sondek). Structures for the Ran-GEF RCC1, for the Ras-GEF Sos, for the Arf-GEFs Arno/Geα2p, the Rho-GEFs Sos, Trio, Pix, and Tiam and Dbs have been solved. To better understand the mechanism of nucleotide exchange structures of the nucleotide-free binary complexes Ras-Sos [14] Arf-Geα2 [12], of the Ran-RCC1 [15], Rac-Tiam [16], and Dbs-Cdc42 [17] complexes with a polyanion in the phosphate binding (P) loop have been solved. Although the details of the interactions are all different, arguing for a variety of kick-out mechanisms, the complexes have structural features in common suggesting mechanistic similarities. The GEFs do not act allosterically but directly interfere with nucleotide binding by inserting residues close to or into the P loop, the Mg^{2+} and/or guanine base binding area which create structural changes that are inhibitory for tight binding of nucleotide. This agrees with binding studies which show that the β-phosphate-P-loop interaction and the Mg^{2+} phosphate interaction are the most important elements for tight binding [5].

Effector B Via Switches and Others

Effectors for GTP-binding proteins are operationally defined as molecules interacting more tightly with the GTP- than with the GDP-bound form. This implies that effector binding involves the switch regions of G proteins, which is borne out by the structures. The Ras-binding domain (RBD) or Ras association domain (RA) has been identified in many

different proteins [18], which may or may not be true effectors [19]. In any case many of the bona fide Ras effectors also bind to proteins of the Ras subfamily such as Rap, R-Ras, and TC21. The structures of several Ras effectors such as Raf, RalGDS, PI3kinase, and recently byr2 [20] either alone or in complex with Ras, Rap, and their mutants have shown that the RBD is a small, well-defined domain with a ubiquitin fold, which binds to Rap/Ras by forming a GTP-binding dependent interprotein β-sheet between both molecules [21]. In the case of the complex with the phosphatidylinositol-specific lipid kinase PI(3)Kγ, the contacts produce structural changes in PI3Kγ which are believed to allosterically influence the catalytic activity [22–24].

Binding of Rac/Cdc42 to its effectors, some of which contain a so-called CRIB (Cdc42/Rac interactive binding) region [25,26] induces major structural changes and has been shown to directly activate the protein kinases PAK (p21-activated kinase), activated Cdc42-associated kinase (ACK), and the scaffold protein WASP (Wiskott-Aldrich syndrome protein). Fragments containing the CRIB domain show no apparent three-dimensional structure, but are structured in complex with C-terminal fragments from the same protein in an autoinhibitory conformation [27,28] or with Cdc42 (see chapter by Mike Rosen) [29,30].

Other structures of effector complexes show a variety of interaction patterns (Fig. 3) the only common feature of which is to include the switch region(s) for the interaction, the exception being the complex between RhoA and an antiparallel coiled-coil fragment from the regulatory region of the protein kinase PKN, where it is not clear why the interaction should be GTP-specific [31]. The complex between Rab3A and its effector Rabphilin-3A involves two modes of binding [32], where one end of Rabphilin-3A contacts the switch region of Rab3A and thus senses its nucleotide status. A second site interacts with the complementarity-determining region (CDR), a site on the surface whose sequence varies among members of the Rab subfamily, and is thus responsible for specificity.

Small GTP-binding proteins such as Arf, Rab, or Ran are involved in a variety of transport processes. Ran·GTP binding to nuclear import factors-cargo complexes is necessary to release cargo on the nuclear side of the nuclear pore. Structures of the Ran complexes with importin-β and transportin show the switch I- and II-mediated interaction with the helical repeat motif of the factors [9,11]. Additional structural studies of a complex of importin with cargo [33] or with components from the nuclear pore [34] have suggested that the cargo-loaded transport factors can bind simultaneously to the nuclear pore but that interaction with Ran·GTP in the nucleus sterically interferes with binding of both, suggesting how Ran terminates the import reaction. The structure of the Arf-related protein Arl2 in complex with its effector PDEδ showed the latter to have a RhoGDI fold with an empty hydrophobic pocket [13], which together with biochemical studies suggested it to be involved in the transport of farnesylated proteins.

The only unifying principle that we find in the structures of effector complexes is that they involve the switch regions.

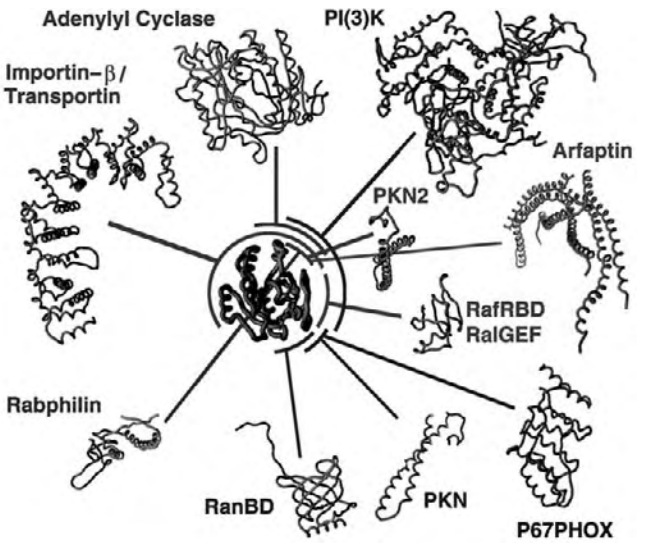


Figure 3 Interaction with effectors is mediated via different surface regions of GTP-binding proteins (see reference [3]). The structures were aligned on the G domain in the middle, and the effectors are removed from the interface in the direction indicated by the line. Involvement of the switch I and/or switch II region in the interface is the only common feature of these complexes. For PKN, the two variations found in the crystal structure are shown.

Other than that they show that the effectors have a variety of shapes of folds and interact in many different ways such that the different effectors cover almost the complete surface of the G domain (Fig. 3). We can distinguish between effectors, which contain a preformed binding domain and show no major structural change on binding, and those involving a large conformational change on binding to the GTP-binding protein. In the former case experimental evidence points toward (only) a recruiting function as the major signal transduction mechanism [24], whereas the other clearly involves (additional) allosteric regulation of the effector.

GAP Proteins and the GTPase Reaction

The GTPase reaction for most Ras-like proteins is slow and would not be suitable for most biological signal transduction processes where inactivation is complete within minutes after GTP loading. It is not surprising then that GTPase activating proteins (GAPs) have been discovered for all major forms of these proteins [35,36]. They increase GTP hydrolysis by several orders of magnitude. As with GEFs, the structures of GAPs for various (sub)family Ras proteins are different, although an evolutionary relationship between Ras- and RhoGAP has been suggested [37,38].

Biochemical experiments showed that the active site arginine of heterotrimeric Gα is supplied in "trans" by RasGAP [39]. Structures of Gα·GDP in the presence of aluminum fluoride had shown that the latter is in a planar conformation and thus seems to mimic the transferred phosphate of the reaction [40,41]. In those structures both the arginine and a conserved glutamine stabilize the conformation of the transition state mimic. The structures of RasGAP and RhoGAP in complex with their respective G proteins in the presence

of AlF_x showed an intrinsic Gln and a so-called arginine finger supplied by GAP into the active site [42–44]. The structures also give an explanation for the inability of oncogenic mutants of Ras to hydrolyze GTP. The mechanism of inserting an arginine finger into the active site of Rho proteins is also used by some bacteria that insert these toxins into eukaryotic cells [45,46].

It has been discussed whether the mechanism of GAP-assisted GTP hydrolysis applies to all G-domain proteins [35]. This is clearly not the case. Rap proteins have a Thr, and protein synthesis factors have a histidine in place of the catalytic glutamine. For Arf, the presence or absence of an arginine of ArfGAP participating in catalysis is disputed [47,48]. Furthermore, in a recent structure of the trimeric complex between Ran, RanGAP, and a Ran-binding protein, no arginine was found to be participating in catalysis [49].

Conclusions

A large number of structural studies on small G proteins showed that there is a conserved module with a canonical structure and switch mechanism that can be considered as a tema con variazoni. The variations are derived from insertions into and additions to the canonical G domain and from a variety of regulators and effectors that are different for different types of G-binding proteins. The mechanisms by which GEFs and GAPs stimulate the otherwise slow intrinsic nucleotide dissociation and GTP hydrolysis have been worked out in some cases and suggest some underlying common principles in spite of the multitude of differences in detail. Interactions with effectors show a similar multitude of interactions. How these interactions generate the biological response of the particular system remains to be established in most cases with more structural studies on complexes of small GTP-binding proteins.

Acknowledgment

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CHAPTER 248

Conventional and Unconventional Aspects of Dynamin GTPases

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Introduction

Dynamin is unique among GTPases for its large size (~96 kDa), its multidomain structure, its low affinity for GTP, its relatively high intrinsic rate of GTP hydrolysis and most strikingly, its ability to self-assemble into rings and helical stacks of rings and its very robust (≥100 min^{-1}) assembly-stimulated rates of GTP hydrolysis. These unique properties have led to the suggestion that dynamin functions, unlike any other GTPase superfamily member, as a mechanochemical enzyme that encircles the necks of deeply invaginated coated pits and generates the force necessary to drive scission. Recent structural data show that the GTPase domain of dynamin is, however, quite conventional. Moreover, dynamin overexpression affects many cellular processes, in addition to its effects on endocytosis, including actin dynamics, MAP kinase activation, transcriptional activation, and changes in cell morphology. Re-evaluation of the current data, from a new perspective, may suggest a broader role for the dynamin subfamily more akin to other regulatory GTPases.

Dynamin is one of the youngest members of the GTPase superfamily, having been discovered in the late 1980s. As a young upstart, it has proven to be unconventional and textbooks have rushed to include models for dynamin function as a fission GTPase—the first example of a GTPase functioning as a mechanochemical enzyme. However, more recent evidence suggests that, like signaling or regulatory GTPases, dynamin overexpression can effect many aspects of cell physiology, including actin dynamics, cell morphology, transcription, and even cell death. Dynamin's function in endocytosis has been extensively reviewed in references [1–3]. Here, with special emphasis on often overlooked aspects, I will review some of the features that set dynamin apart from its GTPase cousins, place it in the context of its siblings in the dynamin subfamily of GTPases, and describe dynamin effects that are not easily reconciled with its "textbook" function as a fission GTPase.

Common and Unique Features of Dynamin as a GTPase

Dynamin is a Multidomain Protein with a Conventional GTPase Domain

Atypically for GTPase family members, dynamin is large (~96 kDa) and has a modular domain structure (Fig. 1) consisting of an N-terminal GTPase domain, a middle domain

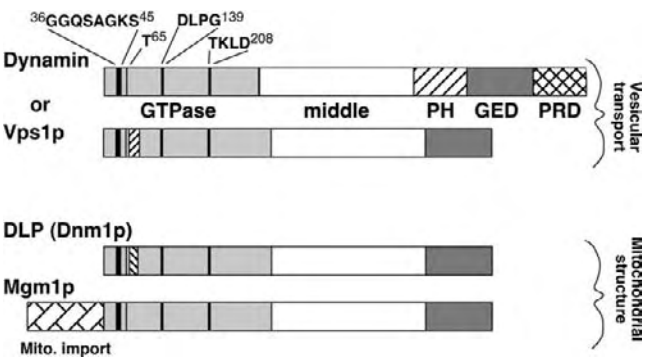


Figure 1 Domain structure of the only three evolutionarily conserved dynamin family members. The conserved elements of the GTPase binding site are indicated for dynamin-1.

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of unknown function, a PH domain that binds $PI4,5P_2$, a GTPase effector domain (GED) that functions in dynamin self-assembly and assembly-stimulated GTPase activity, and a C-terminal proline and arginine-rich domain (PRD) that interacts with numerous SH3 domain-containing partners [4]. The recently solved three-dimensional structure of the 316 amino acid GTPase domain of *Dictyostelium* dynamin A [5], which is 60% identical to the GTPase domain of human dynamin-1, reveals it to have a typical, ras-like core GTPase domain fold (Fig. 2) with several distinguishing features. The first is an insertion, which is quite divergent among dynamin family members (see Fig. 1), that adds two additional strands to the core 6-stranded β-sheet and a variable length extended loop (Fig. 2). It is positioned immediately distal to T65 (in dynamin-1) which coordinates with γ-phosphate on bound GTP, at a region equivalent to switch I of other GTPases. Its position, its variability, and the high content of charged residues suggest that this loop could be involved in interactions with downstream partners, like other GTPases. The loop between β3 and α2 that constitutes the switch II region of the GTPase (element 3 of the GTP binding site), also undergoes GTP-dependent conformational changes and is considerably longer in dynamin than in ras. A third insert is located near the C terminus of the GTPase domain and constitutes two α-helices which extend out from and run perpendicular to the core β-sheet. The fourth distinguishing feature is that the N and C terminus of the GTPase domain come in close contact with each other through an additional N-terminal α-helix and an extended C-terminal α-helix. Interestingly, together these two terminal helices form a hydrophobic groove, which was occupied in the solved structure by hydrophobic residues from the N-terminal fusion partner used in the dynamin construct, the catalytic domain of *Dictyostelium* myosin II [5]. The author's speculation, that this groove might normally be a docking site for the GED domain of dynamin is consistent with results from the dynamin-related mx proteins which identified a hydrophobic residue within GED that is essential for self-assembly and assembly-stimulated GTPase activity [6], and results from two-hybrid analysis mapping the GED-GTPase interaction site in dynamin to the C-terminal regions of the GTPase domain [7]. Importantly, this model for GED-GTPase interaction is inconsistent with that based on the structure of GBP-1, another interferon-inducible GTPase [8]. However, comparison of the GTPase domain structures of GBP1 and dynamin A, together with their lack of sequence similarity would strongly

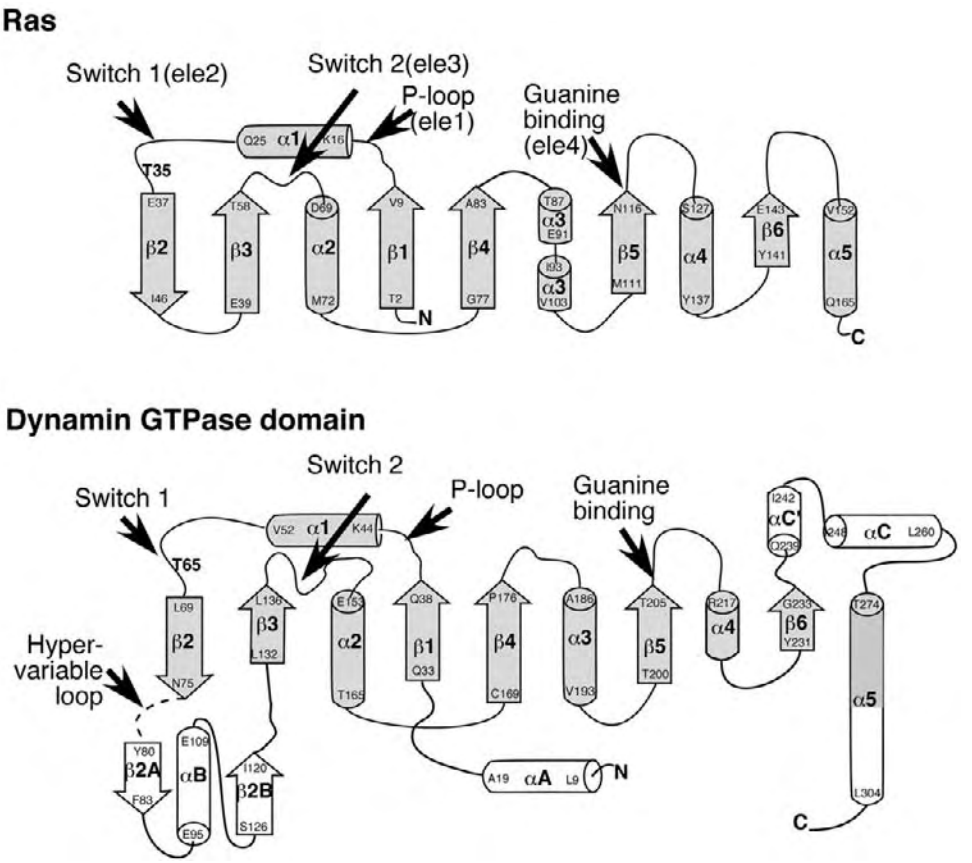


Figure 2 Topology of dynamin GTPase domain compared to Ras. The models for ras and dynamin are redrawn and modified from Wittinghofer, A., *GTPases*, Vol. 24, 244–310, Oxford University Press, New York, with permission and Niemann, H.H. *et al*., *EMBO J*., 20, 5813–5821, 2001, with permission, respectively. The location of the four GTPase binding site elements are indicated.

argue that they represent distinct GTPase subfamilies and, therefore, that the structure of distal domains of GBP-1 may not reflect those of dynamin-family members.

Dynamin's Unconventional GTPase Activity

Although dynamin's GTPase domain is structurally quite conventional, there are several important distinguishing features of its GTPase activity that set it apart from most GTPase family members. The first is dynamin's relatively low affinity for guanine nucleotides (2.5 μM for GTP and ~40-fold weaker for GDP) and the very rapid dissociation rates for both GTP (2.1 s^{-1}) and for GDP (93 s^{-1}) [9]. Thus, unlike most other GTPases, nucleotide exchange is not rate-limiting for dynamin's GTPase activity. The second is dynamin's relatively fast intrinsic rate of GTP hydrolysis. Reported measurements of this rate have varied considerably probably due to variable amounts of dynamin aggregation or self-assembly under different assay conditions. However, when assayed in high salt to inhibit self-assembly, more consistent rates (0.01–0.03 s^{-1}) are reported [10,11]. This rapid intrinsic rate is comparable to that measured for trimeric G proteins; however, the steady-state GTPase activity of trimeric proteins is greatly limited by the very slow dissociation rates of GDP. Together, these unusual properties of dynamin's GTPase activity would suggest that *in vivo*, in the absence of as yet unidentified regulatory factors, dynamin would rapidly cycle between the GTP-bound and unoccupied state, occasionally hydrolyzing bound GTP.

Dynamin has an Intrinsic GAP Domain

As alluded to above, perhaps the most unusual property of dynamin is its ability to self-assemble into rings and helical stacks of rings. Depending on the template provided, self-assembly stimulates dynamin's GTPase activity >100-fold [9,12–14]. This assembly-stimulated GTPase activity is mediated by GED, which functions as an assembly-dependent GAP domain [15]. Thus, dynamin, unlike other GTPase family members, encodes its own GAP; however, like other GTPases, GED appears not to function intramolecularly, but is only activated by intermolecular interactions that occur between dynamin molecules upon self-assembly.

The mechanism of GED-stimulated GTPase activity is unknown. Mutagenesis studies had identified an arginine residue in GED (R725), which when mutated to alanine, appeared to specifically inhibit assembly-stimulated GTPase activity while not affecting dynamin's ability to self-assemble upon dilution into low-salt buffers or on a microtubule template [15]. These findings led to the suggestion that R725 might function as a catalytic residue akin to the Arg-finger in rasGAP. However, a more recent study has shown that dyn(R725A) is capable of near wild-type stimulated GTPase activity when assayed on lipid tubule assembly templates, and instead revealed an assembly defect in the R725 mutant [16]. Consistent with this result, we have found that dyn(R725K) exhibits wild-type self-assembly and GTPase activity (S. Sholly and S.L. Schmid., unpublished results), a finding inconsistent with a role for R725 in catalysis. Given that dynamin's intrinsic rate of GTP hydrolysis parallels that of trimeric G protein α subunits, it is possible that GED functions more in analogy to RGS-type GTP-activating proteins (GAPs) that stabilize and position catalytic residues already in place in the GTPase domain (reviewed in reference [17]).

Dynamin's Function in Endocytic Vesicle Formation

The rapid temperature-sensitive inhibition of endocytosis seen in *Drosophila* bearing mutations in *shibire*, the ortholog of dynamin, remains the strongest and most compelling evidence for dynamin's essential role in endocytosis [18]. In mammalian cells, endocytic clathrin-coated vesicle formation is inhibited by overexpression of dominant-negative mutants of dynamin. One striking example is the finding that HeLa cells overexpressing human dyn(G273D), corresponding to the shibire-ts1 allele, exhibit rapid and reversible temperature-sensitive inhibition of receptor-mediated endocytosis [19]. Several mutations have been made at conserved residues in the nucleotide-binding site within dynamin's GTPase domain (see Fig. 1), which by analogy to other GTPases are predicted to disrupt dynamin's ability to bind GTP. Overexpression of these "dominant-negative" mutants [e.g., dyn(K44A) or dyn(S45N)] potently inhibits receptor-mediated endocytosis and leads to the accumulation of invaginated clathrin-coated pits on the cell surface [16,20–22]. Other endocytic events, such as internalization of caveolae [23,24] and phagocytosis [25] are also inhibited, but the internalization of bulk fluid or membrane markers mediated by a constitutive, but poorly understood, clathrin-independent endocytic process is unaffected [20,26]. In stable HeLa cells induced to express dyn(K44A), the mutant also blocks the retrograde transport of the plant toxin ricin from late endosomes to the trans-Golgi network (TGN) [26]. Other membrane transport events, such as trafficking from the TGN to the cell surface, transport from the TGN to late endosomes or delivery of endocytic tracers to lysosomes are unaffected in these cells [20,27].

Two Models for Dynamin Function in Endocytic Vesicle Formation

While there exists considerable evidence establishing a critical role for dynamin in endocytic clathrin-coated vesicle formation, its exact function remains a matter of dispute [3]. One prevailing model (Fig. 3, model 1) suggests that after self-assembly around the necks of invaginating coated pits, dynamin functions as a mechanochemical enzyme to generate the force—either by constriction (model 1A [28]) or expansion (model 1B [14]) of the assembled ring driven by rapid GTP hydrolysis—that mediates membrane fission and vesicle detachment. This model, which is consistent with its unique enzymatic properties, places dynamin in a class of its

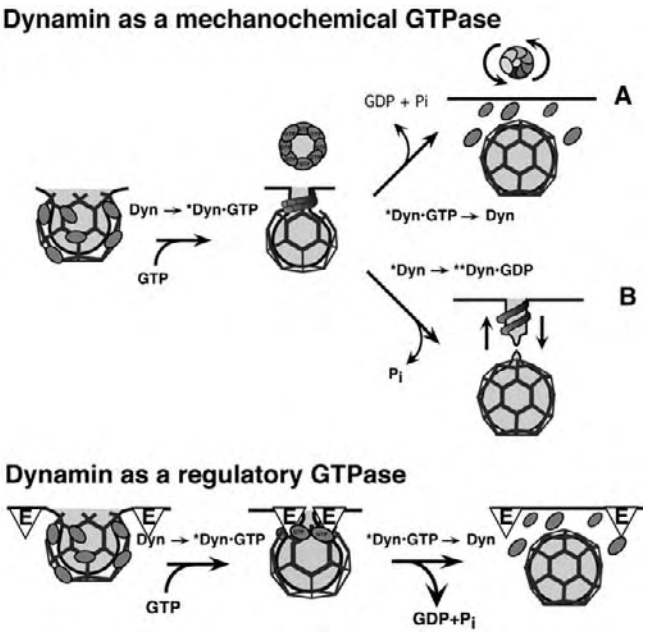


Figure 3 Two prevailing models for dynamin's function in endocytosis. Model 1 asserts that dynamin functions in a mechanochemical manner as either a "pinchase" or a "spring" to sever the necks of invaginated coated pits. Model 2 asserts that in its GTP-bound form dynamin recruits downstream effector molecules that mediated vesicle formation. In both models dynamin must bind and hydrolyze GTP (at least at its basal rate) and undergo GTP-dependent conformational changes (indicated by asterisks).

own as the first example of a mechanochemical GTPase. The second, more recently espoused model (Fig. 3, model 2), is more in keeping with dynamin's membership in the GTPase superfamily, and suggests that dynamin functions, in its GTP-bound form, to recruit downstream effectors, which themselves constitute the vesicle fission/detachment machinery [15]. In this model the collar functions as a geometric sensor and dynamin's GAP, when activated by self-assembly, functions by analogy with all other GTPase superfamily members, as a timer to turn off and recycle the activated GTPase. Naturally, these two models are not mutually exclusive and other models are possible that incorporate features of both extremes.

Testing Models for Dynamin Function *in vivo*: GTPase Domain Mutants

Good faith efforts have been made to test models for dynamin function *in vivo*, but as yet none of the experiments have unambiguously supported one model over the other. One approach is to ask whether GTP hydrolysis by dynamin is required for endocytosis. If dynamin functions like a regulatory GTPase one would predict that an "activating mutant" of dynamin locked in its GTP-bound form would support and perhaps stimulate endocytosis. In contrast, if dynamin functions as a mechanochemical enzyme, its high rates of GTP hydrolysis would be required for endocytosis. Based on analogy to other GTPases, Thr65 of dynamin, which is located in the switch I region of GTPases and is required to coordinate the Mg^{2+} ion essential for GTP hydrolysis, was mutated in an effort to generate such an activating mutant. Overexpression of dyn(T65A) inhibits receptor-mediated endocytosis, suggesting that GTP hydrolysis by dynamin is essential [16].

However, the results are not conclusive, as mutation of this conserved Thr in ras or trimeric G proteins reduces their affinity for GTP by >100-fold [17]. However, ras and Gα GTPases are still able to bind GTP with relatively high affinity and binding is not significantly impaired under *in vivo* conditions. By contrast, given dynamin's already low affinity for GTP, this mutation is more likely to perturb its ability to bind GTP *in vivo*. Moreover, the coordination of this conserved Thr to Mg^{2+} and the γ-phosphate in other GTPases is essential for the GTP-dependent conformational change in the effector loop necessary for interaction with downstream partners. Thus, it is unclear whether GTPases with this mutation are able to efficiently adopt the "active" conformation. Consistent with this interpretation, dyn(T65A) is unable to adopt the GTP-dependent conformation on lipid tubules [16].

Thus the issue of whether GTP hydrolysis by dynamin is essential for endocytosis remains unresolved. Ideally, one needs to mutate an essential catalytic residue, equivalent to Q61 in ras, that does not affect GTP binding or the GTP-dependent conformational change in order to the test whether GTP hydrolysis was required for dynamin function. Unfortunately, candidate residues based on other GTPases are not conserved in dynamin. However, the recently solved structures of the GTPase domain of dynamin A in the unoccupied and GDP-bound state [5] may suggest candidate residues for further mutagenesis.

Testing Models for Dynamin Function *in vivo*: GED Mutants

Given that dynamin encodes its own GAP, another approach to making activating mutants is to identify residues in GED that affect the assembly-dependent GAP activity. Two such mutations, dyn(K694A) and dyn(R725A), were shown to specifically perturb the assembly-stimulated GTPase activity of dynamin *in vitro* [15]. Importantly, these GED mutations do not alter the basal rate of GTP hydrolysis, nor do they effect GTP binding affinities. In contrast to the GTPase mutants of dynamin, overexpression of these GED mutants accelerated the rate-limiting step in clathrin-mediated endocytosis [15]. Dyn(K694A) and dyn(R725A) were overexpressed under the control of a tetracycline-regulatable promoter in stably-transfected HeLa cells and studied using stage-specific endocytosis assays that distinguish the formation of constricted coated pits, an intermediate in endocytosis, from subsequent membrane fission and coated vesicle release [29]. Dyn(K694A) overexpression increased the rate of formation of both constricted coated pits and coated vesicles. Consistent with these findings, expression in yeast of an analogous mutation (K705A) in Dnm1p, a dynamin family member, results in increased mitochondrial fission [30].

Given that the dyn(K694A) mutant was specifically defective in self-assembly, this result is difficult to reconcile with models for dynamin function as strictly a mechanochemical enzyme. Instead, these findings are most consistent with the interpretation that by impairing GAP activity and prolonging dynamin in its GTP-bound form, as yet unidentified, downstream effectors(s) are activated and in turn accelerate the rate of endocytosis. Importantly, dyn(K694A) was impaired but not incapable of self-assembly, and so these results do not rule out a requirement for dynamin self-assembly in vesicle formation. Moreover, results with dyn(R725A) were more difficult to interpret, because, unexpectedly, although overexpression of dyn(R72A) enhanced the rate of formation of constricted coated pits, it had no effect on or was slightly inhibitory for vesicle formation [29]. These results suggested that the dyn(R725A) mutant reduces the rate of membrane fission, while increasing the rate of constricted coated pit formation. Thus, dynamin assembly and assembly-stimulated GTPase activity may be required for the final stages in vesicle detachment. New mutants with more pronounced defects in assembly and assembly-stimulated GTPase activity will be needed to rigorously test models for dynamin function *in vivo.* Interestingly, mutations in mx protein that have abolished self-assembly and assembly-stimulated GTPase activity do not alter the protein's ability to confer viral resistance [6], suggesting that for this dynamin family member, self-assembly is not essential for function.

Dynamin's Siblings: The Dynamin Subfamily of GTPases

Although not the founding member, dynamin was born into a subfamily of GTPases that now bears its name. Members of the dynamin family of GTPases are functionally diverse, and not numerous. That is, unlike other components of the vesicle transport machinery (i.e., arfs, coats, SNAREs, rabs, etc.), there does not appear to be a dynamin analog functioning in every vesicle formation event within the cells. In the yeast, *Saccharomyces cerevisiae* there are only three dynamin family members. These include Vps1p, which is the only dynamin family member involved in vesicular transport in yeast and plays an as yet undefined role in protein trafficking between the *trans*-Golgi network (TGN) and the vacuole; Dnm1p, a cytosolic protein that binds to the outer mitochondrial membrane and plays a role in regulating the size and number of mitochondria; and, Mgm1p, an inner mitochondrial protein also involved in controlling mitochondrial division. These dynamin-related GTPases share high degrees of homology with dynamin's GTPase, middle and GED domains, but they lack the pleckstrin homology (PH) domain and the PRD of true dynamins (Fig. 1).

The *Drosophila* and *Caenorhabditis elegans* genomes also encode only three dynamin family members. These include Dnm1p and Mgm1p homologs, which appear to function like their *S. cerevisiae* counterparts in regulating mitochondrial membrane dynamics [31]. Surprisingly, there are no Vps1p homologs in either organism, instead they both encode a single gene for dynamin. Thus, Vps1p may be the yeast homolog of dynamin, given that both proteins function in vesicular trafficking, albeit at distinct sites. Vps1 mutations have no effect on endocytosis in yeast, whereas dynamin mutations in *C. elegans* and *Drosophila* specifically disrupt endocytosis, without affecting other trafficking pathways.

The issue is more complicated, however, in mammals, which in addition to a Dnm1p homolog (called DLP1 and also known as dymple, DRP1 or DVLP) and a Mgm1p homolog, both of which are associated with mitochondrial membrane dynamics, express three dynamin genes in a tissue-specific manner [31]. Dynamin-1 is exclusively expressed in neurons, dynamin-2 is ubiquitously expressed, and dynamin-3 is expressed primarily in testes, brain, and lung. There are also numerous splice variants of each gene product [2]. Gene duplication in mammals is not uncommon and the simplest interpretation is that the newly evolved isoforms perform specialized functions in specialized cells (e.g., rapid and regulated synaptic vesicle recycling at the synapse). In HeLa cells and MDCK cells, overexpression of dyn2(K44A) functions indistinguishably from dyn1(K44A) in membrane trafficking to specifically and potently inhibit receptor-mediated endocytosis without affecting other vesicular trafficking events [27]. There are conflicting data as to whether specific splice variants of dynamin-2 do [32] or do not [33] localize to the TGN and effect vesicle formation from this compartment. These discrepancies may reflect cell-type differences and/or they may reflect nonspecific effects or incomplete inhibition due to too much or too little overexpression. Knock-out experiments will be needed to unambiguously resolve this issue; however, given that a function for dynamin in the Golgi does not appear to be conserved in *C. elegans* or *Drosophila*, it seems unlikely that it is essential for vesicle formation at these sites.

Mammals also express a fourth, and original member of the dynamin subfamily, the α-interferon inducible mx proteins [34]. The mx proteins are localized in either the cytoplasm or the nucleus and confer cellular resistance to RNA viruses. Recent evidence suggests that one mx family member (mxA) does so by binding to and sequestering the viral nucleocapsid in large aggregates in the cytosol [35]. Thus, of the four dynamin family members in mammals, all of which self-assemble into rings and helical stacks of rings and all of which exhibit assembly-stimulated GTPase activity, only one is involved in vesicular trafficking. This latter point is important when considering dynamin's proposed function as a fission GTPase or vesicle "pinchase" that is integral to the fundamental mechanism of vesicle formation. One might expect such an elegant fission apparatus to be a ubiquitous component of the vesicle formation machinery

Dynamin as a Signaling Molecule

While the mechanisms remain to be established, a growing body of evidence suggests that dynamin (and dynamin

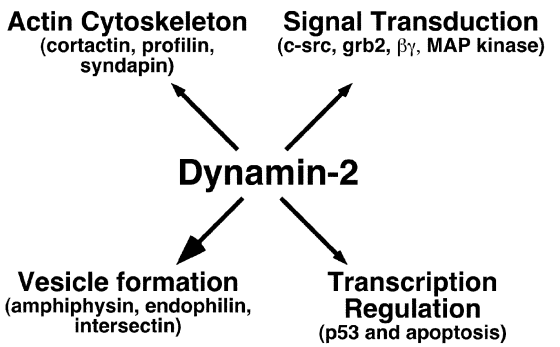


Figure 4 Dynamin as a signaling GTPase. There is now considerable evidence that dynamin can signal to multiple, functionally diverse downstream cellular processes. Some of these signaling pathways are specific for dynamin-2 versus dynamin-1.

family members) may instead, or in addition, function as signaling molecules to control multiple aspects of cell physiology (Fig. 4). For example, dynamin is known to interact with several actin regulatory proteins, including profilin, cortactin, syndapins, and Abp1 [36] and has recently been shown to be associated with sites of active actin assembly/disassembly, including membrane ruffles [37], podosomes [38], and actin comet tails [39,40]. Actin dynamics at these sites are disrupted or altered by overexpression of dyn2 (K44A) mutants suggesting a role for GTP binding and/or hydrolysis in regulating these events. Overexpression of dyn2(ΔPRD) in clone 9 cells causes dramatic changes in overall cell shape [37], again suggesting a more pleiotropic effect than one would expect from a defect in vesicle formation alone.

Dynamin can also effect other signaling pathways, directly or indirectly. Three studies have shown that dyn(K44A) overexpression perturbs G-protein-coupled receptor (GPCR) signaling to MAP kinase family members, independent of its effects on endocytosis [41–43]. Dyn(K44A) overexpression also inhibits high-affinity binding to epidermal growth factor (EGF) receptors [44] and causes reduced acidification of endosomes [45]. While the mechanisms underlying these effects are unknown, these observations are not easily reconciled with a model for dynamin as simply a vesicle pinchase.

Finally, low levels of overexpression of dynamin-2 induces p53-dependent apoptosis in several cell types including HeLa cells, rat-1 fibroblasts, and mouse embryo fibroblasts [46]. Several lines of evidence support the specificity of this effect: (1) <5-fold overexpression of dyn2 induces apoptosis, whereas >250-fold overexpression of the 80% identical dynamin-1 isoform has no cytotoxic effect; (2) dyn2-induced apoptosis requires GTP binding because dyn2(K44A), which inhibits endocytosis, does not induce apoptosis; (3) dyn2 at high levels of overexpression does not induce apoptosis in nondividing cells (e.g., contact inhibited MDCK cells or primary macrophages) or in p53-deficient mouse embryo fibroblasts. Interestingly, a recent report established that dominant-negative DRP1, a mitochondria-associated dynamin family member, can protect cells from staurosporine-induced apoptosis [47]. Together these data suggest that dynamin family members might play a more general role in monitoring membrane dynamics at critical cellular locations—the plasma membrane and the mitochondria—to control cellular homeostasis.

Conclusion and Perspectives

Consider the following re-ordering of the discoveries surrounding dynamin function: (1) dynamin-2 is cloned and found to be a GTPase superfamily member; (2) analysis of the complete genomes of *Drosophila* and *C. elegans* reveal that the only two other dynamin-related GTPases are mitochondrial—there is no "true" dynamin in yeast; (3) in mammals, a fourth dynamin related GTPase is identified as the α-interferon-inducible mx protein, which confers viral resistance; (4) overexpression of dyn2(wt) activates p53 and induces apoptosis, mutants unable to bind GTP do not; (5) overexpression of dyn2(K44A) inhibits MAP kinase activation and alters actin cytoskeleton dynamics; (6) all dynamin-related proteins, even those not involved in vesicular transport, are shown to self-assemble and to have assembly-stimulated GTPase activity; (7) mutants of mx incapable of either self-assembly or assembly-stimulated GTPase activity are fully functional in conferring viral resistance, although GTPase domain mutants are not; (8) dyn(K44A) mutants inhibit endocytosis and assembled dynamin accumulates in GTPγS-treated membranes. Given this set of facts, presented in this order, one might have reached a different conclusion regarding dynamin's cellular function, perhaps one in which dynamin functions more broadly and more in line with rho family regulatory GTPases. While images of "collared" pits are compelling, the function of dynamin GTPase's may turn out to be more than meets the eye.

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CHAPTER 249

Mx Proteins: High Molecular Weight GTPases with Antiviral Activity

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Mx proteins belong to a family of interferon-induced GTPases within the superfamily of high molecular weight GTPases. A unique property of some Mx GTPases is their antiviral activity against RNA viruses. Much progress has been made in better understanding the molecular mechanisms of Mx action over the years. Here we review recent insights into the workings of this fascinating class of large GTPases.

Antiviral Activity of Mx GTPases

Mx, for Myxovirus resistance, has first been described in mice, as an interferon-induced resistance phenomenon against influenza A virus (FLUAV) infection [1]. Subsequently, a variety of Mx proteins from different species were found to have antiviral activity, as shown by transfection experiments with Mx cDNA expression constructs [1,2]. Rodents express nuclear as well as cytoplasmic Mx proteins, whereas most other vertebrates express Mx proteins that accumulate exclusively in the cytoplasm. Interestingly, the antiviral specificity of rodent Mx proteins correlates with their subcellular localization. The nuclear forms (Mx1 in mouse and rat) confer resistance to orthomyxoviruses, FLUAV, and Thogoto virus (THOV), known to replicate in the cell nucleus. In contrast, cytoplasmic proteins (Mx2 in mouse and rat) inhibit multiplication of viruses that replicate in the cytoplasm, such as rhabdoviruses (e.g., vesicular stomatitis virus; VSV) and bunyaviruses (e.g., LaCrosse virus ;LACV) [1]. This suggests that rodents have evolved two distinct types of Mx GTPases to allow control of viruses with different intracellular replication sites.

Humans express a single antiviral Mx GTPase, called MxA. The cytoplasmic MxA has a rather wide antiviral spectrum against different classes of viruses, irrespective of their intracellular site of replication. MxA interferes with the multiplication of members of the bunyaviruses, orthomyxoviruses, paramyxoviruses, and rhabdoviruses [1]. Initially, Mx proteins were assumed to be active against negative-strand RNA viruses only, but recent studies showed inhibitory activity of MxA also against Semliki Forest virus and coxsackievirus B4, two plus-strand RNA viruses, as well as hepatitis B virus, a DNA virus with a genomic RNA intermediate [3–5]. The power of MxA as an intrinsic host defense mechanism is best illustrated in experiments with *MxA*-transgenic mice lacking interferon-α/β receptors. These animals survived an otherwise lethal infection with MxA-sensitive viruses, despite their interferon-nonresponsiveness [6]. Interestingly, Mx proteins without antiviral function also exist, such as human MxB or rat Mx3. It is presently not known whether these Mx GTPases are directed against a different set of microbial pathogens or serve other functions.

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Mouse Mx1 Protein Blocks Primary Transcription of Orthomyxoviruses by Interfering with the Viral Polymerase Activity

The nuclear mouse Mx1 protein inhibits FLUAV and THOV multiplication at the level of primary transcription of the viral genome. It is noteworthy that primary transcription of these viruses occurs in the nucleus of infected cells and is catalyzed by the viral polymerase. The FLUAV polymerase consists of three subunits, PB1, PB2, and PA. Overexpression of the PB2 subunit abolished the antiviral effect of Mx1, suggesting that the PB2 subunit of FLUAV is an Mx1 target [7]. The subcellular localization of Mx GTPases is important for their antiviral activity, as demonstrated by artificial translocation of cytoplasmic Mx proteins into the nuclear compartment. When MxA or rat Mx2 was translocated into the nucleus with the help of a foreign nuclear translocation signal, both proteins gained an Mx1-like phenotype and blocked primary transcription of FLUAV and THOV [8,9].

Human MxA Interferes with Virus Multiplication by Missorting Viral Components

Studies in cell culture showed that cytoplasmic MxA inhibits primary transcription of VSV and measles virus, all of which are known to transcribe their genome in the cytoplasm [1]. Surprisingly, MxA also inhibited primary transcription of THOV which takes place in the nucleus [10]. In addition, MxA inhibits the multiplication of FLUAV, human parainfluenza virus type 3, and bunyaviruses, at later steps in the viral replication cycle [1]. In the case of THOV, we could demonstrate that MxA binds to the nucleocapsids and prevents their transport into the nucleus (Fig. 1A). As a consequence, THOV primary transcription is inhibited [10]. In the case of LACV, we could show that MxA binds to the viral nucleocapsid protein [11]. This interaction leads to the formation of MxA/nucleocapsid protein copolymers. A redistribution of the viral nucleocapsid protein takes place in MxA-expressing cells leading to the disappearance of nucleocapsid proteins from the putative viral replication sites near the Golgi apparatus and to accumulation in the perinuclear area (Fig. 1B). This indicates that the antiviral action of MxA against bunyaviruses is based on missorting of an essential virus component which thus becomes unavailable for the generation of new virus particles. In summary, our analysis of THOV and LACV, representing two viruses with different replication strategies, suggests that MxA acts by interfering with proper transport of critical viral components to their ultimate target compartments within the infected cell.

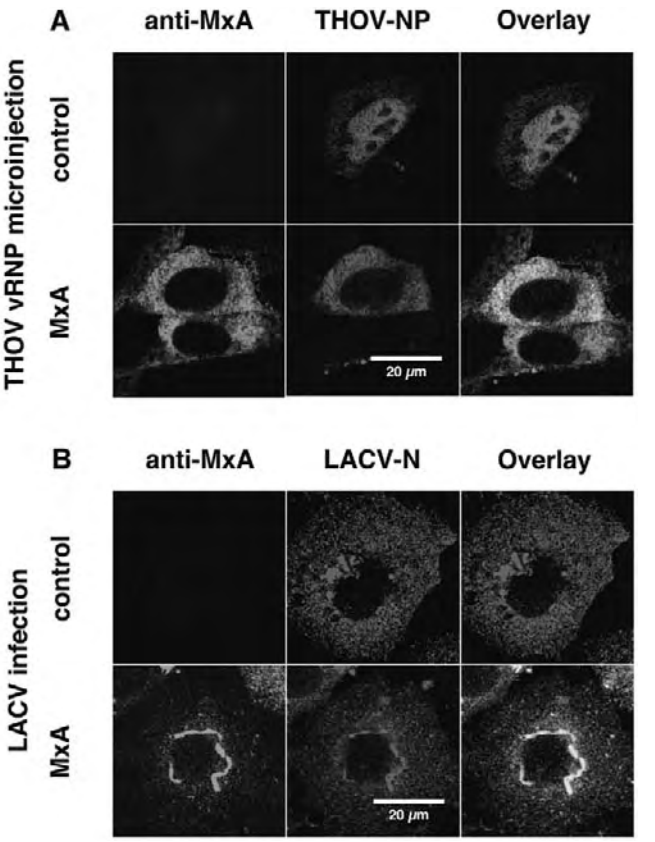


Figure 1 Inhibition of LACV and THOV multiplication by human MxA protein. (A) MxA-expressing or control cells were microinjected with purified viral nucleocapsids and incubated for 2 h. (B) Monolayers of the cells were infected with LACV for 12 h. Cells were fixed and analyzed for MxA and the viral nucleoproteins by double-immunofluorescence as described in references [10] and [11]. The right panels show the superimposition of the two images.

Mx Proteins Belong to the Superfamily of High Molecular Weight GTPases

Mx proteins are large GTPases that differ from small GTPases and heterotrimeric G proteins in their molecular weight of about 70,000 to 80,000, a relatively low affinity to GTP, and a high intrinsic rate of GTP hydrolysis [7]. Accordingly, Mx proteins belong to the superfamily of high molecular weight GTPases known to have mechanochemical properties. The prototype of this superfamily is the mammalian dynamin that is involved in endocytosis at the plasma membrane and other intracellular vesicle trafficking events (see previous chapter and reference[12]). Large GTPases share similarities in their overall domain structure. They contain a conserved tripartite GTP-binding motif within their N-terminal G domain and a less conserved C-terminal part that serves effector functions (Fig. 2A). GTP-binding and/or hydrolysis are necessary for large GTPases to display their force-generating functions [13]. Likewise, GTP binding and/or hydrolysis are required for the antiviral activity of Mx proteins [14].

The crystal structure of human guanylate-binding protein 1 (GBP-1) has been solved [15] and presently represents best the putative three-dimensional structure of high molecular weight GTPases. GBP-1 monomers consist of a globular G domain, a compact central core that is composed of two helix bundles and a long C-terminal helix, which interacts with the central core and the G domain. Figure 2B depicts a

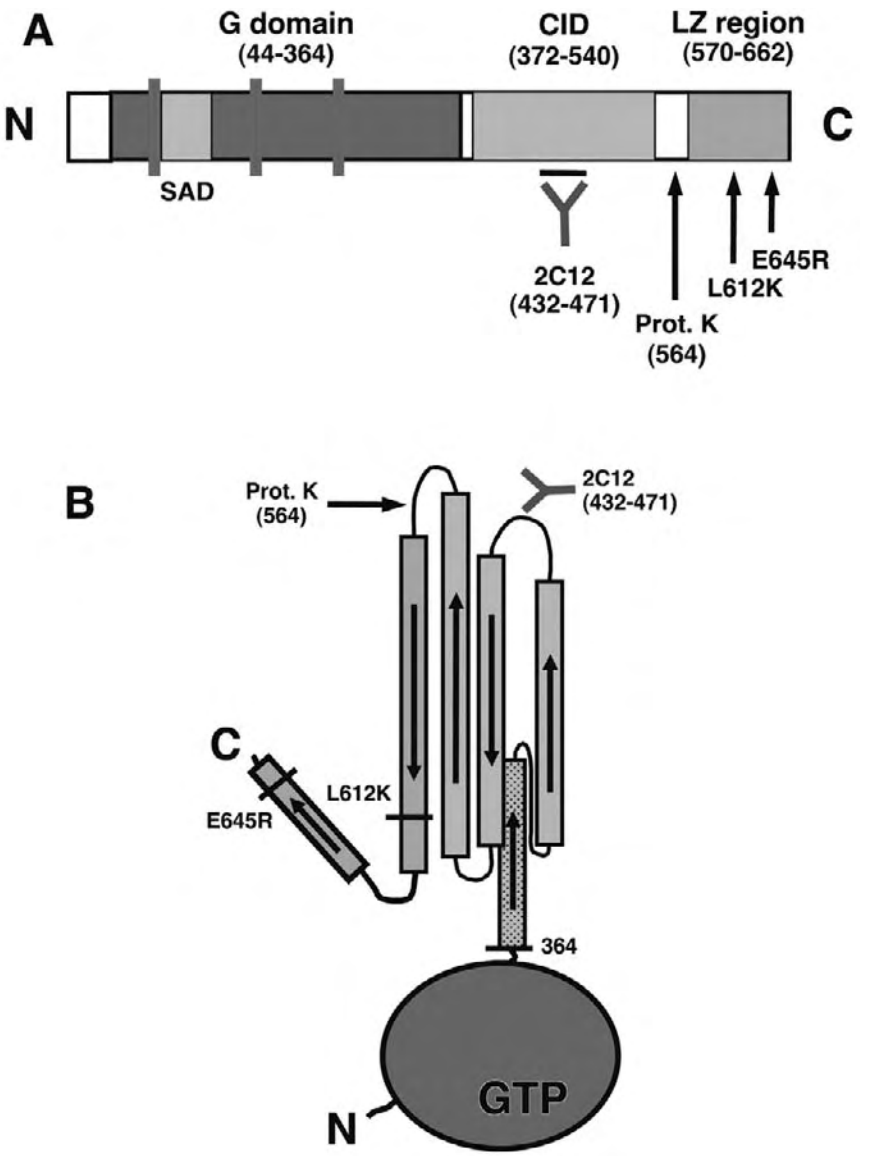


Figure 2 Functional domains of human MxA protein. (A) Schematic diagram of MxA domains and (B) a model of the putative structure according to the three-dimensional structure determined for GBP-1 [15]. The N-terminal globular G domain contains the tripartite GTP-binding element (red bars) and the "self-assembly domain," SAD (light blue box). The C-terminal effector domain contains the "central interactive domain." CID (yellow box), and the leucine zipper (LZ) region (green). The positions of the 2C12 antibody-binding site, the proteinase K cleavage site, and the critical amino acid substitutions L612K and E645R are indicated.

hypothetical model of the structure of human MxA, based on the structure of GBP-1. In this model, MxA consists of a large globular G domain and an extended helical C-terminal domain that controls self-assembly and association with other molecules, such as viral target structures or cellular binding partners.

Three different regions have been identified to be involved in molecular interactions of Mx proteins (Fig. 2A) [16]: (1) an N-terminal "self-assembly" sequence between GTP-binding elements 1 and 2 [17], (2) a "central interactive domain" (CID) [16], and (3) a leucine zipper motif (LZ) [18]. In our model, the central core containing the CID is composed of four helices which interact with the long C-terminal helix formed by the LZ region. This interaction was confirmed by biochemical studies and studies using cellular systems [19]. The CID and LZ region are connected by a putative hinge region that was identified by its accessibility to proteinase K cleavage [20]. The backfolding of the LZ region on CID results in increased GTPase activity, indicating that the C-terminal part acts as a "GTPase effector domain" [20]. Further biochemical studies with the C-terminal half indicated that the effector domain containing the CID and the LZ region is also crucial for binding to the viral target. Likewise, point mutations in the LZ region of MxA, like

MxA(E645R), and rat Mx2 identified the C-terminal effector domain as an important determinant for viral target recognition [8,11,21]. Other members of the superfamily of large GTPases have a comparable molecular structure which is also in agreement with recent reconstructions of electron microscopic pictures of human dynamin-1 [22] and dynamin A of *Dictyostelium discoideum* [23] (see also the previous chapter on dynamin).

What Is the Function of Mx Oligomerization?

Self-assembly of Mx proteins seems to be critical for GTPase activity, protein stability, and recognition of viral target structures. In cells, Mx proteins aggregate into punctate granula [24,25]. *In vitro*, purified Mx proteins form high molecular weight homo-oligomers and self-assemble into ring-like and helical structures [17,18,26]. Homo-oligomerization is essential for the biological function of other dynamin-like GTPases [13]. Dynamin, for example, self-assembles into ring-like and helical structures around membrane invaginations. Hydrolysis of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) then induces conformational changes in these complexes, which generate the mechanochemical force necessary for membrane vesiculation.

A characteristic feature of Mx proteins and other large GTPases is their ability to hydrolyze GTP to GDP in the absence of additional factors like GTPase-activating proteins or nucleotide exchange factors [7]. Mutational analysis and proteolytic digestion of MxA showed that the association of the C-terminal LZ region with the CID is necessary for GTP binding and hydrolysis [20]. Accordingly, it has been demonstrated that oligomerization stimulates GTPase activity of MxA [27]. Homo-oligomerization most likely results from binding of the LZ region of one molecule to the CID of a second neighboring molecule [19]. Mutant MxA(L612K) carries an amino acid exchange from leucine to lysine at position 612 in the proximal part of the LZ region (Fig. 2A). MxA(L612K) has lost both its ability to self-assemble and its ability to hydrolyze GTP [28], indicating that oligomerization is the major regulator of GTPase activity.

Studies in cell culture showed that the monomeric MxA(L612K) is rapidly degraded [28]. In contrast, wild-type MxA is stable, with a half-life of over 24 h. Therefore, we propose that aggregation of Mx proteins prevents their rapid degradation and provides a storage form from which active molecules can be recruited for prolonged periods of time.

As already mentioned, biochemical studies demonstrated complex formation of MxA with the ribonucleoprotein complex of THOV and with the nucleocapsid protein of LACV [10,11]. This interaction only occurred when MxA was in the GTP-bound form, indicating that GTP-MxA is in a conformation able to recognize viral target structures. With LACV we demonstrated that binding of MxA to the viral nucleocapsid protein leads to the formation of protein complexes, most likely by copolymerization of MxA with the viral nucleocapsid protein [11]. We envisage that oligomerization of MxA occurs as a consequence of viral target recognition and is important in mediating the antiviral effect.

Cellular Interaction Partners of Mx GTPases

The antiviral activity of MxA against certain viruses was found to be cell-type specific. In the case of measles virus, MxA inhibited viral transcription in human glioblastoma cells, but not in a human monocytic cell line in which a later step, namely viral glycoprotein synthesis, was affected [29]. Also, the antiviral activity of MxA against Semliki Forest virus was detectable in human cells, but not in MxA-expressing mouse cells [3]. These findings suggested that unknown cellular factors are modulating the antiviral activity of MxA and influence its antiviral specificity for some viruses. Early work by Horisberger revealed an association of MxA with actin and tubulin [30], but the significance of this interaction remained unresolved. It is conceivable that the cytoskeleton plays a role in MxA-mediated relocalization of viral components as observed in infected cells [10,11]. Furthermore, nuclear factors may contribute to the antiviral activity of mouse Mx1 protein against influenza viruses [31]. Interestingly, rodent Mx1 proteins accumulate in distinct dots within the interchromatin nucleoplasm [24]. These dots are in close proximity to subnuclear structures, called promyelocytic leukemia protein nuclear bodies (PML NBs) (Fig. 3) [32,33]. Using a yeast-two-hybrid screen, we found that mouse Mx1 interacts with components of PML NBs, such as Sp100 and Daxx. Moreover, Mx1 interacted with components of the SUMO-1 protein modification system [33]. This is interesting because protein constituents of PML NBs are targets for SUMO-1 modification and SUMOylation of PML has been demonstrated to be crucial for PML NB assembly [34]. Again, the C-terminal effector domain of Mx seems to be responsible for at least some of these interactions (Trost, Kochs, Haller, unpublished results). PML NBs are involved in transcriptional regulation, cell cycle control,

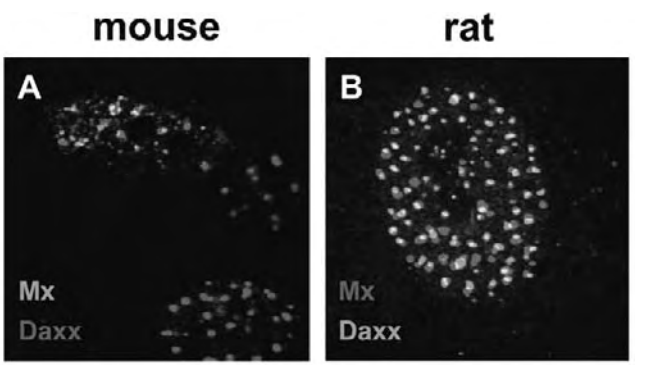


Figure 3 Mouse and rat Mx1 proteins form nuclear dots that are associated with PML NBs. Mouse embryo fibroblasts were transfected with cDNA expression constructs coding for mouse Mx1 (A) or rat Mx1 (B). The cells were fixed 24 h later and analyzed for the accumulation of Mx1 and endogeneous Daxx by double-immunofluorescence. The pictures show the superimposition of the separately recorded images for Mx1 and Daxx.

apoptosis, and antiviral defense [34]. Whether these structures play a role for the antiviral activity of Mx1 is presently being investigated. It will be of interest to see whether the nuclear Mx1 dots are simply storage sites of Mx1, as discussed for MxA, or whether they represent, in fact, specialized nuclear compartments in which the inhibition of the viral polymerase takes place.

Acknowledgment

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SECTION H

Developmental Signaling

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CHAPTER 250

Toll-Dorsal Signaling in Dorsal-Ventral Patterning and Innate Immunity

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The Toll-Dorsal Pathway

The Toll-Dorsal pathway functions in *Drosophila* in the establishment of dorsal-ventral polarity in the early embryo. The same pathway also controls the innate humoral and cellular immune response in larvae and adults and, besides Dorsal, involves an additional NF-κB/Rel protein, Dif. The NF-κB/Rel pathway is conserved in flies and vertebrates. In mammals it functions in the immune and inflammatory responses, and it is critical for cell growth and survival. Moreover a large number of mammalian tumors are associated with misregulation of the NF-κB/Rel proteins. Dorsal, like all Rel proteins, is retained in an inactive state in the cytoplasm through direct interaction with a IκB protein, Cactus. The ventral signal, transmitted through the transmembrane receptor Toll, destabilizes Cactus and controls the formation of a ventral-to-dorsal nuclear Dorsal gradient that, through the specific activation of zygotic genes, results in the formation of the dorsal-ventral axis.

Maturation of the Toll Ligand

Dorsal-ventral asymmetry is set-up in the egg chamber midway through oogenesis and is controlled mainly by EGF signaling [1]. This asymmetry is transmitted to the embryo through the interaction of two groups of genes. One group is expressed specifically on the ventral side of the follicle cells that surround the oocyte and secrete the egg-membranes. The other group is expressed in the oocyte and their products are secreted into the perivitelline space, between the egg or early embryo and the egg membranes. The interaction of the proteins encoded by the two groups of genes culminates in the maturation and cleavage of the ligand, Spatzle, which activates the Toll-Dorsal signaling pathway.

Spatzle is thought to diffuse and form a gradient in the perivitelline fluid. The extent of the Spätzle gradient is controlled by the upstream genes expressed in the ventral follicle cells and is refined by Spätzle autoregulation [2]. The Toll trans-membrane receptor is uniformly distributed in the plasma membrane of the early embryo and is asymmetrically activated by Spätzle.

Toll Signaling Establishes the Embryonic Dorsal Gradient

In early embryos Dorsal is uniformly retained in the cytoplasm bound to its inhibitor IκB protein, Cactus. The graded activation of the Toll receptor induces the nuclear translocation of the NF-κB-Rel homolog Dorsal in a gradient in the early embryo (Fig. 1A). The highest level of protein is present in the ventral nuclei and the concentration decreases laterally until on the dorsal side no Dorsal protein is present in the nuclei. The Dorsal gradient represents the first step in setting up the ultimate fate of each cell along the axis.

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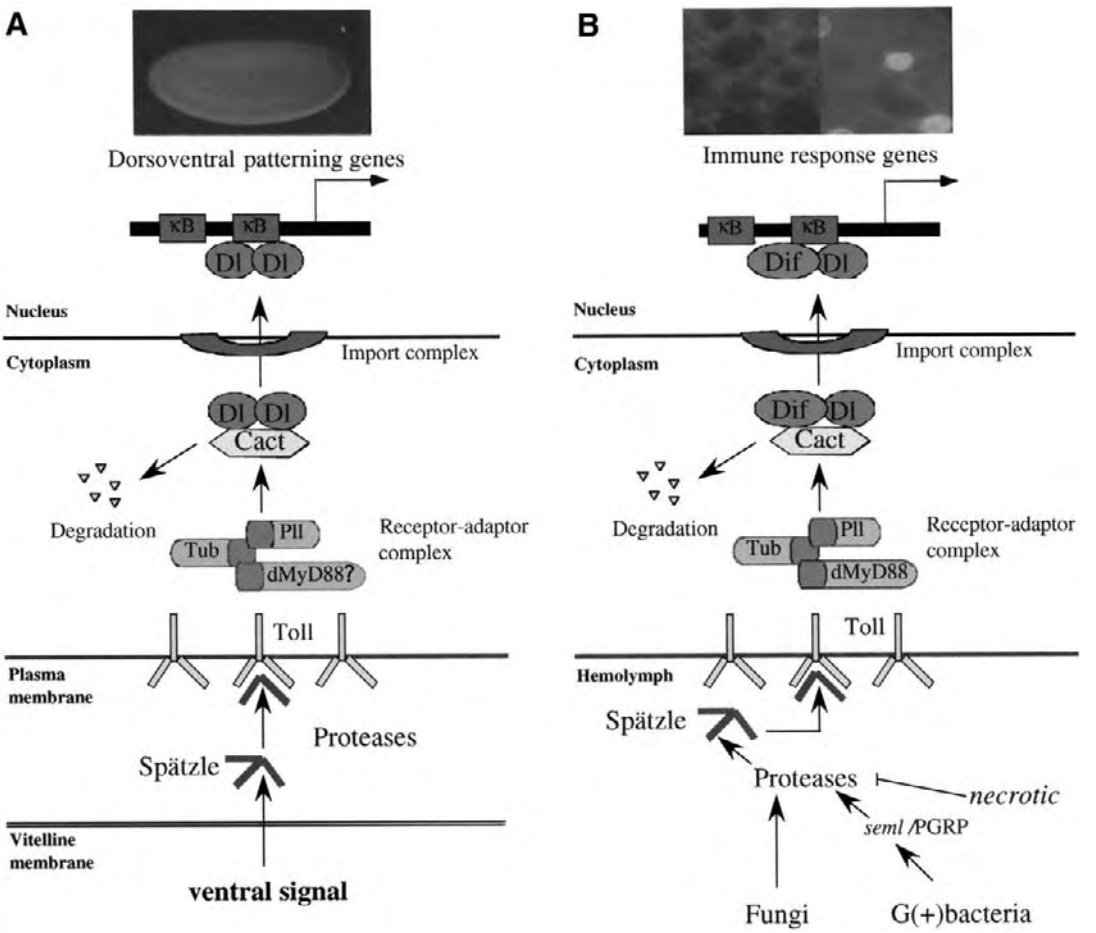


Figure 1 The Toll-Rel pathway in the early embryo and in immune response. (A) The top panel shows a blastoderm stage embryo, anterior to the left and dorsal side up, exhibiting a Dorsal-GFP gradient. Next, the steps in the Toll-Dorsal pathway occurring in the early embryo are depicted. Tub = Tube, Pll = Pelle, Cact = Cactus, Dl = Dorsal, KB = κB binding sites in DNA. (B) The top panel shows Dorsal-GFP in unchallenged (left) and bacterially challenged (right) larval fat body. Next, the steps in the Toll-Rel pathway occurring in the immune response are depicted. Dif = Dorsal-related immunity actor.

Cells with high levels of Dorsal protein will become mesoderm, while cells with no Dorsal protein will form the dorsal hypoderm, with ventral and lateral fates in-between.

The products of the *tube* and *pelle* genes relay the signal from Toll to the cytoplasmic Dorsal-Cactus complex. The Tube encodes a novel adaptor protein while Pelle functions as a serine-threonine kinase. Genetic and biochemical experiments suggest that the transmission of the Toll signal to the Dorsal-Cactus complex is mediated through the formation of a multimeric complex involving Tube, Pelle, Dorsal, Cactus, and possibly additional proteins [3]. Toll signaling results in the phosphorylation of Cactus and targets it for degradation. The same signal also controls Dorsal phosphorylation, essential for the high levels of nuclear protein and the establishment of the gradient [4]. The kinases involved in these phosphorylation events are not known.

In addition to the Toll-mediated degradation of Cactus, there is a signal-independent mechanism for degrading Cactus that also influences the Dorsal nuclear gradient. This degradation is probably controlled by an additional signaling pathway involving the maternally supplied TGFβ protein, Dpp [5]).

Dorsal Regulates the Function of Zygotic Genes

The Dorsal gradient subdivides the embryonic axis into distinct domains by setting the expression limits of key zygotic genes that are responsible for initiating the differentiation of various tissues. Dorsal interacts directly with κB binding sites and activates promoters of different genes. In the same cells it also functions as a represser by recruiting co-repressors. The control of expression of genes in specific domains along the dorsal-ventral axis is controlled by the levels of Dorsal protein and the number and affinity of Dorsal binding sites in the target promoters [6].

The Intracellular Pathway Is Conserved in the *Drosophila* Immune Response

Fungal or gram-positive bacterial infections elicit a specific response in both adults and larvae that involves Spätzle, Toll, and the intracellular components of the pathway (Fig. IB) [7].

Gram-positive and gram-negative bacterial infections are sensed by distinct pattern recognition proteins called peptidoglycan recognition proteins (PGRPs). PGRPs are highly conserved in organisms from insects to mammals. In the Toll-mediated immune response pathway Spätzle activation is under the control of a different set of genes than in the early embryo. The interaction between the microorganism and the PGRP is likely to occur in the circulating hemolymph and triggers a serine protease inhibitor (serpin)-controlled proteolytic cascade to activate Spätzle. Once activated, Toll interacts with death domain proteins: DmMyd88 and Tube, two adaptor proteins, and the kinase Pelle. This receptor adaptor complex relays the signal to the Cactus-Rel protein (Dif and/or Dorsal) complex. Dif is the predominant transactivator in the antifungal and anti-gram-positive bacterial infection. Dorsal acts redundantly with Dif in larvae but has no function in the adult immune response. Dif and Dorsal control the transcription of the antimicrobial peptide genes Drosomycin and Metchnikowin. In addition, a compound Rel protein, Relish, with an N-terminal Rel homology domain and a C-terminal IκB-like domain, is crucial for the response to gram-negative bacterial infection. While it is also inactive in the cytoplasm and translocated to the nucleus upon signaling, this signal is not transmitted through Toll.

Nuclear Import of Rel Proteins

Although the nuclear import of Rel proteins is a key step in the formation of the nuclear gradient and in the immune response, little is known about the factors involved. Proteins destined for nuclear import containing a nuclear localization sequence (NLS) are transported through nuclear pore complexes (NPCs) in a multistep process [8]. The NLS of Dorsal is essential and sufficient for nuclear import [9] and in conjunction with the signal-dependent phosphorylation of Dorsal it could provide a substrate for a specific import in α/β, which has not been identified thus far. Some of the nuclear pore proteins (Nups) forming the NPC have been shown to interact with importins. Mutant analysis has shown that both Nup88 or *mbo* and nuclear transport factor-2 (NTF-2) are involved in the import of the Rel proteins. NTF-2 is an essential gene in yeast, *Caenorhabditis elegans*, and *Drosophila*. Dorsal, NTF-2, and Nup88 form a complex necessary for nuclear import [10,11]. The NLS in the NF-κB/Rel proteins and the proteins identified thus far as important for nuclear import in *Drosophila* are all highly conserved. It is therefore likely that the same mechanisms are functioning in vertebrates.

The NF-κB/Rel pathways are some of the best known signaling processes and play central roles in development, immune response, and cancer. Because of their high level of conservation from flies to humans, further study of the Toll-Dorsal (Rel) pathways in flies will continue to complement our understanding of events in vertebrates.

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CHAPTER 251

Developmental Signaling: JNK Pathway in *Drosophila* Morphogenesis

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Introduction

For a cell to be able to distinguish and respond appropriately to extracellular signals, intracellular information transfer must be precise and reliable yet versatile. One key mode of signal transduction is the use of sequential phosphorylation events mediated by modules of protein kinases. The mitogen-activated protein kinase (MAPK) cascade is a highly conserved linear module that threads information from the cell surface to the nucleus to modulate transcription factor activity and thus gene expression [1]. Several related but functionally distinct MAPK modules have evolved that transduce a variety of extracellular signals. Genetic and molecular studies in *Drosophila melanogaster* over the last several years have revealed that components of stress signaling MAPK pathways, namely the Jun NH2-terminal kinase (JNK) and p38 kinase signaling modules, are functionally conserved and participate in numerous processes during normal development [2].

Loss-of-function genetic studies using Drosophila have identified the JNK signal transduction cascade as one of the key modulators of morphogenetic events. The *Drosophila* model system is providing a number of new insights into the general organization of the JNK pathway, the identification of new components and their epistatic relationships, and the various biological roles of the signaling activity. In the context of normal development, JNK signaling has been linked to the movement and fusion of epidermal cell sheets during dorsal and thorax closure, and to the formation of epithelial tubes during oogenesis [3]. Besides the use of JNK signaling for developmental events, accumulating evidence suggests that activation of the JNK pathway can be induced by stress and injury, as it is in mammalian systems.

The Paradigm of JNK Signaling: Dorsal Closure

In a process that has been compared to mammalian wound healing, dorsal closure of the fly embryo occurs when hundreds of ectodermal cells undergo coordinated cell elongation and movement to close over the dorsal side of the embryo. [4]. The opening is covered initially by a squamous epithelial tissue called the amnioserosa. Coverage of the area occupied by the amnioserosa is achieved by contraction of amnioserosa cells concomitant with dramatic cell elongation within the ectoderm [5,6]. Upon completion of dorsal closure, the opposing ectodermal epithelia meet and fuse at the dorsal midline; this fusion serves to internalize the amnioserosa and to surround the embryo in a continuous protective epidermis [7].

JNK signaling is precisely regulated throughout dorsal closure. Prior to initiation of closure, JNK signaling is transiently active in a broad dorsal domain; this activity requires the product of the *u-shaped* (*ush*) gene, a protein related to mammalian FOG (friend of GATA), which appears to be permissive for JNK activation [8–10]. JNK signaling then

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becomes restricted to the ectoderm by the negative action of *hnt*, which downregulates JNK signaling specifically in the amnioserosa [8,9]. Thus, although the nature of the signal that activates the JNK pathway is still unknown, expression of transcriptional targets of the pathway reveal a spatial refinement of activation, by the start of dorsal closure, to the leading row of migrating cells at the epithelial front. There, JNK pathway activation mediates changes in gene expression, accumulation of a contractile band of actin and myosin, and movement of the epidermis toward the dorsal midline [5,11–13]. Loss of or failure to activate JNK signaling results in defective cell shape changes, failed closure, and lethality [14].

Figure 1 shows a current model of JNK signaling in dorsal closure. One of the molecules that is thought to be activated early in the JNK pathway is the small GTPase, Rac1 [15]. Genetic epistasis studies in the fly are consistent with the proposed biochemical mechanism: Rac1, together with a JNKKKK, *msn*, activates the JNKKK *slpr*, a mixed lineage kinase, through direct interaction with the Rac-binding CRIB domain [16–19]. MLK catalytic activity is stimulated, which by analogy, leads to phosphorylation of Hep (JNKK) and then Bsk (JNK) [19–22]. JNK substrates include, but are not limited to, dJun, a component of the jun/fos AP-1 transcription factor [23]. As a result of AP-1 activation, transcriptional targets of JNK signaling are expressed. Among those targets are Dpp, a secreted morphogen related to the BMP/TGFβ family of signaling molecules, and Puc, a dual specificity phosphatase that provides crucial negative feedback regulation of JNK signaling via dephosphorylation of the JNK, Bsk [12,13,15,24,25]. The precise role of *dpp* expressed in leading cells is not well understood; however, Dpp signaling may control the speed and direction of leading edge cell motility through the GTPase, Cdc42 [3,26].

Given that loss-of-function mutations in any of the component kinases leads to a loss of target gene expression and a failure of dorsal closure, it has been suggested that JNK signaling regulates differentiation and function of the leading cells in which it is activated [9,27]. The special properties of these leading cells, where JNK signaling is highest, are essential for the cell biological mechanics that underlie the morphogenetic movements of dorsal closure. These properties include: (1) the assembly of a contractile actomyosin cable that provides tension for the epithelial front as it moves forward, (2) assembly of focal complexes presumed to regulate adhesion and possibly signal transduction, and (3) protrusion of actin-rich filopodia that may sense the correct segmental position of the approaching contralateral epithelium [5–8].

Until recently, it was not clear if the cytoskeletal changes observed in the leading ectoderm were controlled by JNK signaling directly or through a transcriptional mechanism.

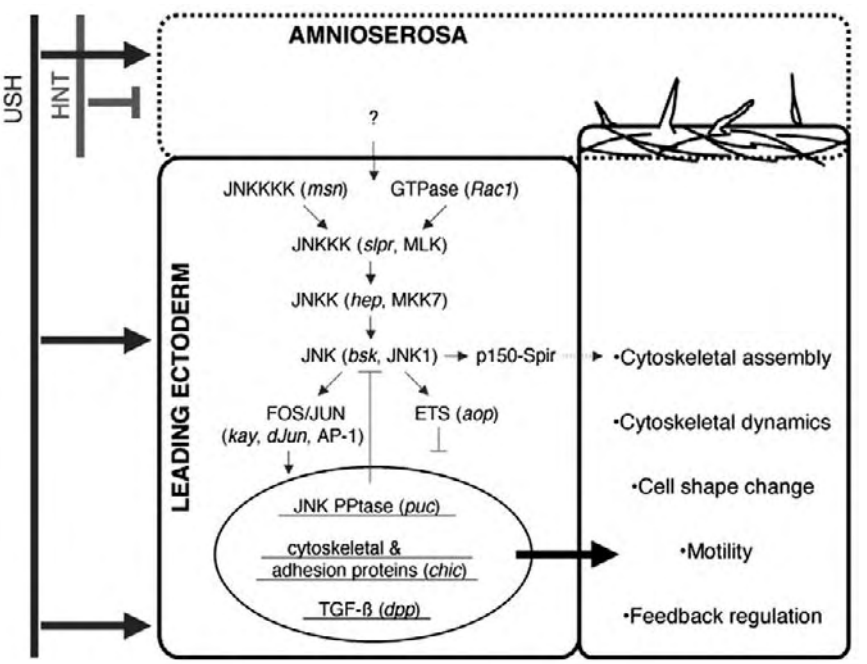


Figure 1 A model of JNK signaling during tissue morphogenesis in *Drosophila*. Prior to dorsal closure, JNK signaling is active in both the amnioserosa and the dorsal ectoderm, requiring the function of U-shaped (Ush), related to mammalian Friend of GATA (FOG) proteins. Hindsight (Hnt), a nuclear Zn-finger protein, participates in restricting JNK signaling to the leading cells of the ectoderm by repressing signaling specifically in the amnioserosa. In the leading ectoderm, JNK signaling is mediated by a kinase cascade, which results in activation of the FOS/JUN (AP-1) transcription factor and regulation of gene expression. The effectors of JNK signaling (both direct and via transcription) execute functions critical for tissue morphogenesis including cytoskeletal deployment and modulation, i.e., assembly of a contractile actomyosin network and extension of cell protrusions.

The expression of the Dpp morphogen in leading cells led to the speculation that Dpp signaling could mediate the majority of the downstream cellular events [13]. Transcript analysis by the serial analysis of gene expression (SAGE) method in embryos with either constitutive-active or blocked JNK signaling has provided compelling evidence that coordinated regulation of many genes encoding actin-binding proteins and cell adhesion molecules is essential for the execution of dorsal closure [28]. These data show that cytoskeletal reorganization driving morphogenetic movements can be mediated through JNK-dependent changes in gene expression. In addition though, biochemical methodology revealed a direct interaction between activated JNK and p150-Spir, a protein that contains an actin-binding motif with similarity to known cytoskeletal regulators of the WASP/WAVE family [29]. Thus, in some cases, the ability of JNK signaling to regulate cell shape change and motility may be direct, without a requirement for changes in transcription.

Thorax Closure

Adult tissues of the fly are generated from precursor cells (called imaginal cells or imaginal discs) that are set aside during embryogenesis and undergo proliferation and eventual large-scale morphogenetic movements during metamorphosis. Genetic analysis of semilethal *hep* (JNKK) and *kay* (dFos) zygotic mutant animals revealed an important role for JNK signaling in the spreading and fusion of sets of matched, laterally-positioned disks as they come together to meet dorsally [30,31]. In a scenario that parallels embryonic dorsal closure, Hep and dFos regulate Puc phosphatase expression in cells that adopt a position at the leading margin of the spreading discs. Ectopic puc expression, induced under conditions where imaginal cells are programmed to express an activated form of the Cdc42 GTPase, is also *hep*-dependent, suggesting that in this system Cdc42 may be the relevant upstream activator during disc morphogenesis [30]. In contrast to dorsal closure, *dpp* is not under transcriptional control by the JNK pathway, even though *dpp* and *puc* are co-expressed in a subset of disc cells, notably those at the leading margin [30,31].

Follicle Cell Morphogenesis

The somatic follicle cells that surround the germline consist of several distinct populations that, over the course of oogenesis, take on characteristic morphologies and migration patterns. The JNK signaling pathway has been implicated, through genetic mosaic analysis, in regulating a late-stage morphogenetic process whereby the respiratory appendages of the eggshell are built up from two populations of dorsal anterior follicle cells [32–34]. Dorsal appendage morphogenesis is impaired when mutant cell clones of *slpr*, *hep*, or *bsk* are induced prior to the convergence of the appendage primordia [34] (B. E. Stronach, unpublished). Short, thin appendages result from a failure of the clustered preappendage cells to fully elongate. Overexpression of the negative JNK regulator, Puc phosphatase, phenocopies the JNK loss-of-function effect on dorsal appendage outgrowth [34]. Together, these data suggest a model similar to that for the role of JNK signaling in dorsal closure; in both processes maintenance of the levels of JNK activity are critical to achieve and maintain proper epithelial cell shape change and migration [33]. Like thorax closure, but in contrast to dorsal closure, *dpp* is not under transcriptional control by Jun in follicle cells. During oogenesis Dpp does not appear to mediate the late morphogenetic events of dorsal appendage formation; in fact, Dpp acts before JNK signaling to regulate the A/P position of the appendage primordia [34].

A New Paradigm: Planar Cell Polarity

Epithelial planar polarity refers to the uniform orientation of cells or cell groups within the plane of an epithelium, observable in the adult fly as oriented bristles on the thorax, hairs on the wings, and facets of the compound eye [35]. Mutations in genes required for the development of planar polarity, such as the *frizzled* (*fz*) receptor, result in random orientation of bristles, hairs, or ommatidia [36]. Genetic mosaics and dominant interaction studies have uncovered the backbone of a signaling pathway required for establishment of planar polarity; this pathway shares several components with, yet is distinct from, the canonical Wg/Wnt pathway [37,38]. The emerging model places the JNK cascade downstream of Fz1 receptor activation. JNK activity is thought to be stimulated through a pathway that includes Disheveled (Dsh), a PDZ-containing adaptor molecule, Misshapen (Msn), related to the yeast Ste20 kinase, and the RhoA GTPase [3,35]. This linkage of Fz/Dsh signaling to JNK activation is also being investigated as a general mechanism underlying convergent extension in vertebrate embryos [39,40].

Cellular Stress Response and Wound Healing

To date, genetic analysis of the JNK pathway in *Drosophila* has revealed developmental requirements throughout the life cycle of the fly from embryogenesis to metamorphosis. Given the widespread use of the JNK signaling pathway in mammals in response to environmental and systemic stresses, such as an inflammatory response, it is important to ask if the *Drosophila* JNK pathway is similarly inducible by stress or injury. It has been suggested that the mechanics of dorsal closure movements resemble, to a large extent, the process of tissue repair during mammalian wound healing [30,41,42]. Recent investigations have probed the requirement for JNK pathway proteins during the response of flies to wounding or the response of fly cells to toxic stresses [43–45]. These studies reveal that *puc*, a transcriptional marker for activation of the JNK pathway, becomes upregulated at the site of tissue injury in the adult

fly in a *Dfos*-dependent manner [44]. Moreover, the temporal pattern of *puc* expression is correlated with cell shape changes and movement of the wound margins to close the injury. In contrast to the noted requirement for Dpp in many of the processes that require JNK, adult tissue wounds do not appear to induce *dpp* marker expression, suggesting a Dpp-independent mechanism of healing. Whether one of the other TGFß-related ligands is upregulated upon injury remains an open question. The JNK-activating signal resulting from injury also remains elusive. Although the molecular identities of JNK-activating signals for numerous JNK-dependent processes are largely unknown, there must be a variety of developmental, inducible, and environmental signals, since markers of JNK signaling activity can be observed under various conditions, e.g., tissue closure, injury, infection, and chemical stress. Future work aims at identifying the relevant signals.

Perspectives

Most of what we have learned from studying JNK signaling in *Drosophila* comes from the dorsal closure paradigm. Further studies on the function of the pathway in other processes (polarity, stress signaling, immunity) are likely to reveal different routes of JNK activation and insights on signaling. Characterization of JNK signaling in various morphogenetic processes throughout development reveals several common themes. Cumulative evidence supports the notion that JNK signaling can modulate cytoskeletal organization and cell adhesion [46]. This would provide a basis to explain the observation that JNK signaling promotes the differentiation of margin cells to establish a boundary between columnar and squamous epithelia or to define the properties of a "free" motile edge [30]. Cooperation between the JNK pathway and the Dpp pathway is a predominant, but not obligate, theme in various morphogenetic events of *Drosophila* epithelia. The role of Dpp, whether or not its expression is transcriptionally coupled with JNK activity, seems to be involved in modulating the dynamic nature of cytoskeletal assemblages beneath the cell surface and in cell projections [46]. The uncoupling of JNK signaling and *dpp* expression in several instances, however, raises the interesting possibility that there may be tissue-specific regulators that act in combination with JNK-inducible factors to specify unique morphogenetic outcomes in a tissue- or context-dependent manner.

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CHAPTER 252

Wnt Signaling in Development

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Introduction

Development is controlled by an interplay of many signaling molecules. Among these, Wnt proteins comprise a large family of secreted proteins with key roles in intercellular signaling throughout the animal kingdom. This chapter is dedicated to reviewing present knowledge about Wnt signaling in invertebrate and vertebrate development, beginning with a brief overview of the components of the Wnt signaling pathway. More details about this signaling cascade can be obtained from the Wnt homepage (www.stanford,edu/~rnusse/) and the references therein. Wnt genes were discovered from two different areas of research. In 1982, Nusse and Varmus [1] identified a proto-oncogene, called *Int-1*, by mouse mammary tumor virus integration into it in mammary tumors. The molecular cloning of the *Drosophila* segment polarity gene *wingless* (*wg*) revealed that *Int-1* and *wg* are orthologs [2], and both terms (W+nt) were combined in a common nomenclature of Wnt genes. By now, roughly 100 Wnt genes have been isolated in different species ranging from hydra to man. Information collected from genetic analysis in *Drosophila* and *Caenorhabditis elegans*, cell and molecular studies in mammalian cells, and functional overexpression experiments in *Xenopus*, as well as loss-of-function analysis in mice, has provided a detailed picture about the molecular components which transduce Wnt signals. In general, there is a high complexity of positive and negative regulation of Wnt signal transduction in each cellular compartment. At the cell membrane, binding of Wnts to their cognate receptors, Frizzled (Fz) proteins, is modulated by co-receptors or inhibitors. In the cytoplasm, Wnt signals can diverge into different molecular cascades, e.g., the best known canonical Wnt/β-catenin pathway, the planar polarity pathway, and the Wnt/Ca^{2+} pathway. The canonical Wnt/β-catenin signaling pathway provides by far the most comprehensive picture of Wnt signaling. Here the central component is β-catenin, which upon Wnt signaling enters the nucleus and functions as a transcriptional co-activator with members of the TCF/LEF-1 family of transcription factors. Wnt signaling results in the activation of specific target genes, which again involves an interplay of repressors (groucho, Sox) and activators (p300, TBP) and the coordinated action of other signaling pathways, e.g., TGFβ signaling (regulation of Twin in *Xenopus*), or retinoic acid receptor stimulation (regulation of cdx1 in mouse). The activation of Wnt/β-catenin target genes controls cell proliferation and cell fate decisions and regulates various morphogenetic events during the development of various organisms.

Wnt Signaling in Invertebrate Development

The *Drosophila wg* gene was discovered in the classical genetic screen for zygotic lethal mutations affecting the larval cuticle pattern [3]. Genome sequencing revealed a total of seven *wg* genes in the genome. The *wg* gene belongs to the class of segment polarity genes, which mediate intercellular interactions regulating parasegmental periodicity and cell fates in the larval cuticle [4]. Additionally, the adult appendages—legs and wings—are formed from sheets of epithelial cells in imaginal discs in which the dorsal-ventral (DV) and anterior-posterior (AP) axes are defined as the disc grows during larval development [5,6]. The AP and DV patterning of the imaginal discs is directed through organizing centers and morphogen gradients of the secreted signaling proteins Wingless, Hedgehog, and Decapentaplegic (reviewed in [7]).

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Studies in *C. elegans* have shown that Wnt signaling acts very early in nematode development (at the four-cell stage) to induce endoderm formation. This is brought about by relieving transcriptional repression and partially by aligning the mitotic spindle in a responding cell along the AP body axis [reviewed in 8]. The outcome is the polarization of the bipotential precursor cell (EMS) producing one daughter that makes endoderm and one for mesoderm [9,10]. In the absence of the polarizing Wnt signal both daughters form mesoderm, a phenotype represented by seven different mutants, *mom-1-5* (for more mesoderm), *lit-1* (for loss of intestine), and *pop-1* (for posterior pharynx-defective). Cloning and sequencing of these genes revealed that they code for components of the Wnt signaling cascade [reviewed in 8]. There are 5 Wnt genes in *Caenorhabditis elegans*. In the invertebrate phylogenetic tree components of the Wnt pathway have been described in the diploblastic Cnidaria (e.g., Hydra) [11], and even in the slime mold *Dictyostelium* homologs to β-catenin (Aardvark) and GSK3β have been found (reviewed in [12]).

Wnt Signaling in Vertebrate Development

Our knowledge about the Wnt signaling cascade in vertebrate development began with pioneer work in *Xenopus laevis*. Injection of Wnt1 mRNA into ventral blastomeres of early *Xenopus* embryos led to duplication of the body axis, and this was subsequently used as a read-out to explore the details of this signaling cascade [13]. The siamese twin tadpoles obtained after ventral injection of mRNA for Wnt/β-catenin pathway members were strongly reminiscent of the experimental animals Hans Spemann and Hilde Mangold [14] had produced by transplanting the dorsal blastopore lip to the ventral side of a host newt embryo. It became apparent that the Spemann-Mangold organizer, which when transplanted had this remarkable ability to induce a secondary body axis, is induced by target genes of the Wnt/β-catenin signaling cascade (reviewed in [15]). This underlines the importance of the Wnt/β-catenin signaling cascade in the induction of the primary body axis. The endogenous Wnt molecule inducing the primary body axis is still not identified, but at least 16 Wnt genes have been described in *Xenopus*. Also in chicken and zebra fish the Wnt genes apparently have similar activities during early development, with 11 and 12 identified genes, respectively.

Conventional and conditional gene knockouts in the mouse have demonstrated the important function of the Wnt/β-catenin signaling cascade in higher vertebrate development in inducing the primary body axis and directing cell fate. Both Wnt3 and β-catenin have been functionally inactivated, leading to the arrest of the mutant embryos in gastrulation due to the inability to form a primitive streak and hence mesoderm and endoderm. Evidence that the Wnt/β-catenin signaling pathway is directly involved in Spemann-Mangold organizer formation in the mouse, here called the node, came from both gain- and loss-of-function experiments [16,17]. More recently, conditional gene inactivation of β-catenin supported the existence of a bipotential mesendodermal precursor cell in the mouse embryo similar to the EMS cell in *C. elegans* embryos [18]. In addition to these Wnt functions in early development, knock-out studies of different Wnt family members have revealed their involvement in organ formation, mid- and hindbrain patterning, and limb development [19–23]. Genome sequences from mouse and human revealed a total of 19 Wnt genes in each species.

Wnt/β-Catenin Target Genes

Based on the mechanisms that lead to gene activation, one can distinguish between direct and indirect target genes. Direct target genes are defined as genes with TCF/LEF-1 binding motifs in the gene regulatory regions, where the functional importance of these motifs in β-catenin-mediated gene activation has been demonstrated. The transcriptional activation of such direct target genes does not require *de novo* protein synthesis. In contrast, indirect target genes are not regulated by TCF/β-catenin complexes, and their activation depends on other factors and requires *de novo* protein synthesis.

Because the unphysiological activation of the Wnt/β-catenin pathway leads to tumor formation, much effort has been made to identify target genes of this pathway in tumorigenesis (reviewed in [24]). Expression profile analysis of cells with an activated Wnt/β-catenin pathway led to identifying direct target genes involved in cell cycle control, e.g., c-Myc [25], Cyclin D1 [26,27], transcription factors including Tcf-1 [28], c-jun and fra-1 [29], or cell surface components such as claudin-1 [30] and MMP-7 [31,32].

The first Wnt/β-catenin developmental target genes were identified via a candidate gene approach correlating gene expression temporo spatially with the known action of Wnt/β-catenin in morphogenetic processes. The *Xenopus* genes *siamois* and *Xnr3*, originally cloned in expression screens for dorsalizing genes, turned out to be direct target genes of the Wnt/β-catenin pathway [33–36]. Direct transcriptional regulation was also reported for *X. fibronectin* [37], *connexin43* [38], *twin* [39], and *engrailed-2* [40]. Developmental mutants proved very helpful in identifying direct Wnt/β-catenin target genes, e.g., *nacre* [41] and *bozozok* [42], in zebra fish or *Dpp* [43] and *Ubx* [44] in *Drosophila*. Although valuable, this candidate gene approach has obvious limitations, making a broader search for developmentally regulated target genes desirable.

In this respect the use of ES cells represents an interesting alternative [45]. It was shown that mouse ES cells express all the necessary components to respond to a Wnt signal. Hence, by seeding ES cells on a feeder layer of NIH3T3 fibroblasts expressing different Wnts, it was possible to screen for expression of Wnt/β-catenin target genes (Fig. 1). *T-brachyury* [45] and *Cdx1* [46] are examples of genes found to be directly regulated by the Wnt/β-catenin signaling

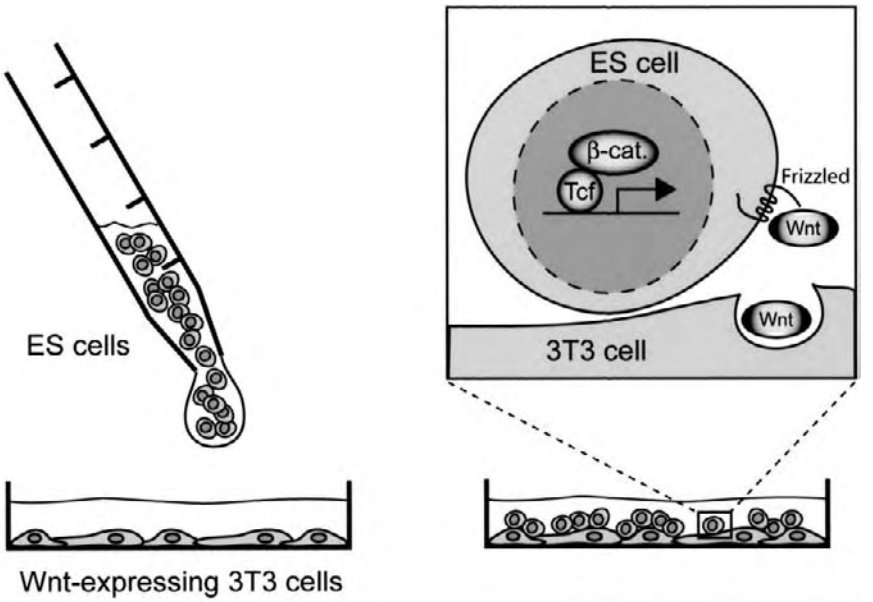


Figure 1 Scheme of the ES cell co-culture system suitable to search for Wnt/β-catenin target genes. (Adapted from Arnold, S. J. *et al*., *Mech. Dev.,* **91**, 249–258, (2000). With permission.) ES cells expressing a Wnt/β-catenin reporter gene (gfp driven by TCF-binding motifs) are seeded on 3T3 cells expressing various Wnts. ES cells receiving a Wnt signal as monitored by gfp expression can be separated and analyzed for Wnt/β-catenin target gene expression.

pathway, using this ES cell co-culture system. The T-box gene *T-brachyury* codes for a transcription factor involved in mesoderm formation [47–49]. Cdx1 is a homeodomain-containing transcription factor with important roles in anterior-posterior patterning [50] and likely in intestinal development [46,51]. The ES cell co-culture system in combination with microarray analysis should allow screening systematically for a large number of potential Wnt/β-catenin target gene candidates.

In conclusion, the Wnt/β-catenin signaling pathway is evolutionarily highly conserved on a biochemical level and regulates many morphogenetic processes during development. From the direct target genes found thus far, it is apparent that this pathway regulates gene expression of a large variety of cellular components, i.e., transcription factors, cytoplasmic and cell surface molecules, and growth factors.

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CHAPTER 253

Hedgehog Signaling and Embryonic Development

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The Hedgehog Proteins: Generation and Distribution

The hedgehog mutation was originally identified in a *Drosophila* genetic screen as one of the segment-polarity genes important in fly development [1]. Cloning of the hedgehog gene (*hh*) revealed a secreted peptide whose expression is confined to Engrailed expressing cells and is dependent upon Wingless expression. It is now clear that Hh plays a vital role in the development of multiple organ systems in the fly and vertebrates [2]. In mammals there are three Hh proteins named Sonic Hh (Shh), Desert Hh (Dhh), and Indian Hh (Ihh). Dhh appears to be most closely related to *Drosophila* Hh, while Shh and Ihh are more closely related to one another. Production and diffusion of these factors in different tissues determines proper development of multiple organ systems.

Hh proteins are synthesized as precursor proteins of about 45 kDa. The C-terminal portion of the Hh precursor has autoproteolytic activity and cleaves Hh into a C-terminal peptide of about 25 kDa with no known function and an N-terminal fragment (Hh-N), which constitutes the biologically active portion of Hh [3]. During autoprocessing, a cholesterol moiety is coupled to the C-terminus of Hh-N, a form which is further denoted Hh-Np [4]. It is thought that the addition of cholesterol helps to retain Hh-Np to cell membranes, thus limiting the range of action of Hh activity. However, in mice engineered to express a form of Shh lacking cholesterol modification (N-Shh), short-range Hh signaling was maintained while long-range signaling was defective, resulting in loss of digits and proper patterning in the developing limb and suggesting differential requirements for cholesterol in Hh signaling [5]. Additional proteins involved in the secretion and diffusion of cholesterol-modified forms of Hh have been identified, such as Dispatched (Disp), which is required for release of Hh-Np from Hh-producing cells, and Tout velu (TTV), which is involved in the biosynthesis of a putative Hh-interacting proteoglycan [6–8].

Hh proteins are further modified by palmitoylation on a highly conserved N-terminal cysteine residue [9]. Mutation of the *sightless/skinny Hh* (*sig/ski*) gene, encoding a transmembrane (TM) acyl transferase, abrogates palmitoylation of Hh-N and results in an Hh-like phenotype, indicating that palmitoylation of Hh is required for some aspect of Hh function [10,11]. In some systems both modified and unmodified forms of Hh show equivalence, indicating that the importance of fatty acid modification may be context dependent [9,12]. Together these data indicate that the biological activity of the Hh proteins is finely tuned through posttranslational modification, affecting its activity and capacity to diffuse.

Transmitting the Hh Signal

Both *Drosophila* and mouse genetics indicate that the transmembrane protein, Smoothened (Smo), is required to transmit the Hh signal [13–15], and another TM protein, Patched (Ptc), negatively regulates Smo in the absence of Hh [16,17]. While it is widely accepted that Hh binds to Ptc [18,19], it is not clear how Hh binding results in downstream Smo activation and signal transduction.

In the conventional model, Ptc binds directly to Smo and represses its activity (Fig. 1A). Upon Hh binding the normal inhibition by Ptc is released and Smo initiates signaling (Fig. 1B). However, recent studies in *Drosophila* have

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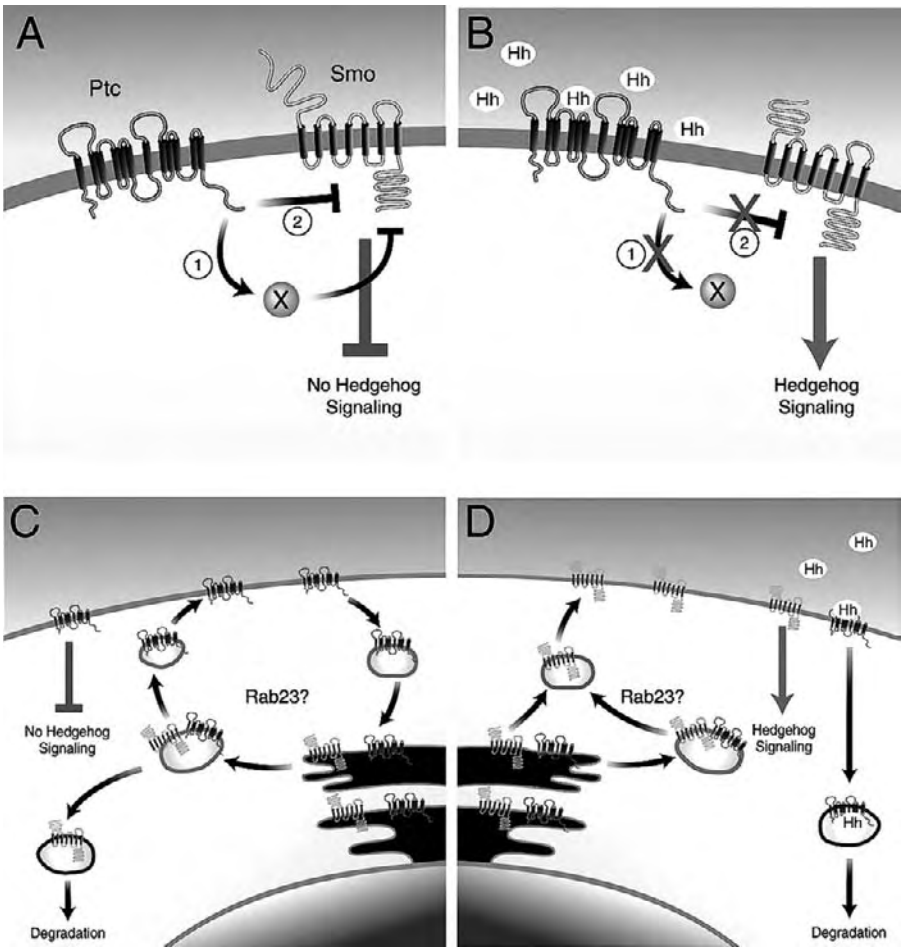


Figure 1 Two models for transmitting the Hh signal through Patched and Smoothened. (**A**) In the conventional model Patched (Ptc; red) inhibits Smoothened (Smo; green) function through (1) direct inhibition of Smo function or (2) an indirect effect, where Ptc acts upon an unknown factor (X) that subsequently inhibits Smo activity. (**B**) Upon Hedgehog (Hh; yellow) stimulation, the repressive function of Ptc is abrogated allowing for activation of Smo and transmission of the Hh signal to downstream targets. (**C**) In a more unconventional model, Ptc (red) acts to repress Smo vesicular trafficking. In the absence of Hh, Ptc and Smo are generated in the endoplasmic reticulum (ER) and are processed through the Golgi. Ptc traffics to and from the cell surface, while Smo may traffic to an unknown subcellular compartment, possibly resulting in Smo degradation, ultimately resulting in a lack of Hh signaling. Rab23 may act to coordinate some aspect of Ptc or Smo trafficking. (**D**) Upon Hh signaling, Ptc binds to Hh stimulation, the Ptc-Hh complex is internalized via endocytosis resulting in the degradation of Ptc. This allows for the transportation of Smo to the cell surface where it can mediate Hh signaling.

suggested that Ptc may not repress Smo activity through a direct interaction, but rather that Ptc inhibits Smo activity from a distance [20–24]. For example, regulation of Smo activity may occur through control of vesicular trafficking by Ptc. SCAP, a protein involved in cholesterol metabolism and trafficking, regulates the activity of SREBP by controlling its movement between the endoplasmic reticulum (ER) and Golgi via a mechanism involving use of a SSD in SCAP [25,26]. Ptc may regulate the activity of its cargo, Smo, in a similar fashion (Fig. 1C and D). Mutation within the Ptc SSD results in the abrogation of Ptc-mediated inhibition of Smo activity [27,28]. Further support for the regulation of Smo transport has come from the *open brain* (*opb*) mutation in the mouse, recently identified as the *Rab23* gene [29], a GTP-activating protein involved in vesiculotubular transport. A model involving the negative regulation of Smo movement to the cell surface by Rab23 has been hypothesized, however, it is also possible that Rab23 aids in the proper localization and function of Ptc.

The main target of Hh activity is a family of zinc finger transcription factors known as cubitus interruptus (Ci) in the fly and Gli in vertebrates. Gli activity is regulated at multiple levels, including nuclear export, proteosome-mediated degradation, and subcellular localization. In vertebrates there are three Ci orthologs; Gli1, Gli2, and Gli3, which may have retained aspects of Ci-155 and Ci-75 function (reviewed in references [2] and [30]). Knockout or transgenic mice of each Gli isoform have been generated and the

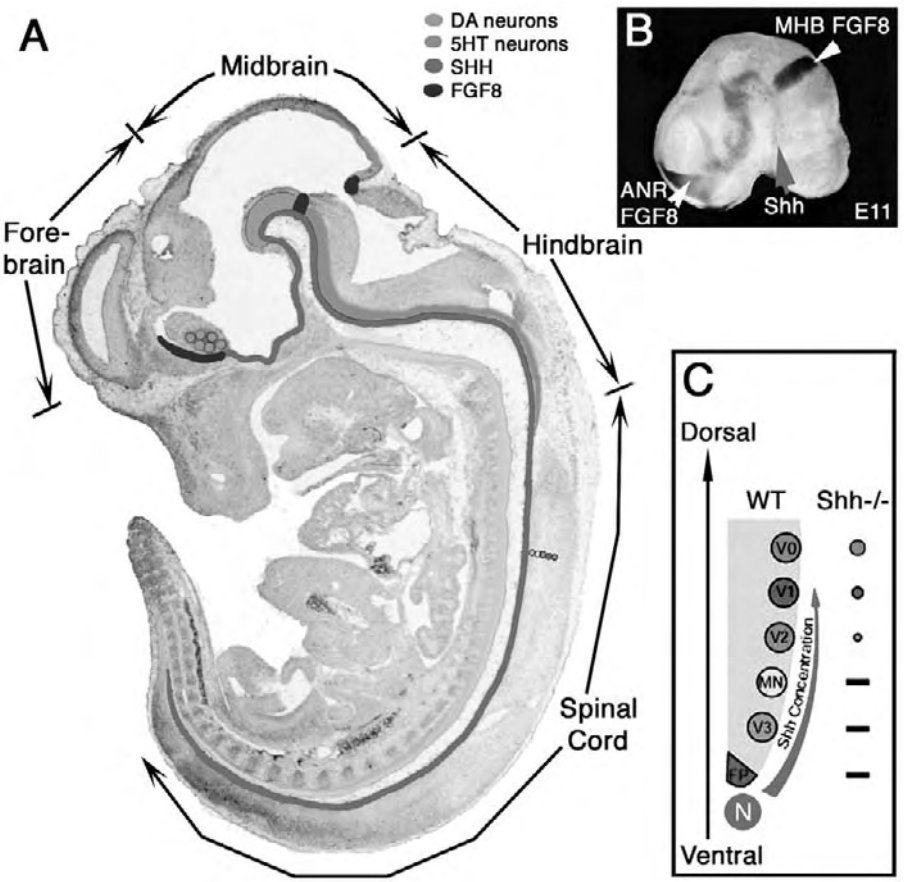


Figure 2 Shh in the developing nervous system and the cell types controlled by SHH. (**A**) Shh (red), FGF8 (dark blue), dopaminergic (DA) neurons (light blue), serotonergic (5HT) neurons (green), and six ventral spinal cord neurons (colored dots with black circles) are diagramed on a sagittal section of a 12-day-old mouse embryo. Shh in the notocord and floor plate is represented by a single line. (**B**) Whole mount *in situ* hybridization of Shh (red) and FGF8 (dark blue) in the brain of an 11-day-old embryo. Shh is expressed in the ventral midline (red arrow), FGF8 is expressed in two organizers—the mid-hindbrain junction (MHB) and the anterior neural ridge (ANR) (white arrow heads). (**C**) The position of six Shh-dependent cell types (color-filled circles) in the ventral spinal cord of wild-type (WT) and Shh mutant embryos ($Shh^{-/-}$), and their relationship to SHH concentration (red arrow). Different cell types are induced by different threshold concentrations of SHH. Higher concentration of SHH induces cells in progressively more ventral locations. FP = floor plate, N = notochord, MN = motor neuron, and V0, V1, V2, and V3 are four classes of interneurons.

phenotypes observed support the idea that Gli2 and Gli3 are critical for normal development, while Gli1 may be redundant for Gli2 and Gli3 function. The interplay between the activities of these isoforms increases the complexity of vertebrate systems dramatically making it difficult to conclusively delineate all of the functions for the different Gli proteins during normal development (reviewed in reference [2] and in Section III of this book).

Hh in Development and Disease

Studies of the normal functions for the Hhs in animal models have helped in our understanding of Hh-related diseases. Many studies have shown that Shh acts as a morphogen in the nervous system, where it is secreted from the notochord and later from the floor plate, patterning neurons along the dorsal-ventral axis of the neural tube in a dose-dependent manner [2] (Fig. 2). In the spinal cord and part of the hindbrain, a fine gradient of SHH with two- to three-fold incremental changes from the source (floor plate and notocord) delineates the ventral neural tube into six distinct domains along the dorsal-ventral axis. The expression of a set of homeodomain and bHLH transcription factors is tightly controlled by different SHH protein concentrations, thus generating six intricate combinatorial transcription factor codes in these domains [31,32]. These codes specify the identities of neural progenitors, which ultimately give rise to six cell types, including the floor plate (FP) cells, motor neurons (MN), and four classes of interneurons, V0, V1, V2, V3[33] (Fig. 2A and C). In the $Shh^{-/-}$ mouse embryos, FP cells, MNs, and V3 neurons are missing, and

the numbers of V0, V1, and V2 neurons are greatly reduced [34,35] (Fig. 2C). In the brains of mouse embryos, SHH acts in concert with another organizer molecule FGF8 to create information grids, which serve as spatial cues for the specification of ventral cell types. Ventral neurons such as dopaminergic (DA) neurons and serotonergic (5HT) neurons are specified in locations where Shh and FGF8 intersect (Fig. 2A and B) [36,37]. Earlier patterning events dictate how neural stem cells respond to these organizer signals, so that DA neurons are induced by SHH and FGF8 in the fore- and midbrain, whereas 5HT neurons are induced by the same set of signals in the hindbrain, because the hindbrain progenitors have been prepatterned by another signal(s) from the posterior [36]. Shh controls cell fates not only by induction, but also by repression. It is found that SHH in the forebrain is required to repress Pax6 expression, thus resulting in the separation of a single eyefield into two retinal primordia [38,39]. This explains the cyclopic phenotype in the Shh-deficient embryos [34]. Late in development, Shh elicits other cellular responses than cell fate specification. For example, in the fetal cerebella, SHH induces proliferation of granule cells while inhibiting their differentiation [40–42].

Shh also acts to determine anterior-posterior (AP) patterning in the developing skeleton, limb bud, and gut tube [2,30]. Shh has recently been shown to act as an angiogenic factor leading to neovascularization and the proliferation of blood cells [43]. In some tissue types, such as the pancreas, Shh acts both as a positive and negative regulator, as its activity is needed for inhibition of pancreatic anlagen formation, but is also needed for specification of the pancreatic β cells [44,45]. Shh is also involved in the morphology of branching structures such as the lung [46]. The importance of Shh is highlighted by the phenotype of Shh knockout mice, which die at birth due to multiple defects, including cyclopia and holoprosencephaly (HPE), as well as other defects in limbs, brain, spinal cord, axial skeleton, and midline structures [34]. Overlapping roles of Shh and Ihh have been identified in heart development as well as specification of left/right (L/R) asymmetry, as observed in Shh/Ihh double knockout mice and Smo knockout mice [47]. The major impact of Ihh is in radial patterning of the gut as well as bone morphogenesis [48,49]. Loss of Ihh function results in a lack of chondrocyte proliferation and differentiation [49]. Dhh is predominantly involved in peripheral nerve sheath and germ cell development, particularly in the development of the male germline and maturation of the testes [50,51].

The patterning functions of Hh are also highlighted in humans where mutations have clearly been linked to developmental disorders, including spina bifida, neural tube defects, and skeletal deformations. For example, mutations in human Shh result in cyclopia and HPE [52]. Downstream of Hh, mutations within Gli3 are found in Grieg's cephalopolysyndactyly [53] and Pallister-Hall syndrome (PHS) [30,54], disorders associated with various abnormalities including polydactyly.

The first indication that the Hh pathway might be involved in tumor formation stemmed from the observation that human Ptc (PTCH) gene was mutated in individuals with Gorlin's syndrome (also known as basal-cell nevus syndrome; BCNS) [55], a familial inherited predisposition to the development of basal cell carcinomas (BCCs), medulloblastomas, and rhabdomyosarcomas [56,57]. Many sporadic BCCs have been associated with mutation within Ptc [58]. In all cases loss of Ptc function leads to constitutive Smo signaling and Hh pathway activation. In addition, point mutations in Smo, which result in constitutively active Ptc-insensitive forms of Smo, have been identified in sporadic BCC [59]. However, most of these tumors show upregulation of the Hh target genes, suggesting that other components of the Hh pathway may be mutated. One such example was identified in Smo where a point mutation, known as the Smo-M2 mutation (Smo-M2), results in a constitutively active form of Smo [59].

Interestingly, treatment of Hh-responsive cells with the teratogenic steroidal alkaloid cyclopamine blocks Hh signaling and Smo activation in cell lines and induces HPE in animal models [60,61]. Treatment of some glioma and medulloblastoma cell lines *in vitro* with cyclopamine has resulted in successful inhibition of cell growth, indicating that cyclopamine or other drugs that specifically block the Hh pathway may be effective in the treatment of Hh-associated tumors [62,63].

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CHAPTER 254

Control of Left-Right (L/R) Determination in Vertebrates by the Hedgehog Signaling Pathway

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Introduction

Although the bodies of most vertebrate animals appear to be almost perfectly symmetrical, profound left-right (L/R) asymmetries exist that are clearly demonstrated by the specific disposition of internal organs (such as the heart to the left side of the chest cavity, or the liver to the right side of the abdomen, just to mention two examples). At another level, there is also asymmetry in the activity of the brain, with specific functions performed by each individual brain hemisphere, resulting in asymmetries in locomotor functions (among which being right- or left-handed is only one well-known manifestation). The normal disposition of organs is called *situs solitus*, and *situs inversus* refers to a complete or near-complete mirror-image reversal of organ disposition. Right or left isomerism usually refers to situations where individual organs are bilaterally symmetric.

Interestingly, this normal (that is, asymmetric) disposition of organs develops in embryos that are initially perfectly symmetrical, at least as far as we can tell from their morphological examination at very early stages of development. Therefore, the problem is to explain how asymmetric animals develop from (apparently) symmetric embryos, and to define the molecular mechanisms that control this process. That L/R development is under genetic control is clear from the existence (in humans as well as in other vertebrates) of congenital syndromes that result in a variety of laterality defects that can severely impair the function of the heart, lungs, liver, and other vital organs.

The problem of L/R specification has enticed biologists for many years, but until very recently a solid molecular entry point that could be used to successfully attack the problem had been missing. The situation changed in 1995, when the first molecular asymmetries were discovered in chick embryos (and later in other vertebrates). This discovery revealed that certain genes were expressed in asymmetric patterns in the developing embryo, and that these asymmetric patterns were somehow translated into asymmetric development of internal organs. Alterations in some of these genes have already been shown to be linked to some of the human syndromes that display laterality defects, further adding to our knowledge of the etiology of L/R defects in humans. Several comprehensive reviews published recently deal with multiple aspects of L/R development from a broader perspective [1–3].

One of the first genes shown to be involved in L/R determination in vertebrate embryos was *Sonic hedgehog* (*Shh*), a member of the *hedgehog* (*hh*) family of genes, which encode secreted factors that play key roles in multiple aspects of embryonic development in both vertebrate and invertebrate embryos. Since general aspects of HH signaling during embryonic development are reviewed by F. de Sauvage elsewhere in this volume, we will focus here only on the aspects of HH signaling relevant to the development of the embryonic L/R axis, especially in chick and mouse embryos. Further discussion on possible species-specific mechanisms can be found in the reviews mentioned above.

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The Discovery of the First Molecular Asymmetries in Vertebrate Embryos and the Role of SHH

In 1995, a team of researchers led by Cliff Tabin and Claudio Stern published a seminal paper that brought into focus key aspects of the molecular basis of L/R determination [4]. The paper demonstrated that asymmetric gene expression did indeed exist in the early embryo, and that it played a key instructive role in the development of the L/R axis in vertebrates. Specifically, it was shown that the *Shh* gene was initially expressed in Hensen's node (the organizer of the chick embryo) without any apparent bias, but that shortly after, *Shh* expression became restricted to the left side of the node by an inferred Activin-like activity (most likely Activin ßB) coming from the right side of the node. Activins belong to the transforming growth factor ß (TGFß) superfamily of secreted factors, another group of signaling factors that play fundamental roles in the development of vertebrate and invertebrate embryos and during tumorigenesis [5,6]. It was shown that application of purified Activin protein to the left side of the node resulted in repression of *Shh* expression, and that application of purified SHH protein (this time to the right side of the node) repressed transcription of the gene encoding the Activin receptor (*ActRIIa*), which depends on the Activin ßB ligand. Therefore, a mutually exclusive regulatory interaction between two secreted proteins with complementary patterns of expression resulted in the "specification" of a right side of the node (producing Activin ßB protein) and a left side (producing SHH protein; Fig. 1A).

Another gene encoding a TGFß factor, *Nodal*, was shown to be expressed on the left side of the chick node under the control of SHH. *Nodal* also has a second domain of expression in the left lateral plate mesoderm (LPM) of the embryo, including cells that eventually will give rise to the organ primordia (Fig. 1A). Importantly, left-specific expression of *Nodal* has been observed in all types of vertebrates examined thus far, correlating absolutely with the development of normal organ *situs*. For example, when *Nodal* is either completely absent or present on both sides, organ *situs* is randomized. When *Nodal* is present exclusively on the right side (which occurs naturally in the *inv* mutant mouse and in some human patients that suffer from specific ciliary dysfunctions), there is *situs inversus*. Therefore, left-specific expression of the SHH target *Nodal* appears to play an instructive role in determining organ *situs* and constitutes a key feature of L/R development that has been absolutely conserved during the evolution of vertebrates. It is important to point out that the correct (that is, left-specific) expression of *Nodal* is not required for the development of the organs, but only for the establishment of their correct L/R asymmetry. When *Nodal* expression is altered, organs still form, but with an altered L/R axis that can result in malformations incompatible with life.

Further work identified additional factors that interact with SHH during L/R determination (Fig. 1A). On the right side of the node, Activin ßB controls expression of another TGFß factor, bone morphogenetic protein-4 (BMP-4), which in turn induces fibroblast growth factor-4 and -8 (FGF-4, -8). Mutual antagonism between BMP-4 and SHH appears to be necessary and sufficient to maintain asymmetric *Shh* expression in the node [7]. Moreover, SHH was shown to induce *Nodal* expression in the left LPM through the control of Caronte (Car), a secreted factor that binds to BMPs and antagonizes their repressive effect on *Nodal* transcription in the left LPM. On the right side of the embryo, FGFs turn off expression of Car, and thus BMPs are free to signal, which keeps the *Nodal* gene repressed on the right [8–10]. *Nodal* expression on the left side of the embryo controls the expression of genes such as *Pitx2*, which direct asymmetric organogenesis by mechanisms that are still poorly understood (see the reviews mentioned before in references [1–3]).

The control of *Nodal* by the HH signal on the left side of the node is likely to involve the activity of the HH reception complex, composed of the transmembrane proteins Patched1 and Smoothened (see F. de Sauvage's review in Chapter 253). Recent results indicate that additional components of the HH signaling pathway also play key roles in L/R determination. For example, protein kinase A (PKA) had been previously shown to act as an antagonist of HH signaling in vertebrates, most likely by phosphorylating GLI proteins, which are key nuclear effectors of the pathway. However, PKA has been recently described to act as a positive regulator of the SHH target *Nodal* during L/R determination in the chick embryo [11]. Consistent with this, the endogenous PKA inhibitor, PKI is more strongly expressed on the right side of Hensen's node, where it inhibits PKA, thus restricting its *Nodal*-inducing effects to the left side of the node [11,12]. Interestingly, the induction of *Nodal* expression by PKA does not require *Car* activity, and thus PKA appears to control *Nodal* through a pathway which acts in parallel to that operated by SHH and Car. Other components of HH signaling pathways are also likely to operate during L/R determination, but their exact roles have not yet been determined.

Finally, it is important to point out that the exact nature of the event that breaks the initial symmetry of the chick embryo remains unknown, although an intriguing hypothesis has been proposed that stresses the role of gap junction communication in this process [13].

The Role of a Composite HH Signal during L/R Determination in the Mouse

In the context of L/R development in the chick embryo, the role of SHH could be defined as that of a "left determinant." That is, SHH becomes restricted to the left side of Hensen's node, it controls expression of *Nodal* in and around the left side of the node, and (through Car) it also controls the *Nodal/Pitx2* domains in the left LPM. Inhibition of SHH activity (achieved, for instance, by exposing the chick embryo to an anti-SHH blocking antibody), inhibits

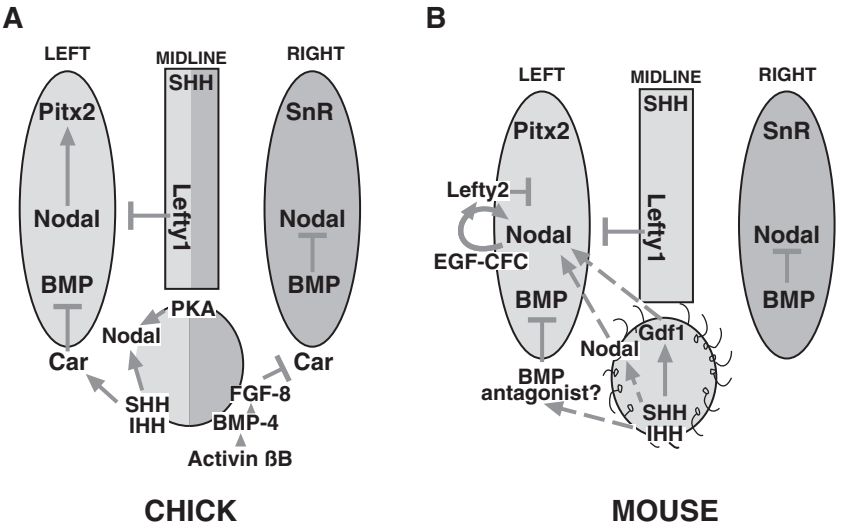


Figure 1 Comparison of the role of HH signaling during L/R determination in chick (A) and mouse embryos (B). At early stages of development of the chick embryo, breaking of the initial embryonic symmetry by mechanisms that are still unknown results in restriction of transcription of *Shh* (and most likely also of *Ihh*) to the left side of Hensen's node (the node is depicted as a circle, and the left side is shown in light gray). On the right side of the node (shown in darker gray), Activin ßB triggers a cascade that activates expression of the *Bmp-4* and *Fgf-8* genes (and also of *Fgf-4*). HH signals display an antagonistic regulatory interaction with both Activin ßB and BMP-4, which contributes to stabilize these side-specific patterns of transcription in the node. In and around the left side of the node, HH signaling mediated by the reception complex formed by the membrane proteins Patched1 (the receptor component) and Smoothened (the transducing component, not shown) results in activation of *Nodal* expression. PKA and other factors may act in parallel to ensure correct *Nodal* expression in the node area. HH signals also operate on the paraxial mesoderm cells adjacent to the node to maintain expression of the *Caronte* (*Car*) gene, which encodes an extracellular antagonist of BMP signaling. The presence of Car protein in the left side of the embryo antagonizes a repressive effect of the BMPs on transcription of the *Nodal* gene in the left LPM (the LPM is depicted as an ellipse), so that Nodal protein is produced and activates its target *Pitx2*. This gene encodes a bicoid-type homeoprotein that plays a key role in the transference of L/R positional information to the organ primordia. In contrast, on the right side of the embryo *Car* expression cannot be maintained, due to the absence of HH signaling and the presence of FGF-4 and/or -8, and thus BMP signals keep the *Nodal* gene repressed in the right LPM. This results in expression of *SnR*, a gene encoding a Snail-related zinc-finger transcription factor that also plays an important role in the determination of organ *situs*, in this case as a "right determinant" [19].

SHH (but not IHH) is also present in the midline (depicted as a rectangle), where it is required for the development of the floor plate, a structure in which the *Lefty1* gene is expressed. *Lefty1* encodes yet another TGFß member that has been proposed to function as a "midline barrier" to the diffusion of left-specific inducing signals from the left to the right side of the embryo (probably through direct interaction with Car and/or Nodal proteins). In cases where development of midline structures is compromised (such as in *Shh*-deficient embryos, for instance), bilateral expression of left-specific genes occurs, most likely due to secondary loss of *Lefty1* expression.

In the early mouse embryo, transcription of the *Shh*, *Ihh*, *Activin ßB,* and *Fgf-8* genes in the node is not restricted to the left side, and a leftward nodal cilia flow (which has been proposed to carry some still unidentified extracellular inducers) results in the transient asymmetrical expression of *Nodal* in the left side of the node. Despite the lack of left-specific restriction of HH signals, deficiency in both SHH and IHH proteins (or, alternatively, deficiency in Smo), completely abolishes *Nodal* and *Pitx2* expression in the left LPM, indicating that HH signaling also acts in the mouse as a left determinant. Interestingly, *Nodal* expression in the node is not abolished, but is nevertheless altered, suggesting that HH signaling does not control *Nodal* directly in the node, but is somehow involved in the specification of its normal domain. Both Gdf1 and Nodal itself play important roles downstream of HH signaling in controlling *Nodal* expression in the left LPM. Indeed, the Nodal protein, in the presence of extracellular cofactors of the EGF-CFC superfamily, has been shown to activate transcription from its own promoter. Exactly as in the chick embryo, Nodal expression in the left LPM activates *Pitx2* expression.

Thus far, no *Car* gene has been identified in vertebrates other than the chick, but it seems likely that a BMP antagonist (or an alternative mechanism of attenuation of BMP signaling) may also exist in the mouse embryo, as illustrated by the fact that mouse mutants deficient in BMP signaling display bilateral expression of *Nodal*. The nature of this putative BMP antagonist remains unknown, as is the chain of events that operate on the right side of the mouse embryo and its possible interactions with HH signaling in the node.

the expression of all these left-specific genes. Moreover, when SHH protein is applied ectopically to the right side of the embryo, it can ectopically trigger the whole program of "left expression."

In the mouse embryo, however, *Shh*, *Activin ßB*, *ActRIIA,* and *Fgf-8* are all expressed in the node, but without any detectable asymmetry (Fig. 1B). *Nodal*, in contrast, displays a (transient) stronger expression on the left side of the node, besides its domain in the left LPM, further confirming it as a bona fide evolutionarily conserved left determinant. But does SHH act as a left determinant in the mouse embryo? Interestingly, recent studies in mouse embryos demonstrate that a second member of the HH family, named Indian Hedgehog (IHH), plays a role in L/R development that is partially redundant to that of SHH [14]. Indeed, the SHH and IHH proteins have been previously shown to display similar activities (and bind to the same receptor) in a variety of assays. Both SHH and IHH are expressed in the mouse node without apparent L/R bias, and *Shh*-null embryos still express IHH, which can perform part of the activities of SHH, thus obscuring the consequences of completely removing all HH signals from the node [15,16]. In fact, in *Shh/Ihh* double mutant embryos, *Pitx2* is completely absent from the LPM, which indicates that a compound HH signaling is absolutely required for the establishment of left-specific gene expression in the mouse embryo. Importantly, this phenotype is comparable to that of *Smo*-deficient embryos. The *Smoothened* (*Smo*) gene encodes the essential transducer of both SHH and IHH signals, and in the *Smo*-deficient embryos, *Nodal* (as well as other left-specific genes) is absent from the LPM. *Nodal* expression is, however, retained in the node, although with variable levels and patterns of expression, and the significance of this observation is still unclear.

These results demonstrate that HH signaling from the mouse node is absolutely required for the activation of left-specific genes in the LPM, exactly as in the chick embryo, even though expression is not restricted to the left side of the node. In contrast with the situation in the chick, however, ectopic HH signaling in the mouse fails to induce ectopic expression of *Nodal*, which suggests that the regulation of *Nodal* by HH is indirect. In this regard, the TGFß family member Gdf1 and the EGF-CFC extracellular factor Cryptic (a cofactor for the Nodal protein) both appear to be targets of HH signaling, and may mediate the effects of HH proteins on the node and the LPM (Fig. 1B). In the mouse, there is also some evidence that the domain of *Nodal* expression in the node is required for expression of *Nodal* in the left LPM, where a positive autoregulatory loop is established. It is important to point out that the same situation of redundancy between SHH and IHH in the node is likely to occur in the chick (Fig. 1A), where the anti-SHH blocking antibody presumably blocks both SHH and IHH proteins.

Why isn't there left-specific expression of *Shh*, *Activin ßB*, or *Fgfs* in the mouse node? It has been postulated that a leftward "nodal flow" driven by nodal cilia (possibly carrying some extracellular inducing factor), is the event that breaks the initial symmetry in the mouse embryo (and most likely also in other mammals), resulting in asymmetric expression of *Nodal* in and around the node [17]. The existence of this putative inducing factor carried by the nodal flow has not been demonstrated yet, but it is clear from the results indicated above that HH signaling in the node is required for the correct regionalization of *Nodal* expression in the mouse node. The reason why *Nodal* is transiently upregulated specifically on the left side of the mouse node is still unknown, and it is also important to point out that it is still unclear whether nodal cilia control the L/R axis in the chick embryo [18].

In conclusion, although the mechanisms that break the initial symmetry may be very different in chick and mouse embryos, the basic role of HH signaling as a left determinant appears to be conserved during evolution, as is the left-specific expression of the *Nodal* gene. Without a doubt, the characterization of additional components of HH signaling pathways and of their roles in the early vertebrate embryo will still provide very valuable information about the mechanisms that control L/R determination during early embryogenesis.

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CHAPTER 255

EGF-Receptor Signaling in *Caenorhabditis elegans* Vulval Development

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A crucial aspect of *Caenorhabditis elegans* vulval development is induction of the vulval precursor cells by the anchor cell of the developing uterus. Precisely three of the six vulval precursor cells are induced by the anchor cell to generate vulval cells. As we shall discuss, this induction uses an epidermal growth factor (EGF)-receptor signaling pathway involving the *C. elegans* EGF-receptor homolog LET-23. There are a number of other cell-signaling events during vulval development including at least three uses of WNT [17,19,48,61], and the LIN-12 (Notch-type receptor) pathway. The LET-23 pathway is also used for a reciprocal induction by which a subset of the vulval cells signal back to the developing uterus [9]. Because vulval induction is relatively easy to observe, it can be used to study EGF-receptor signaling *in vivo*.

The Core LET-23 Signaling Pathway

Proteins necessary for LET-23 signaling during vulval induction were found by analyzing mutants with defective (vulvaless) or excessive (multivulva) vulval induction, or by suppressor mutations altering other pathway components. The order of action of these proteins was inferred from analysis of double mutant combinations, in the presence or absence of the inducing cell. The double mutant experiments relied on use of overexpressed EGF, activated EGF-receptor LET-23, or activated RAS. For example, a gain-of-function mutant version of LET-23 causes vulval development in the absence of the anchor cell. *lin-3* encodes multiple products sharing an EGF domain and a transmembrane domain, but differing in their amino termini and spacing of the EGF and transmembrane domain [27,40]. *let-23* encodes the *C. elegans* EGF receptor subfamily ortholog [1]. *sem-5* encodes a GRB2 ortholog with an SH2 domain and two SH3 domains [14]. *let-341* encodes an SOS ortholog, a Ras guanine nucleotide exchange factor [10,15]. *let-60* encodes RAS [23]. *lin-45* encodes RAF [24]. *mek-2* encodes MAP kinase kinase [35,36] and *mpk-1/sur-1* encodes MAP kinase [37,63]. The genetic data are consistent with a linear pathway from LET-23 to MAP kinase: LET-23 acts downstream or in parallel to LIN-3; SEM-5 acts downstream or in parallel to LET-23; LET-60 acts downstream or in parallel to SEM-5; LIN-45 acts downstream or in parallel to LET-60, etc. Downstream of MAP kinase the pathway is more complicated, with a number of positive- and negative-acting nuclear factors. The LIN-1 ETS-domain protein and the winged helix protein LIN-31 primarily act to inhibit vulval development, but direct targets are not yet known [2,46,58]. LIN-1 and LIN-31 can dimerize and affect each other's activity. Positive-acting transcription factors include LIN-25 and SUR-2 [54,60], which appear to act together. SUR-2 is homologous to a component of a human mediator complex [4], which in mammals and yeast links site-specific DNA binding proteins to RNA polymerase. The hox gene *lin-39* is a positive-acting nuclear factor, while the hox gene *mab-5* acts negatively on the two posterior vulval precursor cells [12,43].

Tissue Specificity

Analysis of the tyrosines in the carboxyl terminal, cytoplasmic domain of LET-23 revealed distinct positive functions in activation of the RAS pathway activity involved in cell fate specification and an inositol 1,4,5 trisphosphate (IP_3)

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pathway involved in ovulation [6,13,39]. The three tyrosines sufficient for RAS pathway have the YXN consensus for GRB2 (SEM-5) binding, and a single tyrosine site can act via SEM-5 in the absence of the other two. By contrast, yet another tyrosine site stimulates the ovulation pathway not via RAS. The coupling of LET-23 to IP_3 is predicted to involve a phospholipase.

A number of cell fate choices during *C. elegans* development also depend on LET-23. For several of these choices, the outcome may depend on the particular hox gene the cells express. The vulval cells express *lin-39*, the male hook precursor cells express *mab-5,* and the P12 cell expresses *egl-5* [32,44,49].

Positive and Negative Regulators

Besides the core signaling components, additional positive and negative regulators of vulva development have been identified. Elimination of LIN-2, LIN-7, or LIN-10 results in a partial vulvaless phenotype identical to elimination of the carboxyl six amino acids of LET-23. The inferred signaling defect correlates with a shift in LET-23 localization from the basolateral to apical surface of vulval precursor cells, which is the side furthest away from the anchor cell [35,53]. Overexpression of LET-23 rescues the vulvaless phenotypes indicating that these genes facilitate signaling by concentrating LET-23 close to LIN-3 [53]. LIN-2, LIN-7, and LIN-10 all contain PDZ domains [29,53,62]. *In vitro* experiments indicate that the PDZ domain of LIN-7 binds the cytoplasmic tail of LET-23 [53], and that LIN-2 promotes ternary complex formation with LIN-7 and LIN-10 [34]. A truncated form of LIN-10 that lacks both PDZ domains has a relatively weak vulval phenotype indicating that at least for LET-23 signaling, LIN-10 PDZ domains are not crucial [62].

Genetic screens for mutations that suppress the multivulva phenotype conferred by a gain-of-function *let-60* allele *(n1046gf)* identified a number of modulators of vulval development. On their own, mutations in *ksr-1* [36,57], *sur-8/soc-2* [49,51], and *sur-6* [52] result in no obvious vulval phenotype. However, they cause *let-60(n1046gf)* animals to become wild-type, and produce highly penetrant vulvaless phenotypes in the presence of weak reduction of function mutations in core components such as *mpk-1* and *lin-45*. *ksr-1* encodes a predicted Ser/Thr kinase related to RAF, but lacks the CR1 and CR2 domains. *ksr-1* likely functions upstream of *lin-1*, and *in vitro* studies suggest that it may interact directly with MPK-1/SUR-1 through an FXFP site during vulval development [31]. Although some *ksr-1* alleles harbor missense mutations in the kinase domain, transgenes mutant in the ATP binding site or the catalytic aspartate have the same rescuing activity as wild-type DNA, indicating kinase activity is not crucial for its function [56]. *sur-8* encodes a protein consisting of 18 tandem Leu-rich repeats similar to yeast adenylate cyclases. *sur-8* mutations do not reduce the penetrance of the multivulva phenotype conferred by a gain-of-function LIN-45 RAF transgene, suggesting it works upstream of RAF activation. SUR-8 interacts with a different part of the LET-60 effector domain then LIN-45 RAF, and not all *sur-8* mutations affect this interaction [51]. *sur-6* encodes a regulatory β subunit of protein phosphatase 2A(PP2A). *sur-6* mutations do not suppress the multivulva phenotype conferred by a gain-of-function LIN-45 RAF transgene, suggesting it also works upstream of RAF activation. *sur-6* mutations produce a strong synthetic vulvaless phenotype with *sur-8*, but not with *ksr-1*, raising the possibility that SUR-6 function is intimately connected with KSR-1. dsRNA interference experiments against the A and C PP2A catalytic core components, also inhibit the multivulva phenotype of *let-60(n1046gf)* [51], suggesting that SUR-6 is a positive regulator of PP2A, and that the phosphatase activity of the complex promotes RAS-dependent vulva development.

Deletion of the SH2-domain containing protein tyrosine phosphatase, *ptp-2*, which is most closely related to SHP-2/corkscrew [21] does not perturb vulval development on its own, but reduces the multivulva phenotypes conferred by mutations in *lin-15* and activated alleles of *let-23* and *let-60*. In addition, a *ptp-2* mutation results in a synthetic vulvaless phenotype in the presence of a weak *sem-5* mutation.

Loci involved in negative regulation of vulva induction have been elucidated through three different approaches. Genetic screens for mutations, which suppress reduction-of-function mutations in the *let-23* pathway, led to the identification of *unc-101* [38], *sli-1* [33,65] *gap-1* [20], and *sur-5* [22]. UNC-101 is a functional homolog of the AP47 medium chain of the trans-Golgi clathrin-associated AP-1 complex. dsRNAi experiments indicate it works redundantly with a second AP47 homolog, *apm-1* [50]. An *sli-1* mutation suppresses a severe non-null allele of *let-23*, but not severe non-null alleles of *let-60*, suggesting it works upstream of RAS [66]. SLI-1 is related to the CBL family of adaptors/E3 ubiquitin ligases. Although both the PTB and RING finger domains are required for full inhibitory activity, SLI-1 displays some activity in the absence of the RING finger, indicating it may have some function independent of E3 ubiquitin ligase activity [66]. Recently, a tyrosine in the C-terminal tail of LET-23, in the sequence NSSRYKETP, was shown to be required for the genetic inhibition of *sli-1* on *sem-5*-dependent vulval induction [66], similar to a ZAP-70 binding site for the CBL PTB domain [42,45], and consistent with a model of direct binding of SLI-1 to LET-23. *gap-1* mutations suppress reduction-of-function mutations in *let-60*, but not *lin-45*, and its encoded protein is most similar to the Gap-1 and Gap-1m RAS GTPase activating proteins in *Drosophila* and vertebrates, respectively, strongly suggesting that it is a direct regulator of LET-60. *sur-5* was isolated as a suppressor of a dominant negative *let-60* allele. Unlike the previously described negative regulators, it has the unusual property of not being able to suppress any of the standard reduction-of-function mutations in the core components, including a weak allele of *let-23*. Moreover, it only suppresses a subset of dominant negative *let-60* alleles. SUR-5 has some identity to acetyl-CoA synthetases, but its mechanism of action is a mystery. The *cdf-1* gene also was identified as a suppressor of activated RAS [5]. Zinc ions inhibit RAS signaling in *C. elegans*, and the cation

diffusion facilitator CDF-1, which lowers cytoplasmic zinc concentration, is thus a positive modulator of the pathway [5].

Genetic screens in sensitized, but phenotypically wild-type backgrounds have identified other negative regulators of vulva induction. Ferguson and Horvitz [18] first defined this approach by isolating mutations in two classes of loci, A and B, wherein any class A mutation caused a synthetic multivulva (synmuv) phenotype in combination with any class B mutation. Using class A or B sensitized backgrounds, a number of additional class A and B mutations have been isolated. *lin-15A* [16,30], *lin-15B* [16,30], and *lin-36* [59] encode novel proteins. LIN-35 is related to the retinoblastoma family of proteins [41], *lin-53* encodes the Rb-binding protein, RbAp48 [41], and DPL-1 is related to the DP family of transcription factors [8]. Reverse genetics indicate that *efl-1/2*, which are E2F-like genes, also have synmuv properties [8]. Genetic [8,30,41,59] and cell ablation [30,55] experiments indicate that the synmuv phenotype is strongly dependent on LET-23 and its downstream signaling components, but not LIN-3. Expression, transgenic, and mosaic analyses suggest that both the vulval precursor cells and the surrounding epidermal syncytium might be a source for an inhibitory signal by these genes [8,25,26,41,59].

Similar to the synmuv screens, *ark-1* was recovered as a mutation that caused a synthetic multivulva phenotype in the presence of a *sli-1* mutation [28]. An *ark-1* mutation also suppresses non-null mutations in the *let-23* pathway. *ark-1* encodes a putative tyrosine kinase most similar to Ack, and yeast two hybrid studies indicate that a C-terminal, proline-rich domain can bind to SEM-5, suggesting its function may be intimately connected to SEM-5. In addition, both SEM-5 and ARK-1 have an inhibitory effect on LET-23 signaling during ovulation, suggesting that SEM-5 might recruit ARK-1 to inhibit LET-23.

A loss-of-function mutation in *lip-1*, a gene predicted to encode a MAPK phosphatase, was generated based on its genomic sequence [3]. This mutation suppresses vulvaless defects conferred by loss-of-function mutations in the *let-23* pathway. LIP-1 transcription is induced by LIN-12 (Notch) signaling [3], and thus might be one way in which Notch signaling is antagonistic to LET-23 signaling [55].

Prospects

One key issue includes the identification of direct targets for the transcriptional response to the MAP kinase pathway. The *egl-17* gene, which encodes a fibroblast growth factor necessary for muscle precursor positioning, is induced by the LET-23 pathway [7], and is one candidate for an immediate target. The hox gene *lin-39* is upregulated by LET-23 signaling [43], and is another candidate. A long list of other candidates has been generated by DNA expression microarray experiments [47]. With the continued discovery of regulators and modulators of this core LET-23 pathway, studies in *C. elegans* will help place these many proteins into their physiological context, including other extracellular signals, tissue-specific effects, and environmental modulation.

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CHAPTER 256

Induction and Lateral Specification Mediated by LIN-12/Notch Proteins

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In this chapter, we describe general and conserved features of the LIN-12/Notch signal transduction pathway. We also describe features of two different binary cell fate decisions mediated by this pathway in *Caenorhabditis elegans*. Finally, we discuss whether the primary role of Notch is to mediate binary decisions between alternative cell fates or to block differentiation.

The LIN-12/Notch Pathway

Receptors of the LIN-12/Notch family are evolutionarily conserved single pass transmembrane proteins. A single *Notch* gene exists in *Drosophila*, whereas two, *lin-12 and glp-1*, have been described in *C. elegans* and four, *Notch-1* to *Notch–4*, in vertebrates (Table I). The ligands for LIN-12/Notch are single pass transmembrane proteins of the DSL family, after canonical proteins from *Drosophila* (Delta, Serrate) and *C. elegans* (Lag-2).

Genetic and biochemical experiments on invertebrates, mainly *C. elegans* and *Drosophila melanogaster*, and more recently on vertebrates, have defined members of the LIN-12/Notch signal transduction pathway (Table I), as well as an original model for signal transduction (Fig. 1). LIN-12/Notch proteins in vertebrates appear to be cleaved during their transit to the cell surface by the protease furin at site 1 (see Fig. 1). Upon activation of LIN-12/Notch by a DSL ligand, the receptor is cleaved both in its extracellular domain (site 2) and within its transmembrane domain (site 3), to produce an active intracellular fragment which translocates to the nucleus. The site 2 cleavage is likely to be mediated by a transmembrane metalloprotease of the ADAM family (see Table I). The site 3 cleavage requires a complex that contains the membrane proteins Presenilin, APH-1, Nicastrin and PEN-2 (reviewed in references [1,2]).

In the nucleus, the intracellular part of LIN-12/Notch associates with a sequence-specific DNA binding protein (known as LAG-1 in *C. elegans*, Su(H) in *Drosophila*, and RBP-J or CBF1 in mammals [3]), with a glutamine-rich protein (SEL-8, for example, see Table I [4,5]), and with another protein called SKIP [6]. This complex, which is likely to include other proteins as well, binds to promoters of target genes, activating their transcription. In addition, the activation complex is believed to contribute to target gene activation by displacing a co-repressor complex previously associated with CBF1 [7].

A number of negative modulators of LIN-12/Notch signaling have been described, which influence the ability of a ligand to signal (Fringe, [8]), the ability of the receptor to transmit the signal (Numb, [9]), or the stability of the receptor (Sel-10, [10]). Crosstalk between the LIN-12/Notch signaling pathway and others, such as the frizzled pathway in *Drosophila* [11] or the Ras pathway in worms [12], has also been described.

Cell-Cell Interactions Mediated by the LIN-12/Notch Pathway

When equivalent cells interact with each other, that process has classically been termed "lateral inhibition," or more recently, "lateral specification" (see the following section). When the signal comes from a different cell type, that process

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Table I Members of the LIN-12/Notch Pathway in *C. elegans*, Drosophila, and Mammals

Role	*C. elegans*	Drosophila	Mammals
Ligands (DSL family)	LAG-2, APX-1, others	Delta Serrate	Delta 1–4 Jagged 1–2
Receptors	LIN-12, GLP-1	Notch	Notch 1–4
Site 1 cleavage	(Furin)	(Furin)	Furin
Site 2 cleavage	SUP-17	Kuzbanian	TACE/ADAM-17
Site 3 cleavage	SEL-12, HOP-1 APH-1 APH-2 PEN-2	Presenilin Aph-1 Nicastrin PEN-2	Presenilin Aph-1 Nicastrin PEN-2
Modulators	(NUMB) – SEL-10	Numb Fringe (Sel-10)	(Numb) Fringe Sel-10
Nuclear complex	LAG-1 SEL-8 (SKIP)	Su(H) Mastermind (Skip)	RBP-J/CBF1 Mastermind SKIP
Selected targets	– *lip-1* *lin-12*	*E(spl)* complex – *(Notch)*	*HES-1,5* – *Notch-1*

Proteins drawn on the same line are equivalents in the three groups shown. Proteins shown in parentheses, e.g. (furin), exist in the organism, but have not been proven to function in that step. Fringe and genes of the E(spl) complex do not have clear orthologs in *C. elegans*; it is not known whether *lip-1* orthologs exist or are targets of the LIN-12/Notch pathway in other organisms. Note that SUP-17, Kuzbanian and TACE are metalloproteases belonging to the ADAM family.

has classically been termed "induction." LIN-12/Notch signaling has been shown to underlie both kinds of interactions in many different systems. The outcome is a binary decision, so that bipotential cells choose between one of two alternative fates depending on whether LIN-12/Notch activity is high or low. We will illustrate the basic principles here using two simple cell fate decisions from *C. elegans*.

Lateral Specification in the AC/VU Decision: A Decision between Two Equivalent Cells

During formation of the somatic gonad of the hermaphrodite, two cells, named Z1.ppp and Z4.aaa, have an equal probability to become either the anchor cell (AC) or a ventral uterine precursor cell (VU; Fig. 2A). In wild-type hermaphrodites, Z1.ppp and Z4.aaa interact with each other, so that only one of the two cells becomes the AC while the other becomes a VU [13,14]. Cell contact appears to be necessary for the interaction to occur correctly, because in mutants that disrupt contact between Z1.ppp and Z4.aaa, both cells become ACs [15].

Genetic studies of mutations in *lin-12* and other members of the LIN-12/Notch pathway have established that in Z1.ppp or Z4.aaa, a low level of *lin-12* activity results in the AC fate, whereas a high level results in the VU fate. Manipulation of the relative level of *lin-12* activity in genetic mosaics [14] and the detailed analysis of *lin-12* and its ligand *lag-2* transcriptional reporter gene expression during the course of the AC/VU decision [16] has revealed that *lin-12* and *lag-2* are initially expressed at comparable levels in Z1.ppp and Z4.aaa. A stochastic initial difference in *lin-12* or *lag-2* activity is further amplified by a feedback mechanism involving positive and negative autoregulatory loops. As a result, there is an increase of *lin-12* expression and a decrease in *lag-2* expression in one cell, and the reciprocal situation (decrease in *lin-12*, increase in *lag-2*) in the other. The cell in which *lin-12* activity is high becomes a VU and the other, in which *lin-12* activity is low, becomes an AC [14,16].

Lateral specification that includes transcription-based feedback loops also appears to operate in cell fate decisions in *Drosophila* and probably in vertebrates [17,18]. We prefer the term lateral specification to lateral inhibition to emphasize the bidirectional nature of the communication (see also reference [19]). Such simple circuits can also be modified by either intrinsic factors or external signaling events so that the element of randomness seen in the AC/VU decision is reduced or eliminated (see, for example, references [9] and [11]).

Inductive Signaling: Germline Proliferation and Meiotic Differentiation in *C. elegans*

The *C. elegans* gonad is organized as two symmetrical U-shaped arms extending distally from a common uterus (Fig. 2B). The distal-most germ cells divide mitotically, and

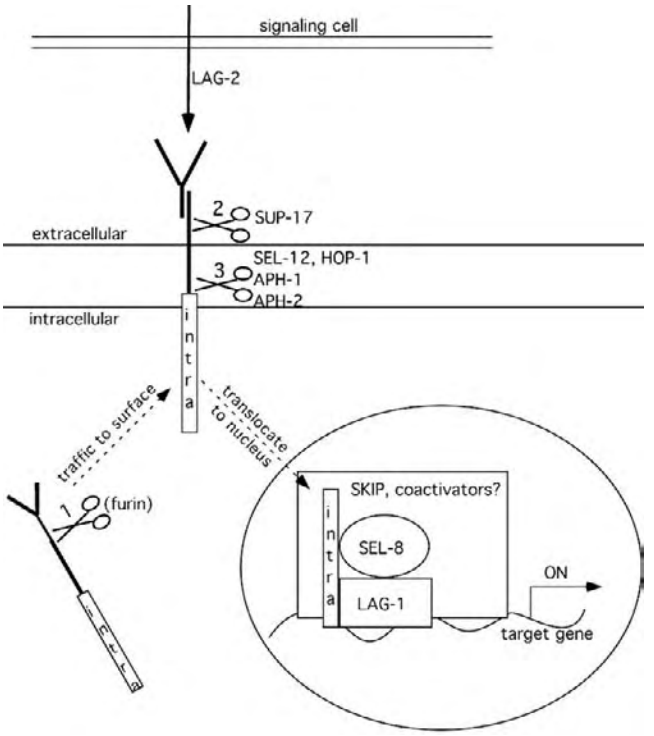


Figure 1 Simplified model of LIN-12/Notch signal transduction. The *C. elegans* names for components are used; see Table I for *Drosophila* and mammalian equivalents. A DSL ligand, such as LAG-2, on the surface of a neighboring cell activates LIN-12/Notch, triggering cleavage in the extracellular domain (site 2) and within its transmembrane domain (site 3). The released intracellular domain translocates to the nucleus, where in a complex with LAG-1, and probably SEL-8, SKIP, and other co-activators, it participates in the transcriptional activation of target genes. The protease furin has been shown to cleave LIN-12/Notch at site 1 during its transit to the surface in mammals [30]; however, this cleavage has not been addressed experimentally in *C. elegans* nor in *Drosophila*.

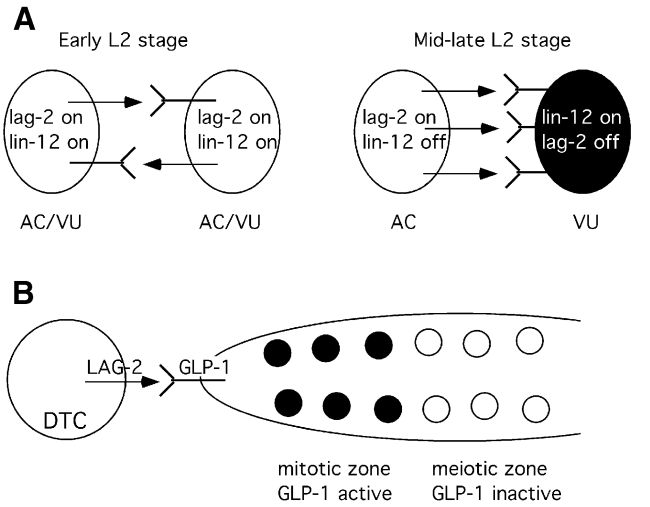


Figure 2 Lateral specification and inductive signaling events mediated by LIN-12/Notch. (A) Lateral specification, the AC/VU decision: Two equivalent cells each have the potential to adopt the AC or VU fates (AC/VU) in the early L2 stage. As the L2 stage progresses, a small variation in ligand or receptor activity is amplified by transcription-based feedback loops, so that activation of LIN-12 in one cell promotes *lin-12* expression and represses (or fails to maintain) *lag-2* expression. Failure to activate LIN-12 has the opposite effect. The presumptive AC is the cell that continues to express *lag-2*, and the presumptive VU is the cell that continues to express *lin-12*. (B) Induction, the mitosis/meiosis decision: The distal tip cell (DTC) of the somatic gonad expresses LAG-2, which activates GLP-1 in the distal portion of the germline syncytium. Distal germline nuclei therefore undergo mitosis, although as they move more proximally, they enter a zone in which GLP-1 is inactive and they begin to enter meiosis.

progress down the arm; at the bend, they enter a transition zone and proceed to meiosis. At the extremity of each arm, there is a somatic distal tip cell (DTC) that regulates the decision of the germ cells between mitosis and meiosis: If the DTC is ablated, the distal germ cell nuclei in that gonad arm enter meiosis precociously [20].

Genetic studies of *glp-1* have established that a low level of *glp-1* activity is required for germ cells to undergo meiosis, whereas high levels of *glp-1* activity promote mitosis [21–23]. The DTCs express the DSL gene *lag-2*, and the distal germ cells express the LIN-12/Notch protein GLP-1 [24,25]. Activation of GLP-1 in the germ cells that lie near the DTC causes them to remain proliferative.

The Role of LIN-12/Notch Proteins: Suppression of Differentiation versus Specification of Binary Cell Fate Decisions

Truncated forms of LIN-12/Notch proteins, such as the intracellular domain, mimic the natural cleavage product and behave like constitutively active receptors [26]. This approach has been important for studying the role and the molecular mechanism of the Notch pathway in vertebrates.

The idea that the role of Notch is to block differentiation was first proposed when activated Notch was thought to inhibit photoreceptor differentiation in *Drosophila* [27]. Furthermore, this view was reinforced by studies of proneural clusters in *Drosophila*, which showed that expression of the achaete-scute neural competence factors are lost in cells in which Notch has been activated [28]. The view that Notch activation inhibits differentiation in *Drosophila* strongly influenced the interpretation of phenotypes caused by expressing the Notch intracellular domain in vertebrates. However, recent work has established that Notch does not block differentiation of photoreceptors, and indeed, plays a direct role in photoreceptor differentiation (for example, see reference [29]). Thus, photoreceptor differentiation in the *Drosophila* eye, like the *C. elegans* examples illustrated above, suggest that activated Notch promotes a fate that is a normal outcome of a binary decision.

These considerations suggest that, while it may be that in some cases the effect of activated Notch is to "block differentiation" there may perhaps be an underlying binary cell fate decision; to differentiate or to remain a stem cell. Indeed, the continued mitotic proliferation of the germline when GLP-1 is constitutively activated in *C. elegans* may be considered a model for this kind of binary decision.

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CHAPTER 257

Notch Signaling in Vertebrate Development

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Introduction

The Notch signaling pathway was discovered in *Caenorhabditis elegans* and *Drosophila* by studying gene mutations that cause equivalent embryonic phenotypes, many of which entail alterations in cell fate [1]. Subsequently, homology cDNA cloning identified the vertebrate counterparts of these genes, paving the way for the discovery that Notch also plays a prominent role in the development of just about every organ system in the vertebrate embryo [2]. The first part of this chapter will describe the structural features of the receptors, ligands, and accessory proteins that mediate Notch signaling in vertebrates. The second part will describe how Notch signaling is used, in many cases in a similar manner, to regulate various aspects of cell fate determination and tissue patterning during vertebrate development.

Components Mediating Vertebrate Notch Signaling

Vertebrate Notch Receptors

The Notch receptors comprise a relatively small family of type I transmembrane proteins with just one member in *Drosophila*, two in *C. elegans*, and four in vertebrates [1]. As illustrated in Fig. 1A, the extracellular domains (ECN) of these receptors share a number of structural similarities, including multiple, tandem copies of a epidermal growth factor (EGF)-like motif that comprises most of the ectodomain as well as tandem copies of a second motif called the Notch/lin-12 repeat. The intracellular domain (NICD) contains a conserved juxtamembrane region called RAM23, six tandem ankyrin repeats (ANK), and a PEST (P) domain located at the carboxy terminus. The different receptors presumably arose during vertebrate evolution by several gene duplications starting from a receptor very similar to *Drosophila* Notch. Thus, mouse Notch1 and 2 contain the same number of EGF repeats as *Drosophila* (36) while Notch3 and Notch4 contain 34 and 29 repeats, respectively. The NICD of both Notch 3 and 4 is also shorter than that of Notch1 and 2. In addition, as expected for genes recently duplicated, the four Notch homologs show some degree of genetic redundancy based on the phenotypes produced by targeted mutations [3]. Nonetheless, the "knockout" phenotype of mouse Notch1 is more severe than that of the other Notch homologs, suggesting that the latter receptors have evolved to serve more specialized functions [4–6]. These specialized functions may have arisen by changes in their developmental expression pattern (for example, see [7,8]), in their preference for activation by the different ligands [9], or in the way that they signal. An example of the latter is Notch3, which may activate downstream targets differently than the other Notch receptors under at least some assay conditions [10].

The vertebrate Notch receptors were also identified as genes linked with several disease states. In certain forms of T-cell leukemia, a chromosomal translocation fuses the intracellular domain of the human Notch1 receptor to the extracellular domain of the β chain of the T-cell receptor [11]. Similarly, in MMTV-induced mammary tumors in the mouse, one of the viral integration sites (INT3) leads to expression of just the Notch4 intracellular domain [12]. Based on the known Notch signaling pathway (see the following section), these observations suggest that constitutive

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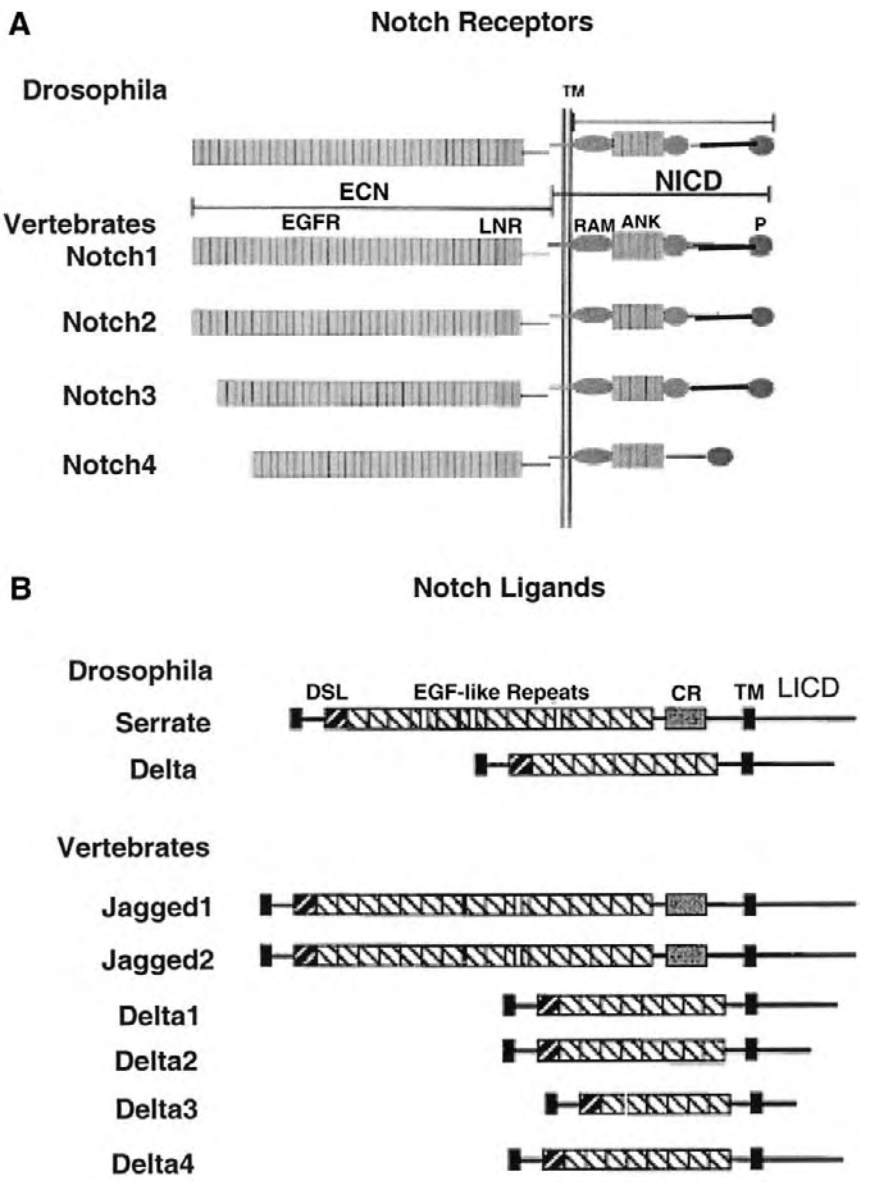


Figure 1 Diagram illustrating the structure of the Notch receptors (A) and ligands (B) in vertebrates relative to their *Drosophila* counterparts. See text for details.

Notch signaling contributes to tumor progression. Finally, an autosomal dominant disease resulting in stroke and dementia, called CADASIL, is associated with missense mutations in the human Notch3 receptor [13], although the consequences of these mutations on receptor activity are not fully known.

Vertebrate Notch Ligands

Ligands for the vertebrate Notch receptors are type I transmembrane proteins that are closely related in structure and sequence to the two ligands, Delta and Serrate, which bind and activate *Drosophila* Notch (Fig. 1B) [14]. The extracellular domains of these ligands are primarily made up of EGF-like repeats like the receptors but are distinguished by a signature divergent EGF-like repeat located at the amino terminus called the DSL domain (Delta, Serrate, and Lag-1). While the Delta-like ligands contain from 6–9 EGF-like repeats, the Serrate-like ligands (referred to mainly as the Jagged ligands) have 16 repeats, as well as a unique cysteine-rich (CR) domain located between the EGF repeats and the transmembrane domain. All vertebrate species have a Delta-like ligand, called Delta1, which seems to be the most closely related to *Drosophila* Delta in terms of structure and function. The other Delta-like ligands in vertebrates, such as Delta3 [15] and Delta4 [16] in the mouse or Delta2 in *Xenopus* [17], are either much more divergent in structure (Delta3), or more restricted in their expression pattern during development (Delta2 and 4). Vertebrates have at least two Serrate-like ligands (Jagged1 and Jagged2) [18,19] that contribute to the development of a number of tissues based on knockout phenotypes in the mouse [20,21]. Like the

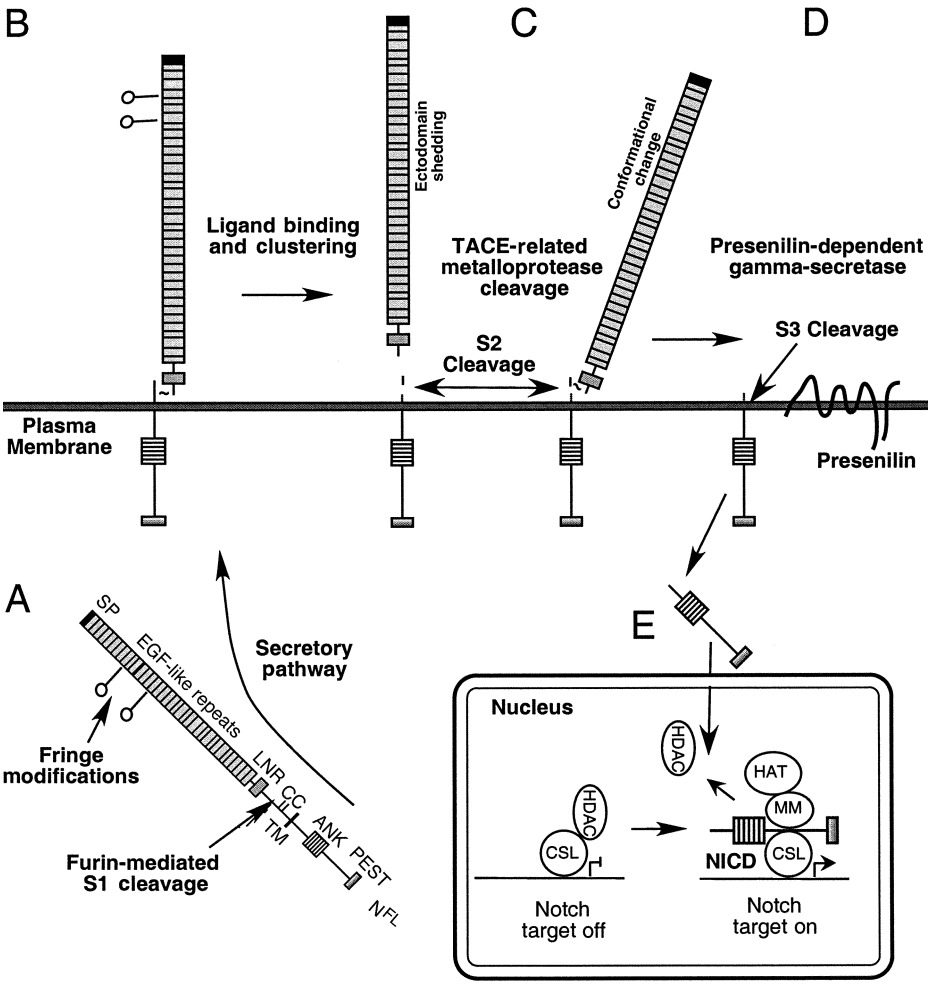


Figure 2 Signal transduction in the Notch pathway. (A) Receptors are modified during secretion by addition of sugar residues by the Fringe proteins as well as by a Furin cleavage that produces a heterodimeric receptor linked by metal bridge. (B) Ligand binding leads to receptor clustering which exposes an S2 cleavage site either by ectodomain shedding or a conformational change (C). Following S2 cleavage, the remaining receptor in the membrane is cleaved at S3 by a presenilin-dependent protease activity. (E) This cleavage releases the intracellular domain that then moves to the nucleus and associates with the CSL proteins to modulate transcription. In the absence of NICD, the CSL proteins repress transcription by associating with co-repressors with histone deacetylase (HDAC) activity. Binding of NICD to the CSL proteins displaces the co-repressor complexes and recruits co-activators including the mastermind (MM) proteins, which in turn recruit proteins such as p300 and CREB binding protein (CBP) with histone acetyltransferase (HAT) activity.

receptors, mutations in the ligands are associated with several disease states. Haploinsufficiency in the *Jagged1* gene is tightly associated with Alagille's syndrome; a human autosomal dominant, developmental disorder characterized by liver, heart, eye, skeletal, craniofacial, and kidney abnormalities [13]. A mutant *Delta3* gene is tightly linked with a skeletal abnormality spondylocostal dysplasia in humans, [22] and is responsible for the skeletal defects seen in the pudgy mouse [23].

Vertebrate Notch Signal Transduction

The mechanism used in vertebrates to transduce a Notch signal is largely the same as that used in *Drosophila* and *C. elegans*, and is one where the NICD is released from the plasma membrane, moves to the nucleus, and serves as a dedicated transcriptional co-activator for the CSL proteins (Fig. 2) [24–28]. CSL is the name given to essentially the same DNA binding protein found in different species (CBF1, vertebrates; SuH, *Drosophila*; Lag 2, *C elegans*), and this particular form of Notch signaling is termed the CSL-dependent pathway. Evidence for a CSL-independent form of Notch signaling exists in vertebrates [29], but the nature of this pathway remains poorly understood. In *Drosophila*, several examples of CSL-independent signaling by Notch have been described including one that is transduced by a cytoplasmic RING domain protein, called Deltex, [30,31], a second involving the abl tyrosine kinase during axon guidance [32], and a third involving the c-Jun N-terminal kinase (JNK) during dorsal closure [33].

Although the components involved in these pathways are found in vertebrates, including vertebrate homologs of Deltex [34–36], their contribution to Notch signaling during vertebrate development remains largely unexplored. These alternative pathways may gain further prominence as Notch signaling is studied in cases other than those involving cell fate choices, such as the reported affects of Notch signaling on the remodeling of neuronal dendrites [37,38].

The idea that NICD moves between the membrane and the nucleus was at first controversial [39] but became more generally accepted with the discovery of other dual-address transcription factors such as SREBP, which are activated by regulated intramembranous proteolysis (RIP) [40]. In the case of Notch, a key step in RIP (Fig. 2D) is carried out by a presenilin-dependent gamma-secretase activity that is also known for its role in processing the beta amyloid protein precursor (βAPP) into soluble peptides that potentially contribute to Alzheimer's disease [41,42]. Mutations in the presenilin homologs in *Drosophila* and *C elegans* disrupt Notch signaling, and subsequent biochemical experiments indicated that this activity cleaves the Notch receptor within the transmembrane domain at a site referred to as S3 [43]. By analogy with βAPP, the S3 cleavage of Notch is thought to occur constitutively upon the formation of a suitable substrate generated by other proteolytic processing events [44]. In the case of Notch, the substrate for S3 cleavage can be generated by cleaving the receptor by a TACE-related metalloprotease at a second site (S2), which lies extracellularly between the transmembrane domain and the conserved paired-cysteines (Fig. 2C) [45–47]. Thus the current model suggests that activation of the receptor induces changes that permit cleavage at S2, resulting in a substrate that is constitutively processed at S3, thereby releasing NICD from the membrane.

How ligand activation regulates cleavage of the receptor at S2 is not fully known, but one insight into this issue comes from the observation that forms of the receptor lacking an extracellular domain are constitutively processed at both S2 and S3 [45]. In the absence of ligand, the S2 cleavage site of the full-length receptor may be masked by interactions with another region in the extracellular domain, most likely the conserved Lin12/Notch repeats. The S2 cleavage site might be exposed during ligand binding through conformational changes that disrupt this inhibitory domain (Fig. 2B). Alternatively, for reasons discussed next, ligand binding might expose the S2 cleavage site by promoting the shedding of the receptor ectodomain (Fig. 2B). The Notch receptors could easily undergo ectodomain shedding because they reach the cell surface as heterodimers that are produced during secretion by cleavage with a Furin-like protease at a site called S1 (Fig. 2A) [48]. Disrupting the metal bridge that holds this heterodimer together exposes the S2 cleavage site [49], but whether or not this is ligand regulated is unknown. Regardless of whether the S2 cleavage is promoted by a conformational change or by ectodomain shedding, the proteolytic processing of the receptor is apparently inefficient, producing only small amounts of NICD that are difficult to detect biochemically, even though they are effective physiologically [50].

Upon release from the membrane, NICD associates with the CSL proteins, thereby converting these DNA binding proteins from transcriptional repressors into transcriptional activators (Fig. 2E) [27]. A prominent group of genes that are upregulated directly by Notch signaling encode basic helix-loop-helix (bHLH) transcriptional repressors [51], such as the HES and HRT genes in mouse [5,24,52], the HER/HRT genes in zebra fish [53,54], and the ESR genes in *Xenopus* [55]. Activation of these genes is dependent on tissue type and developmental context, indicating that Notch transcription in most cases acts in combination with additional transcriptional cofactors [56]. Additional complexity is likely to come from various proteins that are recruited by the Notch transcription complex that both positively and negatively regulate its activity. Further description of the mechanisms by which Notch regulates transcription of these genes via the CSL proteins are reviewed in more detail in Chapter 294.

Regulation of the Notch Ligands

One important feature of the Notch signaling pathway is that all of the known ligands presumably activate receptors on neighboring cells as membrane-bound proteins. Indeed, when secreted forms of the Notch ligands are engineered and expressed *in vivo*, they inhibit Notch signaling [57,58], suggesting that they bind but do not activate the Notch receptors. In cultured cell experiments, soluble ectodomains of the vertebrate ligands are much less effective at activating Notch signaling than membrane-bound ligands expressed on cells and used in co-cultivation assays [19]. Significantly, among the effective soluble Notch ligands are those engineered by fusing the ligand extracellular domain to the FC portion of antibodies, known as ligand-bodies. Ligand bodies activate Notch signaling but are the most effective when crosslinked extensively using FC antibodies or immobilized on plastic surfaces [59,60]. As a practical matter, these reagents have proved to be important tools for activating Notch signaling in many experimental systems [61]. Moreover their properties suggest that a simple monomeric interaction between the Notch ligands and receptors is not sufficient for receptor activation, but that clustering of the normally membrane-bound ligands may be required for signaling [62].

Even though the intracellular domains of the vertebrate Notch ligands (LICD) are not highly conserved in primary sequence, ligands lacking this domain are also potent dominant-negative inhibitors of Notch signaling [63,64]. Recent evidence suggests that one function of the LICD is to facilitate interactions between the ligands and the intracellular machinery that promote endocytosis into endosomes and perhaps other intracellular events that traffic ligands back to the cell surface or to destruction [65]. These trafficking events are regulated by ubiquitination and at least one E3 ubiquitin ligase, called neuralized, has been shown to promote the endocytosis of Notch ligands [66–68]. The function of ligand trafficking, and thus the LICD, in Notch signaling

may be threefold. First, ligand endocytosis may be one means of promoting ligand clustering on the cell surface and thus receptor activation as described above [62]. Second, ligand trafficking could be used to produce asymmetric levels of ligand among populations of cells in cases where Notch signaling regulates cell fate by the process of lateral inhibition (see the following section). Finally, ligands lacking an intracellular domain may accumulate on the cell surface, resulting in a block of Notch signaling, presumably because ligand, at high levels, sequesters the Notch receptor into nonproductive cis-complexes [69]. Thus, ligand trafficking may be needed for the ligand to function in signal-emitting cells, as well as to keep ligand levels in a range that permits the Notch receptor to be activated in signal-receiving cells.

The ability of the ligands to bind and activate the Notch receptors is also regulated by a mechanism first found in *Drosophila* through the analysis of the *fringe* gene [70]. *Fringe* encodes a 1,3 *N*-acetylglucosamine transferase [71,72] that modifies an *O*-fucose linkage on a subset of the EGF-like repeats of Notch, thus enhancing activation of the receptor by Delta but inhibiting activation by Serrate [73]. The vertebrate genome contains three fringe homologs [74] that clearly interact at least in some cases with the Notch pathway [75–77]. Experiments in cultured cells have shown that mammalian lunatic fringe potentiates the ability of mammalian Notch1 to respond to signaling by Delta1 but inhibits the response to Jagged1 [9,78]. Significantly the change in ligand activation produced by lunatic fringe is not at the level of ligand binding, but rather at the level of receptor activation. One area where the fringes regulate Notch activity in vertebrates is during segmentation where they modulate signaling in a periodic fashion (see the following section).

Notch Signaling in Vertebrate Development

A large body of work has implicated the Notch pathway in the development of a variety of tissues in the vertebrate embryo. The ubiquitous contribution of Notch signaling to vertebrate development makes generalization difficult, particularly when, in many cases, the exact function of Notch signaling in a given developing tissue is still poorly understood. Nonetheless a few common themes are clear. First, the Notch pathway is commonly used to control the fate of progenitor cells, often through genetic interactions with the bHLH transcription factors. These interactions can take on many different forms, and examples are discussed in the following section where the dynamic output of Notch signaling is used to produce salt-and-pepper patterns of cell differentiation (neurogenesis) or sharp boundaries of differentiation between two groups of cells (segmentation). Second, during many developmental processes, rapid changes occur in the expression and/or activity of the Notch ligands or in the ability of cells to respond to Notch activation. Examples of this regulation are discussed in the context of lateral inhibition. Finally, Notch signaling is not likely to be limited to the regulation of cell fate, but may also have roles in cell proliferation, death, and morphology. A challenge for the future is to explore these other roles as the Notch pathway is examined in the context of developmental processes as hematopoiesis, vascularization, and limb development.

Notch Function during Neurogenesis

One function of Notch during vertebrate development is to regulate the activity of bHLH proteins that promote the differentiation of neural precursors into neurons. This regulation mirrors almost identically one that was first uncovered by genetic studies in *Drosophila*, where Notch signaling restricts the formation of neuroblasts or sensory organ precursors by regulating the activity of the proneural bHLH proteins [79]. In vertebrates, this interaction has been studied extensively in fish and frog embryos during primary neurogenesis where an early-born population of neurons is generated from neural progenitors located within discrete domains of the neural plate [63,80–82]. Within these domains, expression of bHLH proteins such as the neurogenins initially occurs in a relatively uniform pattern, thus establishing progenitor cells that can give rise to neurons [83,84]. While the neural bHLH proteins promote neuronal differentiation cell-autonomously, they inhibit neuronal differentiation non-cell-autonomously by promoting the expression of the Delta ligands [82,83], which activates Notch signaling in neighboring cells (Fig. 3A). Activation of Notch leads to the upregulation of genes encoding bHLH repressors, such as the ESR genes in *Xenopus* and the HER genes in zebra fish [53,55], which then act to antagonize the expression/activity of the neural bHLH proteins, both in terms of their ability to promote neuronal differentiation and to activate the expression of Notch ligands [51]. Blocking Notch signaling during primary neurogenesis results in a marked increase in the expression of the neural bHLH proteins within each proneural domain, with a corresponding increase in the number and density of neurons that subsequently differentiate. Conversely, increased Notch signaling in gain-of-function experiments represses the expression of the neural bHLH proteins and blocks neuronal differentiation.

Significantly, by restricting the number of progenitors that form neurons, Notch signaling keeps progenitor cells uncommitted, making them available for alternative neural fates, such as the neural crest [84]. Indeed, it is hard to argue that the raison d'être of Notch signaling is to regulate neuron number since neurons are usually overproduced in many regions of the developing nervous system during neurogenesis and then eliminated later on by cell death. It is also not likely that Notch exists to ensure that neurons differentiate in a precise spatial pattern since neurons undergo extensive migration to reach their final destination. Rather, Notch seems to be required to maintain a population of uncommitted precursors, which can contribute to later-born cell types that are generated over the protracted course of neural cell differentiation [85].

Studies of Notch function during secondary neurogenesis suggest that Notch signaling is used widely to maintain cells

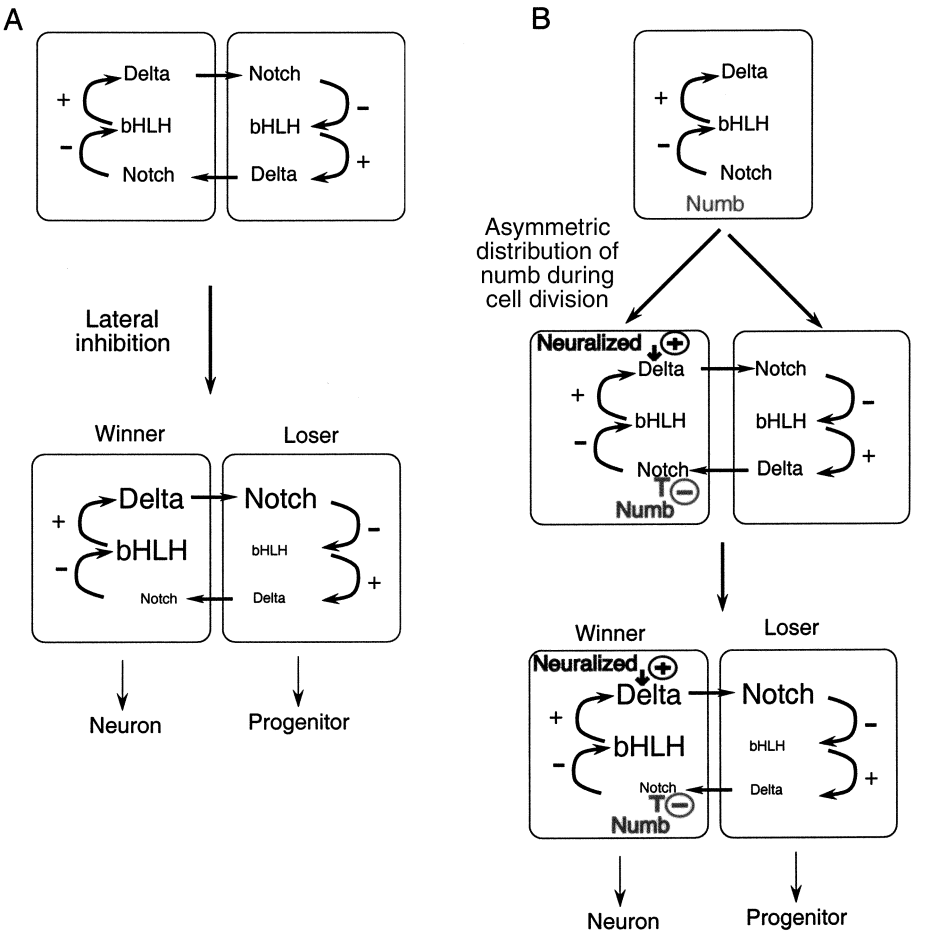


Figure 3 Lateral inhibition. (A) Genetic interactions between the Notch pathway genes and the differentiation bHLH proteins result in winners and losers during lateral inhibition. In this model, bHLH proteins promote neuronal differentiation cell autonomously but also inhibit neuronal differentiation in neighboring cells by activating the expression of ligand activating Notch, which inhibits the activity of the bHLH proteins. As a result, an unstable situation ensues in which some cells will tend to increase both bHLH activity and their ability to inhibit their neighbor (winner) while other cells will do the opposite (loser). (B) Example of mechanisms that preordain the outcome of lateral inhibition. In one case, the cytoplasmic determinate numb is asymmetrically localized to one daughter cell during division. Notch signaling is disabled in the cell that inherits numb, ensuring that this cell wins lateral inhibition while its sister loses. In another case, the protein neuralized is asymmetrically activated in one cell, and promotes Delta signaling by promoting its endocytosis in one cell, thus ensuring that it wins lateral inhibition.

in a progenitor state by preventing their differentiation en masse into neurons [86]. In the retina, loss of Notch function results in an excess production of early-born neuronal cell types at the expense of cell types that would be generated at later stages, while expression of activated forms of Notch activity in the retina represses neuronal differentiation [87–89]. Similarly, functional studies of Notch in neural precursors in the developing forebrain, the cerebellum, or in neural stem cells isolated from either the central or peripheral nervous system also point to a role for Notch in inhibiting neuronal differentiation [61,90–95]. Finally, Notch signaling negatively regulates the differentiation of hair cells in the inner ear as well as the differentiation of a mechanosensory neuron, the neuromast, in the developing lateral line of fish [20,96–98].

In cases where Notch inhibits neuronal differentiation, it seems to act by inducing the expression of the bHLH repressors that inhibit the activity/expression of the proneural bHLH proteins [5,99,100]. As a result, the role of Notch as a regulator of cell fate during neurogenesis has been viewed as permissive rather than instructive in nature. However, in some cases, expression of activated Notch in progenitor cells not only represses neuronal differentiation, but also promotes the differentiation of glial cells. For example, activated Notch promotes the differentiation of a later-born glial cell type, the Müller cell, in the retina [101], radial glial cells in the forebrain [90], myelin-producing glia in cultures of neural crest stem cells [61], or astrocytic glia in CNS neural stem cell cultures [95]. Nonetheless, it remains unclear whether Notch acts instructively in these cases since

the targets of Notch required for glial differentiation are unknown [90].

Lateral Inhibition

During primary neurogenesis, the levels of RNA encoding the Notch ligand and to some extent the receptor change rapidly among progenitor cells. These changes have been attributed in part to the fact that the bHLH proteins not only promote neuronal differentiation but also promote the expression of the Delta-like ligands [82]. Activation of Notch therefore not only inhibits neuronal differentiation but also the ability of that cell to produce ligand and inhibit its neighbors (Fig. 3A). In principle, these interactions produce a dynamic negative feedback loop, called lateral inhibition, which amplifies differences in ligand expression between neighboring neural progenitor cells and generates winners that go on to form neurons (Fig. 3A)[102,103]. At one extreme, lateral inhibition has been proposed to act as a self-organizing selection process that uses the negative feedback loop to generate a salt-and-pepper pattern of ligand expression and differentiation from an initially equivalent population of neural progenitors (Fig. 3A). However, at the other extreme, lateral inhibition has been proposed to reinforce a bias that arose by another mechanism. An example of the latter has been described in *Drosophila*, whereby an asymmetrically localized, cytoplasmic determinant encoded by *Numb* has been shown to determine the fate of two daughter cells by regulating Notch signaling (Fig. 3B) [104,105]. In a similar manner, the vertebrate homologs of Numb, numb, and numb-like, may also determine cell fate by regulating Notch activity in the nervous system [106–109]. Another example is the protein neuralized which potentially promotes Delta activity, thus ensuring that certain cells consistently win lateral inhibition (Fig. 3B).

Notch and bHLH Factors

The role of Notch in regulating the differentiation of progenitor cells by targeting the activity of bHLH proteins is not restricted to the developing nervous system. Activated forms of Notch inhibit myogenesis in cultured myoblasts and this phenomenon has served as a model system for exploring potential inhibitory interactions between Notch and both myogenic bHLH proteins and associated transcription factors such as MEF2 [29,110–113]. Upregulation of MyoD and subsequent myogenic differentiation can also be blocked in chick embryos by Notch in gain-of-function experiments [114], but whether or not this regulation normally plays a physiological role *in vivo* is still unexplored. In the developing pancreas, there is strong genetic evidence showing that endodermal cells differentiate into an endocrine cell fate in response to a bHLH protein, neurogenin 3, and this differentiation step is inhibited by Notch signaling [115–117]. In a manner similar to that observed during neurogenesis, disabling Notch signaling, or the putative Notch target gene HES1, greatly increases the expression of neurogenin 3, resulting in a premature production of endocrine cells. Thus, the pancreas is another case where cell differentiation is promoted by a bHLH protein but inhibited by the Notch pathway [118].

Notch Function during Vertebrate Segmentation

During lateral inhibition, Notch signaling is used on a cell-by-cell basis to control cell fate. However, the study of Notch signaling in invertebrate embryos has also emphasized its role in patterning larger tissue domains such as when it is used to regulate the size of veins [119] or used to specify the margin in the developing *Drosophila* wing disc [120]. In an analogous manner, Notch signaling is also used in vertebrate embryos to pattern larger tissue domains, a particularly striking example of which occurs during segmentation of the mesoderm into somites.

Somites are the repeating, metameric building blocks of the vertebrate body plan that arise in early development from two bilateral sheets of cells, called the paraxial, or presomitic, mesoderm (PSM), which lie on both sides of the notochord [121]. Somites do not form en masse, but in an anterior to posterior progression, one unit arising at a regular interval every 30–90 minutes depending on the species. Because the process is progressive, a "differentiation wavefront" has been proposed that determines the point along the anterior-posterior (A/P) axis where paraxial cells mature and form a somite (Fig. 4A). However, to divide this differentiation process into a periodic pattern a segmental clock has been proposed which oscillates on and off during each segmental cycle, thus specifying the boundary between one somite and the next [121].

A role for the Notch pathway in somitogenesis was first evident from defects in somite morphology observed in mice with targeted mutations in the Notch1 gene or in the CSL protein, RBPjk [6,122]. Subsequently, the expression pattern of Notch pathway genes within the PSM revealed the formation of a segmental pattern of gene expression that prefigures the formation of somites. Indeed, based on this pattern, one could identify 1–3 segments, depending on the species, within the presomitic mesoderm (S0 to S-3 in Fig. 4A) [17]. Moreover, further analysis has produced a wealth of evidence indicating that Notch signaling is required for generating this segmental patterning, and that it contributes to this process somewhat as a component of the segmental clock [123]. First, the pioneering work of Pourquie and colleagues showed that genes encoding bHLH repressors oscillate in expression within the undifferentiated paraxial mesoderm with a periodicity corresponding to one segmental cycle (Fig. 4A) [124–127]. While it is not yet clear in all cases whether these genes are direct targets of Notch-mediated transcription, evidence from mouse and zebra fish indicate that their oscillatory expression is dependent on Notch signaling [127–129]. Secondly, Notch signaling seems to be activated synchronously within the paraxial cells during each segmental cycle. In fish and frog, the expression of RNA encoding the Notch ligand cycles off and on during

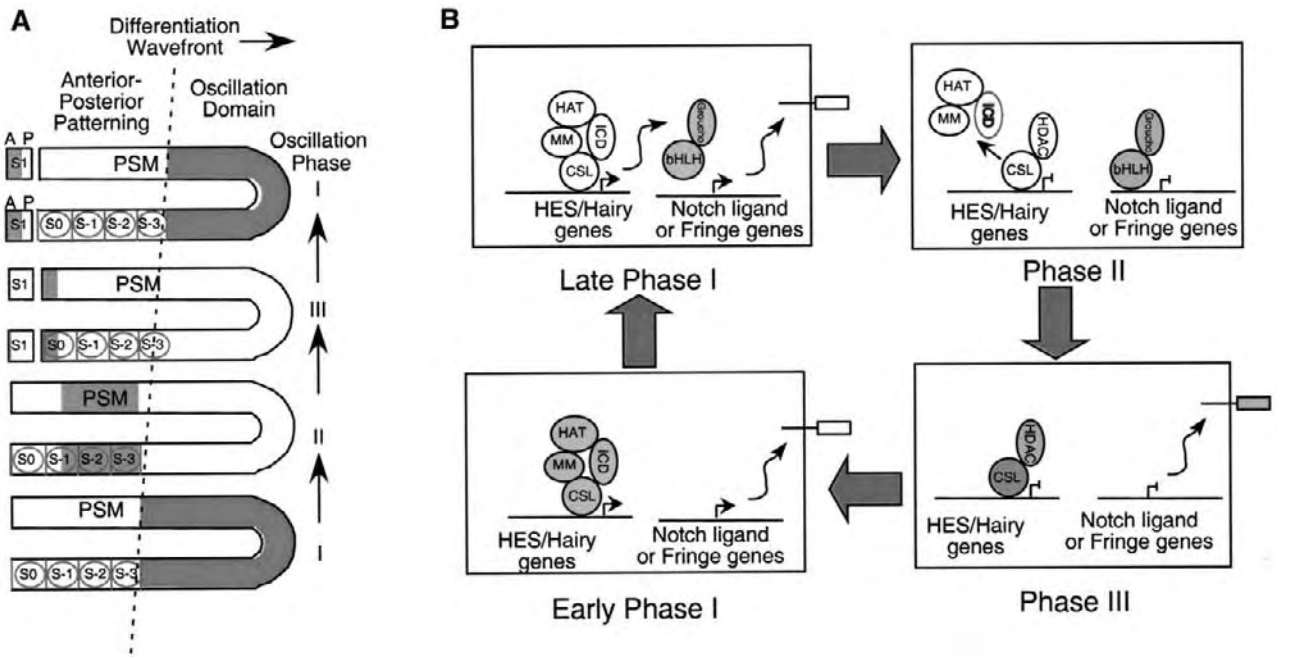


Figure 4 Model for Notch signaling during segmentation. (A) Somites form from the presomitic mesoderm (PSM) at regular intervals, with the most recently formed somite called S1. Each somite is divided into an anterior (A) and posterior (P) half (green shading). Notch signaling potentially plays two roles when segments (S0–S3) are generated within the PSM. In the more caudal region of the PSM (oscillatory domain), Notch signaling oscillates on and off during each segmental cycle (blue shading). This oscillation has been proposed to be part of the segmental clock that establishes segmental boundaries (purple lines). In the more rostral portion of the PSM (anterior-posterior patterning), Notch signaling may also act to subdivide segments into two halves by undergoing a dynamic refinement from a broad domain to a narrow half-segmental domain (red shading). (B) Model for the oscillation in Notch signaling that underlies the segmental clock. In this model, the bHLH repressors form a negative feedback loop that generates an oscillation much the same way the transcriptional repressors underlie oscillation in gene expression during circadian rhythms. A key element in the model shown is that the expression of a bHLH repressor (bHLH-groucho) is activated during each segmental cycle via Notch signaling, which then turns off the expression of genes required for Notch signaling, such as the Notch ligands or the fringe genes.

each segmental cycle within the undifferentiated paraxial mesoderm [130]. In contrast, ligand RNA expression is relatively uniform in the paraxial mesoderm of mouse and chick, but cycling is observed instead in the expression of RNA encoding the Notch modifier lunatic fringe [77,131]. Finally, mutations in these various components of the Notch pathway produce defects in somitic boundaries, indicating that the oscillating expression of these genes is critical for proper segmentation [76,77,126,129,132]. The exact role of Notch signaling in the segmental clock is still a matter of debate. One possibility is that the Notch pathway is the clock itself in which oscillations in gene expression are generated by Notch activation of the repressor bHLH proteins which then negatively feed back to shut down their own transcription by repressing the expression of the ligands or the fringe genes (Fig. 4B) [128]. Alternatively, Notch signaling may be downstream the target of a master clock, which drives the cyclic expression of components in the Notch pathway, perhaps as a means of synchronizing the phase of the clock among paraxial cells [130].

Establishment of boundaries during somitogenesis is tightly associated with the division of each somite into an anterior and posterior half, which is marked by the restricted expression of Notch ligands [121]. This A/P somite polarity has important functional consequences since it imposes segmental organization on the spinal cord and peripheral nervous system [133]. Loss of somite A/P polarity occurs in many cases where Notch signaling is disrupted during somitogenesis, indicating that Notch may also have a secondary role in polarizing somites along the A/P axis [121]. Significantly, the Notch pathway may contribute to A/P polarity by regulating the expression of the Mesp-like bHLH proteins [134,135]. Thus, the output of Notch signaling seems to specify domains of bHLH gene expression and thus patterns of differentiation during segmentation, in a manner that is similar to that seen during neurogenesis.

Notch Signaling in the Limb

The limb is another prominent example in vertebrate development where the contribution of Notch signaling to tissue patterning has been examined in some detail. In this case, Notch signaling contributes to the formation of a structure, called the apical epidermal ridge (AER), which forms at the boundary between the dorsal and ventral limb epidermis [120]. In chick limbs, Notch signaling may be modified during this process by a vertebrate homolog of *Drosophila* fringe, called *radical fringe*, which is expressed in the dorsal but not ventral limb epidermis [136–138]. When ectopically expressed ventrally using retrovirus vectors,

radical fringe induces ectopic AER cells as does an activated form of Notch, while a dominant-negative form of the CSL proteins inhibits AER formation. It remains uncertain, however, whether these findings extend to the mouse where targeted mutations in *radical fringe* have no affect on limb development, although the situation is further complicated by potential genetic redundancy with the two other mammalian fringe genes, *lunatic* and *manic* [139]. Expression of the Notch ligand *Jagged2* is localized to the AER, and mice with targeted or naturally occurring mutations in this gene develop with syndactyly in both fore- and hindlimbs [140,141]. Additional work is needed to determine whether Notch signaling is a necessary factor in generating the AER, or whether it only has a secondary role in its maintenance and/or function.

Notch Signaling and Lymphoid Development

The hematopoietic system is an impressive example of cell diversification, and thus it is not surprising that the Notch pathway may have multiple roles in regulating cell fate as hematopoietic progenitors become progressively restricted in their developmental potential. The best evidence in this respect is for a role in Notch signaling in directing the differentiation of lymphoid-restricted progenitors into a T-cell versus a B-cell fate as these cells pass through the thymus and bone marrow, respectively. In mice where Notch1 is conditionally ablated in lymphocytic progenitors using the CRE-LOX system, T-cell development is inhibited [142] with a corresponding increase in intrathymic B cells [143]. Conversely, expressing activated Notch in bone marrow progenitors promotes extrathymic development of T cells and represses the development of early B cells [144]. A major unresolved issue is why Notch signaling is preferentially promoted in the thymus where T-cell development occurs [145] but repressed in the bone marrow during B-cell development. One likely target of Notch signaling within the T-cell lineage is the gene encoding one chain of the pre-TCR receptor whose expression then reinforces a T-cell fate [146], but the targets of Notch signaling that repress B-cell development are unknown.

Several lines of evidence indicate that Notch signaling influences the subsequent diversification within the T-cell lineage where progenitors are restricted further between a $\gamma\delta$ or $\alpha\beta$ fate or between the CD4 versus CD8 T-cell fate [147,148]. While most attention has been given to the role of Notch in the CD4/CD8 choice, how Notch affects this T-cell subdivision remains controversial [149–152]. The phenotypes reported for alterations in Notch signaling in this context may differ due to experimental differences in the forms of activated Notch used and how these forms were expressed in developing thymocytes. Nonetheless, one idea with far-reaching implications produced by this work is that Notch signaling may not be acting in the CD4/CD8 switch to influence cell fate per se, but rather to regulate such processes as lineage-specific cell death or differentiation. If this is indeed the case, then the targets of Notch signaling may be more extensive than previously appreciated, and inappropriate expression of such targets may potentially contribute to oncogenic transformation when the Notch pathway goes awry [153].

Notch Signaling during Vascular Development

Another venue where Notch signaling may control developmental processes other than simple cell fate choices is vascularization [154]. Indeed, one of the major reasons why mutations in the Notch pathway lead to embryonic lethality is defects in vascularization. These defects are likely to be due, in part, to a misspecification of angioblasts along an arterial or venous fate, thus producing profound defects in the how the vascular network is established. In zebra fish, loss of Notch signaling in fish embryos leads to a loss of artery-specific markers and a corresponding ectopic increase in cells that express venous markers [155]. Conversely, activated Notch signaling suppresses the expression of markers of venous differentiation. A potential direct target of Notch signaling during this process is a bHLH repressor encoded by the gene *gridlock*, which is also required genetically for establishing an arterial over a venous fate [54]. Further evidence for a role in vascular development comes from targeted mutations in various components of the Notch pathway in the mouse. Severe vascular defects associated with angiogenesis occur in mice that are double-mutant for both Notch1 and Notch4 [3]. Misexpressing the Notch4 intracellular domain specifically within the endothelial lineages produces a striking disorganization of the vascular network, indicating that Notch signaling acts in part by regulating some aspect of angioblast differentiation [156]. Both the Jagged1 and Delta1 knockout mice die at early embryonic stages with defects in vascular morphology [21,157] and a fourth ligand, Delta4, is expressed even earlier in the developing arterial system [3,16]. Thus, Notch signaling is likely to be required within the endothelial cells during the processes that elaborate the vascular network. Whether Notch functions simply as a regulator of cell differentiation or whether it has multiple roles during vascularization remains to be determined. Given that several Notch ligands and receptors are expressed with varying degrees of genetic overlap during vascular development, the role of Notch signaling during this process is likely to be quite complex.

Aspects of Notch Signaling in Other Organ Systems

The discussion thus far has focused mainly on the prominent examples of where Notch signaling has been shown to act during vertebrate development. However, this list is incomplete given the variety of tissue defects that have been described in various mouse mutants or combination of mutants in components in the Notch pathway. For example, mice with a hypomorphic mutation in Notch2 die from kidney defects that are most apparent within the developing glomeruli, and also show defects in various aspects of heart and eye development [158]. Various combinations of mutant

forms of Notch2 and Jagged1 result in liver defects and other abnormalities that mimic those found in Alagille's syndrome [159]. In addition, the early lethality of the mouse mutants in many components of the Notch pathway has precluded the analysis of the pathway in tissues that develop at later stages, a problem that can be overcome by generating conditional knockouts. Based on both gene expression and functional studies, there is already a clear indication that Notch signaling has additional diverse roles in vertebrate development, including the patterning of the forebrain [160], the differentiation of oligodendrocytes into myelinating cells [161], the differentiation of epidermal stem cells into keratinocytes [162], and the formation of the lung [163].

Summary

Over the last ten years, the Notch pathway has emerged as one of the key signaling pathways operating during vertebrate development. In many cases, Notch signaling is used widely in vertebrate development to regulate cell fate often by activating the expression of bHLH repressors which in turn regulate the expression and/or activity of bHLH activators. These interactions can determine patterns of differentiation that range from salt-and-pepper to segmental. The role of Notch is not likely to be exclusive to cell fate decisions, but emerging evidence suggests that it might regulate a variety of cellular events ranging from differentiation, morphogenesis, and cell death to proliferation. A key goal of future studies is to identify the various gene targets of the CSL-dependent pathway that mediate these events, or to elucidate parallel pathways in which Notch signals by a CSL-independent mechanism if they exist. In addition, the diversity of Notch signaling is likely to depend in part on functional diversity of ligands and receptors, about which we still know very little. Finally, the Notch signaling pathway is likely to contribute to various human diseases, but more needs to be done to identify the nature of these lesions and how they alter cell physiology.

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CHAPTER 258

Reiterative and Concurrent Use of EGFR and Notch Signaling during *Drosophila* Eye Development

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Notch and epidermal growth factor receptor (EGFR) pathways are conserved throughout evolution and function together in many different developmental contexts. During the specification of the *Drosophila* eye, these two pathways either function in synergy or in opposition to achieve different outcomes. Similarly, in some instances, EGFR activation causes a serial activation of the Notch pathway through the control of the Notch ligand Delta, while in others, the two pathways act together on the same enhancer element in parallel to control target gene expression. We propose that the combinatorial effects of these two pathways have evolved as a network that generates a significant diversity in output with a small number of components used in a reiterative fashion.

Introduction

Intercellular signaling plays a critical role in the establishment of the body plan and cell fate during the development of multicellular organisms. The variation in the cell fates adopted is quite extensive; yet the number of conserved signaling pathways that mediate a majority of cell-cell interactions is extremely limited [1]. These few signaling pathways are used reiteratively and evoke specific responses depending upon the context in which they function. Understanding how this context is created within an equipotent group of cells is fundamental to our understanding of the role of cell-signaling in fate specification during development.

The *Drosophila* compound eye consists of a large number of ommatidia (facets), each containing a fixed number of cells: eight photoreceptor cells (R cells), four non-neuronal cone cells, three classes of pigment cells, and a bristle complex [2]. The fate of these cells is not derived by clonal mechanisms but through cell-cell communication [3]. The *Drosophila* eye develops from a sheet of epithelial tissue called the eye imaginal disc. In the third larval instar an indentation called the morphogenetic furrow (MF) initiates at the posterior tip and sweeps anteriorly across the disc. As cells emerge out of the furrow they attain the competence to respond to signaling pathways and initiate differentiation in a precise order (Fig. 1). The photoreceptors are the first cells to differentiate, followed by the cone cells and the pigment cells. This led to the hypothesis that unique signals from differentiated cells will sequentially induce the precursors of later developing cell types [4]. The molecular basis for such a combinatorial model is now becoming clear. The biggest surprise is that the signals involved are not very specific and that a small number of common signaling pathways and transcription factors can combine in different ways to generate a tremendous diversity of readouts [5–8]. The EGFR and Notch signaling pathways are prominently involved in this process. All components of these pathways, except the ligands, are ubiquitously expressed. The spatiotemporal

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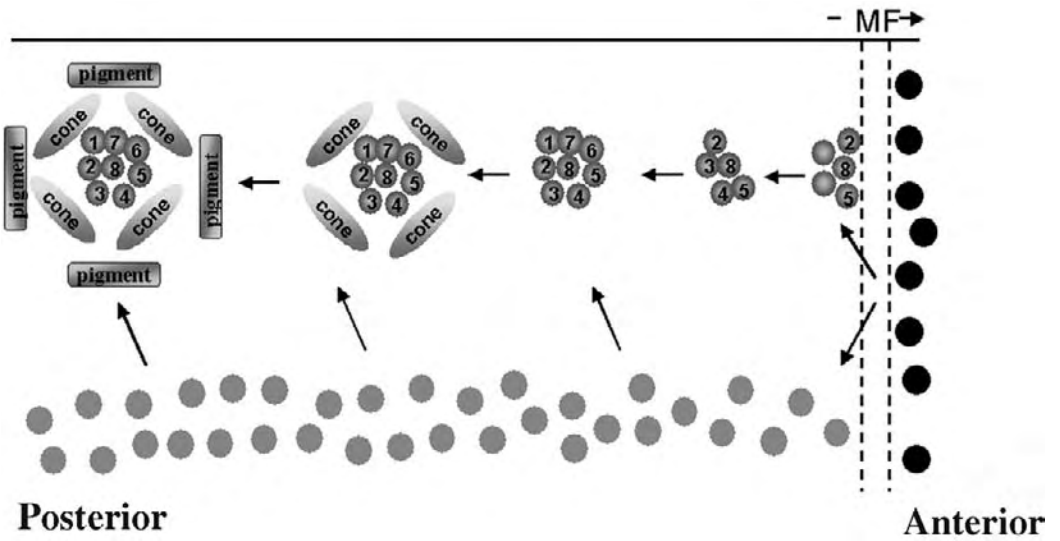


Figure 1 A schematic representation of cell fate specification during eye development. Cells ahead of (anterior to) the morphogenetic furrow (MF) are pluripotent and undergo extensive proliferation. The MF marks the onset of cell fate specification. The precluster cells (R2–R5, R8) differentiate first from cells ahead of the furrow. Cells that do not join the precluster undergo a single round of mitosis and become precursors to the rest of the cells in the ommatidial cluster (R1, R6, R7, Cone, and Pigment cells).

control of the ligand determines the cells in which a mosaic pattern of activated transcription factors is generated. In this chapter we briefly discuss steps in *Drosophila* eye development in which the Notch and EGFR pathways combine in different ways to generate unique outputs.

Establishment of the Eye Primordium

The *Drosophila* eye and antenna both arise from a single primordium at stages 14–15 of embryonic development [9], and only later the posterior region of this common primordium differentiates into the eye disc while the anterior region gives rise to the antenna. Expression of either a dominant-negative version of Notch or an activated version of EGFR in the common primordium converts the eye tissue into antenna [10]. This interplay of signals is important for the expression of Eyeless (DPax-6) in the aspect of the tissue that will give rise to the eye. Eyeless encodes a conserved transcription factor containing a Paired and a homeodomain and functions as a pivotal control point for a network of genes that establish eye fate [11]. Although the directness of the signaling control is not yet clear, it seems that Notch signaling is required for the expression of Eyeless, while EGFR antagonizes Notch-mediated Eyeless expression [10,12]. A balance between these two pathways is crucial for the specification of the eye primordium. It seems that such a balance is invoked repeatedly to create unique cell fates.

Proliferation and D/V Patterning

The eye disc primordium undergoes extensive proliferation during the later embryonic stages, as well as in the first and second larval instars. During late stages of the second larval instar, the eye disc primordium is patterned along the dorso-ventral (D/V) axis [13]. The segregation between the dorsal and ventral regions is not along traditional compartmental boundaries. Rather, the dorsal and ventral halves of the eye disc express distinct ligands for Notch. Cells in the ventral region express Serrate while those in the dorsal region express Delta (Dl) [14]. These ligands function differentially with the Fringe protein such that they activate Notch only at the boundary. This promotes the specification of a D/V organizer, which initiates cell proliferation and patterning along its edges [14]. Loss of EGFR at this stage will also cause a block in cell proliferation; however, its possible role in the specification of cell fate at this early stage remains to be established. In a comparable situation in the wing disc, EGFR functions with Notch during D/V boundary specification to control both proliferation and cell fate specification [15].

Morphogenetic Furrow and R8 Specification

Cells ahead of the morphogenetic furrow are undifferentiated and undergo several rounds of mitosis. These cells express all the genes that specify them as eye tissue and can adopt one of many possible differentiated fates upon the reception of an appropriate signal. Ectopic activation of EGFR and Notch in clones of cells ahead of the furrow causes them to differentiate as R cells, suggesting a readiness and capacity of these undifferentiated cells to respond to these signals [16,17].

The signaling pathways most closely linked to the initiation and progression of the morphogenetic furrow are Hh and Dpp (TGFβ) [18]. As the furrow progresses along the largely oval shaped disc it needs to be continuously reinitiated at the lateral edges so that it extends across the increasing width of the disc. This reinitiation requires Wingless (Wnt)

signaling, and also the function of EGFR and the Notch pathways [19–21]. Loss of EGFR or Notch causes loss of furrow initiation at lateral edges and activation of EGFR and Notch at the lateral edges ahead of the furrow causes ectopic furrow initiation [21]. This is therefore an example of the EGFR and Notch pathways working cooperatively. In contrast, during the differentiation of the founder cell, R8, at the morphogenetic furrow, EGFR and Notch signals function in an antagonistic manner. EGFR has been proposed to be required for the establishment [22] and maintenance [23] of R8 cell fate, and these cells are lost in mutations that abolish the signal. In contrast, loss of Notch at the morphogenetic furrow causes overspecification of R8 cell types [24]. This is because Notch functions in a lateral inhibitory process at the furrow that helps restrict the number of R8 cells and generate regular spacing between the clusters. This lateral inhibition by Notch is mediated by the activation of repressor proteins of the E(spl) complex, which block the expression of the proneural gene *atonal* and the specification to an R8 cell type [25].

R-Cell Specification

Cells within the morphogenetic furrow are arrested in the G1 phase of the cell cycle. As they exit out of the morphogenetic furrow they follow one of two possible paths. A subset of these cells form "preclusters" consisting of the first five R cells (R2–R5, R8), and those that do not join the precluster undergo a terminal round of mitosis and continue to be pluripotent undifferentiated cells [26]. These cells express a distinct set of transcription factors (e.g., Lozenge) not found in the undifferentiated cells ahead of the furrow from which they are derived [27]. The remaining cell types within an ommatidium (R1, R6, R7, Cone, and Pigment cells) arise from the undifferentiated cells behind the morphogenetic furrow. This second round of recruitment completes the ommatidial cluster and is referred to as the second wave of morphogenesis in the eye [3]. The EGFR pathway is required for the G2/M transition during mitosis that gives rise to the precursors for the second wave [28]. Additionally, EGFR function is also required for maintaining viability and differentiation of all R cells. In contrast, during this later step of R-cell differentiation the function of Notch seems to be more specific. The Notch signal specifies the R4 cell and distinguishes it from R3, a process that is essential for the proper rotation of the ommatidial cluster with respect to the midline (equator) of the eye disc [29,30]. Furthermore, the Notch signal is required for the specification of the R7 cell fate as in its absence R7 cells are converted to an R1/6 type [31].

Sequential Linkage between Notch and EGFR Pathways

Although the Notch signal is required in only a subset of R cells for their proper specification, the Notch ligand Dl is expressed at high levels in all R cells [32]. The Dl protein functions in R cells to induce the later arising cone cells [8]. Interestingly, the expression of Dl in R cells is under the control of EGFR. A series of gain and loss-of-function studies indicate that loss of EGFR signaling causes a loss of Dl expression and ectopic activation of the EGFR pathway causes increase expression of Dl [33]. The EGFR-related activation of Dl requires the usual downstream components Ras, Raf, and MAPK but also two novel nuclear proteins Ebi and Strawberry notch (Sno) [33]. Ebi encodes an F-box/WD-40 repeat containing protein [34] and Sno codes for a novel nuclear protein [35]. The regulation of *Dl* in photoreceptor cells upon EGFR signaling requires the disruption of a Su(H)/SMRTER repression complex and involves the function of ubiquitin-mediated proteolysis and nuclear export of the co-repressor protein, SMRTER. This study highlights a mechanism whereby the EGFR and Notch pathways are linked in a serial manner. Activation of EGFR triggers a proteasome pathway leading to de-repression of *Dl*, which in turn can activate the Notch signal to be received by the neighboring cone cell precursors (Fig. 2).

Parallel Linkage between EGFR and Notch

The four non-neuronal cone cells are recruited to the ommatidial cluster after the R cells have joined. These cells express specific transcription factors such as D-Pax2, Prospero, and Cut, which are required for proper specification of their fate. The mechanisms that govern the specific expression of these factors have been uncovered in some detail. The expression of D-Pax2 and Prospero depend upon EGFR signaling, Notch signaling, and the transcription factor, Lozenge (Lz), expressed in the pool of undifferentiated cells behind the morphogenetic furrow [27]. Loss-of-function mutations in any one of these three components will cause a

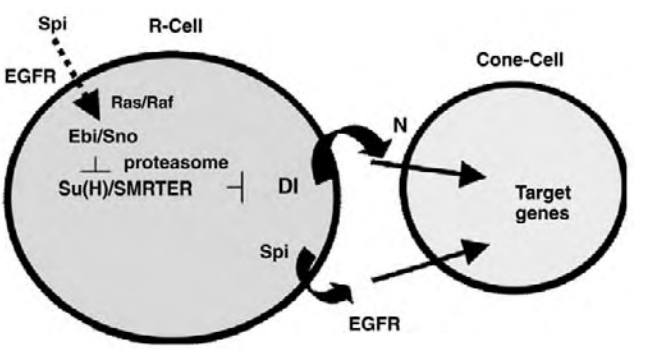


Figure 2 Sequential and parallel link between Notch and EGFR in cone cell specification. In R cells the EGFR and Notch pathways are linked sequentially. Activation of EGFR by its ligand Spi activates Ras/Raf/Sno/Ebi and leads to de-repression of the *Dl* locus by blocking its Su(H)/SMRTER-mediated repression. Dl protein expressed in R cells activates Notch signaling in cone cell precursors. These cells express the transcription factor, Lz, and also receive the EGFR signal from R cells. A combinatorial integration of the EGFR and Notch signals activates target genes required for cone cell specification.

complete loss of D-Pax2 and Prospero expression [8,36] (Nagaraj and Banerjee, unpublished). A 350 bp minimal enhancer element within the *D-Pax2* locus recapitulates its expression in the four cone cells in each cluster of the eye disc. Sequence analysis of this enhancer revealed functional binding sites for Lz and for Pointed and Su(H), the nuclear effectors of EGFR and Notch pathways, respectively. *In vivo* mutational analysis of these binding sites established that they are essential for the expression of D-Pax2 in the cone cells. Furthermore, ectopic activation of combinations of Notch, EGFR, and Lz using cell-specific drivers will cause D-Pax2 expression in different combinations of cell types in the eye disc that do not normally express this protein [8]. These results established that EGFR and Notch signals function combinatorially to specify cell fates among a group of pluripotent precursors.

Pigment Cell Differentiation and Apoptosis

The cooperativity and antagonism between EGFR and Notch is also evident in pigment cell differentiation. Cell ablation studies show that cone cells provide an inductive signal for the differentiation of the primary pigment cells [37]. In this function, Notch works combinatorially with EGFR as activation of EGFR is also needed for the specification of primary pigment cells. In contrast, the EGFR and the Notch pathways antagonize each other in promoting the survival of the secondary and tertiary pigment cells [37]. While EGFR promotes survival, Notch signaling in these cells has been proposed to promote cell death. The mechanisms by which these pathways function to regulate apoptosis remain to be established.

Conclusion

This review highlights how EGFR and Notch pathways function together in different ways within the same tissue and within small temporal windows to promote a diversity of cell fate specification events. These pathways can be linked to function in either a synergistic or an antagonistic manner; they can combine in series or in parallel to generate different outcomes. It is unlikely that these two pathways are chosen randomly to function together in so many different developmental decisions. Rather, this suggests the co-evolution of the two pathways to generate an efficient network involving possibilities for crosstalk. This intricate interplay between the EGFR and the Notch pathway can be seen from worms to man. During the induction of vulval fate in *C. elegans*, the anchor cell provides the EGF signal and induces the closest vulval precursors to differentiate into primary fate. This cell in turn uses the Notch signal to influence its neighbors to differentiate into secondary cells [38]. In mouse, differentiation of hematopoietic cells requires RTK and Notch signaling for proliferation and cell fate specification [39]. Finally, in tumor progression, Ras activation causes expression of Dl [40] similar to that observed in the developing *Drosophila* eye [33]. It is likely that the lessons learned from *Drosophila* will be useful in interpreting interactions between these pathways across species.

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CHAPTER 259

BMPs in Development

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Introduction

Genes encoding bone morphogenetic proteins (BMPs) and their signaling components are found in many animal phyla, suggesting an ancient function as morphogens in metazoan development [1]. Where it has been possible to make comparisons (e.g., dorsal-ventral patterning and restriction of neuronal fate) there is a remarkable conservation of the roles of BMP signaling components in establishing and interpreting BMP gradients. A full discussion of the roles of BMPs in development is beyond our scope. Rather, we discuss recent concepts that have emerged from analysis of the BMP pathway in the fruit fly (*Drosophila*) embryo. We relate these concepts to the analysis of BMP function in vertebrates.

A fundamental task of the developing embryo is to inform individual cells of their relative positions through either direct cell-cell interactions, or soluble molecules that act as morphogens. To qualify as a morphogen, the factor must act directly on distant cells, generate a gradient of signaling activity, and elicit different patterns of gene expression at different concentrations [2,3]. Strong evidence for activity as a morphogen is available for only a few secreted proteins. BMP family members are among the best characterized examples [2–4].

Gradients of BMP Activity

BMPs satisfy the most critical requirement for morphogens: They act in a concentration-dependent manner to elicit differential gene expression [2,4]. For example, in *Drosophila,* neural cells form ventrally where BMP signaling is absent, ectoderm forms laterally where intermediate levels of BMP signaling take place, and peak levels induce the most dorsal tissue, the amnioserosa (Fig. 1). The most direct method to determine where BMP signaling pathways are active is the use of antibodies that specifically detect the active (phosphorylated) forms of Smads (pMAD and pSmad, in *Drosophila* and vertebrates, respectively). BMP activity gradients have been seen along the *Drosophila* and *Xenopus* dorso-ventral (D/V) axis, in the wing disc of the fly, and in the chick [5–9]. A common finding is that the shapes of the BMP ligand and activity gradients are considerably different. For example, along the *Drosophila* D/V axis, although pMAD levels form a gradient with highest levels dorsally, mRNAs for the ligands DPP and SCW are uniform dorsally (DPP) or are ubiquitously expressed (SCW) (Fig. 1) [8]. This finding underscores the substantial level of control over the shapes of BMP activity gradients that occurs extracellularly via ligand interactions with antagonists, and at the intracellular level by regulating BMP receptor and Smad degradation.

Establishing BMP Ligand Gradients

Many mechanisms are employed to establish BMP gradients, even within a single species. The propagation of endogenous BMP ligand gradients has not been studied directly in vertebrates, but in *Xenopus*, the TGFβ family member activin forms a gradient by diffusion [2]. On the other hand, BMP4 does not diffuse freely, perhaps due to its ability to bind proteoglycans [10], which are abundant in the extracellular matrix. In the *Drosophila* wing disc, the DPP gradient is controlled by receptor-mediated endocytosis [11]. DPP binds to its receptor (Thickveins, Tkv), and repeated cycles of endocytosis and re-secretion transport DPP from its source. DPP is also distributed extracellularly, and binding of DPP to Tkv limits its diffusion [6]. Little is

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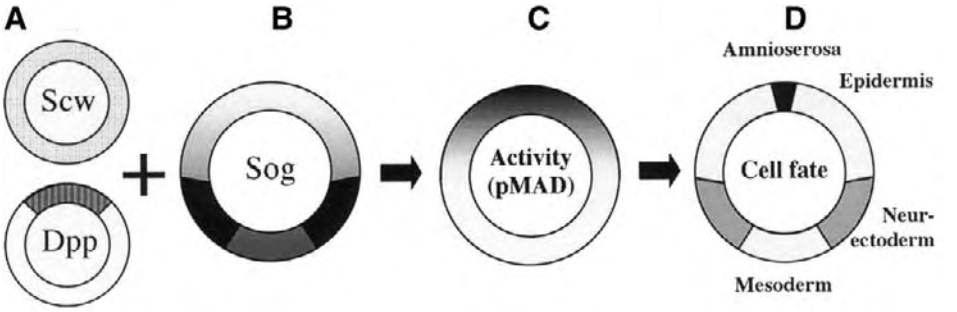


Figure 1 Formation of the BMP activity gradient along the *Drosophila* D/V axis. (A) mRNAs for the ligands are distributed uniformly (Scw) or broadly on the dorsal side of the embryo (Dpp). (B) Sog, the Scw/Dpp antagonist, is distributed in a ventral high-dorsal low gradient due to its expression in ventrolateral regions (black), and destruction in the dorsal region. (C) As a result of the Sog gradient, Dpp and Scw are antagonized ventrally, leading to a dorsal high-ventral low gradient of BMP signaling activity, as monitored by distribution of pMAD. (D) Different cell types are specified along the D/V axis. The amnioserosa is the most dorsal cell type.

known about how levels of BMP receptor expression are regulated, but BMPs can repress expression of their receptors [12], which limits the duration of responsiveness to BMP signals and prevents sequestration of BMPs close to the source of expression.

Extracellular Modifiers of BMP Activity

Antagonists that act by binding BMPs in the extracellular space play a pivotal role in shaping BMP gradients, and a large number of such antagonists have been identified [13]. The best characterized example, conserved through evolution, is the Short Gastrulation (Sog) protein, and its vertebrate homolog Chordin (Chd). They are the primary architects of the BMP activity gradients that specify the D/V axis in vertebrates and invertebrates (reviewed in references [13] and [14]).

Controlling the availability of these antagonists allows the embryo to create a BMP activity gradient despite uniform expression of the ligands. Sog/Chd is cleaved by the protease Tolloid (Xolloid in *Xenopus*). Along the *Drosophila* D/V axis, Sog is expressed in a ventral to dorsal gradient that opposes the gradient of DPP activity [9] (Fig. 1B). As a result of the Sog gradient, SCW is antagonized ventrally (where Sog levels are highest), but is active dorsally, where Sog is removed by endocytic degradation and cleavage by Tolloid [9]. Similar mechanisms are likely operational in vertebrates. For example, in zebrafish, the BMP2/4 homolog Swirl is not asymmetrically distributed, but it patterns the entire D/V axis through localized expression of zChd [15].

Twisted Gastrulation (Tsg), a protein that interacts with Sog/Chd and BMPs to affect the activity of Tolloid/Xolloid, brings an additional level of regulation. Tsg acts as an antagonist by forming a complex with Sog and SCW [16–19]. This promotes cleavage of Sog into a truncated form, Supersog, that can inhibit DPP in addition to SCW [16–19]. Thus, Tsg serves to steepen the Sog activity gradient, leading to more potent antagonism of BMP activity.

The consequences of this complex formation are not fully understood, and BMP binding proteins may not act solely as antagonists. For example, genetic studies indicate that Tsg and Sog have positive effects on BMP signaling, perhaps by mediating ligand diffusion (reviewed in references [9] and [14]). Moreover, in *Xenopus*, Tsg is multifunctional, acting as both a BMP agonist and antagonist depending on experimental conditions [18–20]. In addition, loss-of-function studies suggest that many antagonists have redundant functions that will be uncovered only through construction of double mutants. For example, although unrelated structurally, the antagonists Noggin [21] and Chd have a redundant function in induction of anterior neural tissues in mammals [22].

Interpreting the Gradient-Role of BMP Receptors

BMP signal transduction is initiated by formation of complexes of type I and type II receptors, leading to Smad phosphorylation. It is clear that different type I receptors activate common signaling pathways. How can such a simple system lead to differential gene expression? First, BMPs trigger different responses when different numbers of receptors are activated [2]. Second, different BMP receptors have different affinities for specific BMP ligands. Thus, two groups of cells might respond differently to a single BMP concentration because they express different type I BMP receptors.

A striking example of this latter mechanism is the synergy observed between some type I receptors. In *Drosophila*, Thickveins (Tkv) can act independently of the Saxophone (Sax) type I receptor, but signaling through Sax takes place only when Tkv is active. Moreover, Tkv and Sax strongly synergize when co-expressed, and this synergy is required for formation of the amnioserosa (reviewed in [4]). Evidence for a synergistic relationship has also been found in mammals [23]. BMPRIA and BMPRIB are structurally related to Tkv and function equivalently in that constitutively active (ca) forms induce chondrogenesis independently.

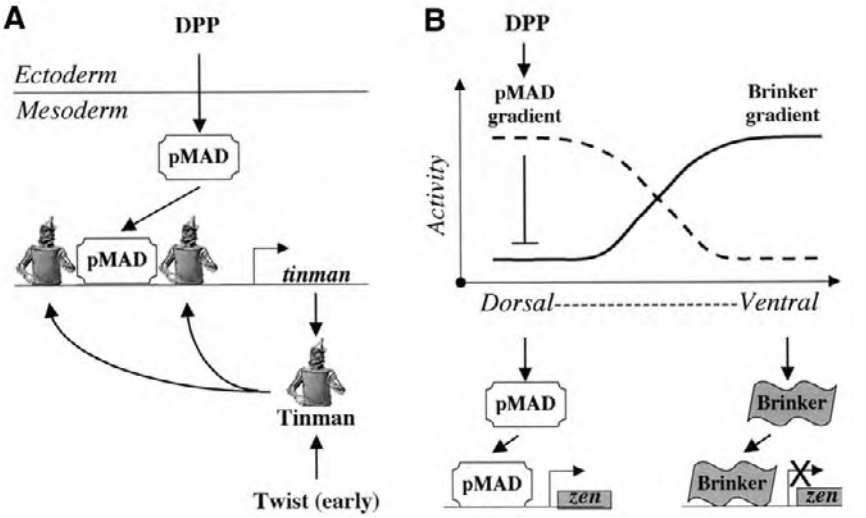


Figure 2 Regulation of target genes by BMP signaling. (A) The *Drosophila tinman* gene is expressed in dorsal mesoderm. At early stages, *tinman* is induced by Twist. The mesoderm subsequently migrates, and mesoderm located dorsally is exposed to high levels of DPP produced by the overlying ectoderm, resulting in high levels of pMAD activity. pMAD binds directly to the *tinman* promoter and cooperates with Tinman to maintain high levels of *tinman* expression. (B) Brinker is a transcriptional repressor of DPP target genes. *Brinker* expression is repressed by DPP. Hence, Brinker is present in areas of low pMAD activity. pMAD binds to the *zen* promoter to promote *zen* transcription. Brinker competes for binding and represses *zen* transcription. This mechanism would sharpen the boundary between *zen*-expressing and nonexpressing cells.

In contrast, ALK1 and ALK2 are structurally related to Sax, and ca forms of these receptors cannot induce chondrogenesis. However, ca BMPRIA or ca BMPRIB synergizes with ca ALK2 to promote chondrogenesis [23]. The mechanistic basis for the synergy is unknown, but may involve formation of a more stable receptor complex, differences in Smad activation, or effects on Smad cofactors.

Differential Gene Activity in Response to BMP Signal Transduction

The mechanisms by which changes in levels of activated Smads (pSmads) lead to changes in patterns of gene expression is an area of intensive investigation (reviewed in [24]). The ability of pSmads to interact with a myriad of transcriptional regulators is responsible for the dose-dependent effects of BMPs on gene expression [25]. The developmental history of the responding cell (i.e., its competence) determines which specific co-activators and co-repressors are present, thus defining the nature of the response. An example is the induction of *tinman* in dorsal mesoderm in *Drosophila* [26] (Fig. 2). The bHLH protein Twist activates *tinman* in the entire trunk mesoderm. During gastrulation, some of this mesoderm migrates to a position under the DPP-expressing dorsal ectoderm. The *tinman* promoter contains binding sites for Tinman and MAD, and in response to DPP, pMAD acts synergistically with Tinman to maintain *tinman* expression.

Different BMP target genes are activated by different levels of pSmads. One molecular explanation for this is that they have binding sites that recognize pSmad-containing complexes with different affinities. Another mechanism involves competitive binding of pSmads and repressors. For example, the *zen* promoter in *Drosophila* contains overlapping binding sites for pMAD and the repressor Brinker [27] (Fig. 2B). *zen* is active in the dorsal mesoderm, where the high levels of pMAD outcompete Brinker for binding to the *zen* promoter; DPP signaling also represses transcription of *Brinker*, leading to a sharp boundary of *zen* expression [28].

Intracellular Negative Regulation of BMP Signaling

BMP signaling pathways are subject to several levels of negative intracellular regulation that impact developmental outcomes. One of these involves inhibitory Smads (I-Smads) that stably associate with BMP receptors and/or other Smads to interfere with signaling. Expression of I-Smads is induced by BMP signaling, generating a feedback inhibition loop [25].

An additional level of modulation occurs at the level of Smad stability. Smurfl and Smurf2 are Smad binding proteins that promote ubiquitin-mediated proteolysis of Smads and ligand-activated receptors (see references in [8]). The *Drosophila* cognate DSmurf is essential for establishing proper spatial control over DPP signaling, as *DSmurf* mutants exhibit expanded domains of DPP activity, leading to lethality [8]. In vertebrates, Smurfs may play an additional role; overexpression of Smurfl not only decreases signaling through BMP pathways, but also enhances signaling through the TGFβ pathway [29]. This may occur through

relief of competition for the common signal transduction component, Smad4 [30]. Competition between TGFβ and BMP pathways for limiting signal transduction components may be a widespread mechanism for modulating responsiveness to BMPs in vertebrates, as a number of examples of such competition have been described [31,32].

Lessons from Loss-of-Function Studies in Mammals

Over 15 different BMPs have been described in mammals, and expression of at least one is seen in almost every tissue type and stage of development, reflecting the universal importance of BMP pathways in vertebrates. Mutant strains have now been produced for most of the ligands, receptors, and Smads. Another useful approach has been to overexpress BMP antagonists or dominant-negative receptors in transgenic mice [33]. It has not been possible to uncover evidence that BMPs act as morphogens in mammals owing to the complexities of mammalian development and the lack of suitable markers. However, several important features have emerged from genetic analyses in mammals. First, they confirm widespread roles for BMP pathways in gastrulation, axis determination, and organogenesis. Second, the similar phenotypes of some mutant strains reveal the framework of the BMP pathways controlling specific developmental events. An example is the finding that mutations in the ligand nodal and the receptor ActRIIB are associated with defects in left/right asymmetry [34]. Another example is the nearly identical phenotype of mice lacking *Gdf5* or *BmprIB*, which suggests that GDF5 is a ligand for BMPRIB *in vivo* [35,36]. Third, mutant phenotypes are often more restricted than predicted from patterns of expression. One explanation that is consistent with analyses of double mutants is functional redundancy. For example, *Bmp7–/–* or *BmprIB–/–* mice have minor skeletal defects, but double mutants exhibit severe defects in long bones [35]. Hence, BMP7 and a different BMP (that acts through BMPRIB) have redundant functions in skeletal development. Surprisingly, ligands that act redundantly by genetic criteria are often not co-expressed in the affected tissues [37]. The molecular basis for the ability of ligands with nonoverlapping patterns of expression to compensate for each other genetically is not known, but may involve altered expression of other BMP ligands and/or signaling components, and reflects inductive interactions between adjacent tissues.

Conclusions

BMPs act as morphogens in both vertebrates and invertebrates. The distribution of BMP ligands often does not predict the shape of the BMP activity gradient. Clearly, ligand availability is highly regulated at the extracellular level by numerous BMP binding proteins. Many of these binding proteins can act both positively and negatively. How this occurs at a molecular level is not completely understood. Tissue-specific loss-of-function studies are needed to determine which BMP binding proteins act as positive and/or negative effectors of BMP signaling in particular developmental events.

Receptor synergy appears to play an important role in shaping BMP activity gradients in *Drosophila* and vertebrates. Additional studies will be required to delineate the mechanistic basis for this synergy, and to assess its role in specific developmental events in vertebrates. Our knowledge of how BMP signaling regulates the expression of specific target genes is increasing. It is clear from the large number of transcriptional regulators known to collaborate with Smads that multiple growth factor signaling pathways are integrated to control the nature of the response to the BMP signal. Understanding this integration on a molecular level will be challenging, but vital to our understanding of all developmental decisions in which BMPs participate.

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CHAPTER 260

Neurotrophin Signaling in Development

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Introduction

The formation of the vertebrate nervous system is characterized by widespread programmed cell death, which determines cell number and appropriate target innervation during development. The neurotrophins, which include nerve growth factor (NGF), brain-derived growth factor (BDNF), NT-3, and NT-4, represent an important family of trophic factors that are essential for survival of selective populations of neurons during different developmental periods.

The neurotrophic hypothesis postulates that during nervous system development, neurons approaching the same final target vie for limited amounts of target-derived trophic factor [17]. In this way, the nervous system molds itself to maintain only the most competitive and appropriate connections. Competition among neurons for limiting amounts of neurotrophin molecules produced by target cells accounts for selective cell survival. Two predictions emanate from this hypothesis. First, the efficacy of neuronal survival will depend upon the amounts of trophic factors produced during development. Second, specific receptor expression in responsive cell populations will dictate neuronal responsiveness.

Neurotrophins exert their cellular effects through the actions of two different receptors, the tropomyosin-related kinase (Trk) receptor tyrosine kinase and the p75 neurotrophin receptor ($p75^{NTR}$), a member of the tumor necrosis factor (TNF) receptor superfamily. On one level, neurotrophins fit well with the neurotrophic hypothesis, as many peripheral neuronal subpopulations exhibit a predominant dependence on a specific neurotrophin during the period of naturally occurring cell death (Fig. 1). However, the biological reality appears much more complex. In the central nervous system, the overlapping expression of multiple neurotrophin receptors and their cognate ligands allows for the creation of diverse connectivity, which extends well into adulthood; even in the periphery complexities remain, such as the molecular mechanisms underlying the retrograde signal, a pathway that must efficiently transmit information over long distances, at times over a meter. The role of the neurotrophin system in development has been reviewed [12]. This chapter will focus upon new views concerning ligand-receptor interactions, signal transduction, and retrograde transport in the peripheral nervous system.

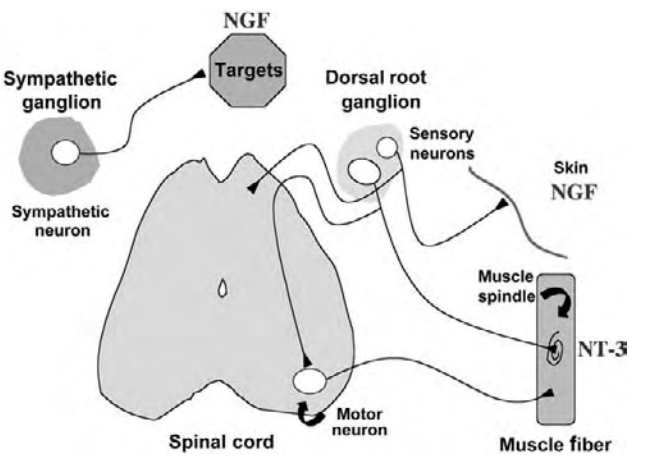


Figure 1 Neurotrophins serve as target-derived survival factors. NGF and NT-3 display specific survival and differentiative effects upon sympathetic and sensory neuron populations in the peripheral nervous system.

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The Neurotrophin Ligands

The neurotrophins are initially synthesized as precursors or pro-neurotrophins that are cleaved to release the mature, active proteins. The mature proteins form stable, noncovalent dimers and are normally expressed at very low levels during development. Pro-neurotrophins are cleaved intracellularly by furin or pro-convertases utilizing a highly conserved dibasic amino acid cleavage site to release carboxy-terminal mature proteins of approximately 13 kDa. These extensively studied mature proteins mediate neurotrophin actions by selectively binding to members of the Trk family of receptor tyrosine kinases to regulate neuronal survival, differentiation, and synaptic plasticity. In addition, all mature neurotrophins interact with $p75^{NTR}$, which can modulate the affinity of Trk:neurotrophin associations.

Neurotrophins promote cell survival and differentiation during neural development. Paradoxically, they can also induce cell death. $p75^{NTR}$ serves as a pro-apoptotic receptor during developmental cell death and after injury to the nervous system. Increases in $p75^{NTR}$ expression are responsible for apoptosis in embryonic retina and sympathetic neurons during the period of naturally occurring neuronal death [3,7]. Whereas BDNF binding to $p75^{NTR}$ in sympathetic neurons causes rapid cell death, NGF binding to the TrkA receptor on the same neurons provides a survival signal. In the context of neurotrophin processing, pro-neurotrophins are more effective than mature NGF in inducing $p75^{NTR}$-dependent apoptosis [16]. These results suggest that the biological action of the neurotrophins can be regulated by proteolytic cleavage, with pro-forms preferentially activating $p75^{NTR}$ to mediate apoptosis and mature forms selectively activating Trk receptors to promote survival.

Neurotrophin Receptors

One way of generating more specificity during development is by imparting greater discrimination of ligands for the Trk receptors. NGF binds most specifically to TrkA; BDNF and NT-4 to TrkB; and NT-3 to TrkC receptors. The $p75^{NTR}$ receptor can bind to each neurotrophin but has the additional capability of regulating a Trk's affinity for its cognate ligand. Trk and $p75^{NTR}$ receptors have been referred to as high- and low-affinity receptors, respectively. However, this is not correct since TrkA and TrkB actually bind their ligands with an affinity of 10^{-9}–10^{-10} M, which is lower than the high-affinity site ($K_d = 10^{-11}$ M). Also, the precursor form of NGF displays high-affinity binding to $p75^{NTR}$. Trk-mediated responsiveness to low concentrations of NGF is dependent upon the relative levels of $p75^{NTR}$ and TrkA receptors, and their combined ability to form high-affinity sites.

Although $p75^{NTR}$ and Trk receptors do not appear to bind to each other directly, there is evidence that complexes form between the two receptors [5]. As a result of these interactions, increased ligand selectivity can be conferred onto Trks by $p75^{NTR}$. NGF and NT-3 both can bind to TrkA, but $p75^{NTR}$ restricts signaling of TrkA to NGF and not to NT-3 [4]. Sympathetic neurons express TrkA and $p75^{NTR}$ receptors and depend upon NGF for survival. In the absence of $p75^{NTR}$, NT-3 compensates for a lower level of NGF *in vivo*. Though NT-3 binds weakly to TrkA, the survival effects of NT-3 can be attributed to activation of TrkA receptors in the absence of $p75^{NTR}$. These observations support a role for $p75^{NTR}$ in enhancing the specificity of Trk receptors for neurotrophins *in vivo*. Hence, $p75^{NTR}$ and Trk receptors may interact to provide greater discrimination among different neurotrophins.

In addition to increasing ligand-receptor affinity and selectivity, $p75^{NTR}$ contains a death domain sequence similar to the intracellular domains of the Fas and p55 TNF receptors [18]. Several examples of $p75^{NTR}$-mediated cell death now exist; these events appear to depend upon cell-specific interactions with adaptor proteins. It has been generally assumed that the mechanism of $p75^{NTR}$-mediated cell death is similar to apoptotic signaling by the TNF receptor and Fas. However, a number of experimental findings indicate that cell death by $p75^{NTR}$ occurs quite differently from that of other TNF receptor family members [6,10]. First, regions of $p75^{NTR}$ other than the death domain are responsible for apoptosis. Second, whereas recruitment of caspase-8 to the death domains of the p55 TNF and Fas receptors is critical for the initiation of apoptosis, other caspases are responsible for $p75^{NTR}$-mediated killing of oligodendrocytes.

What are the reasons for having a Trk receptor that mediates neuronal survival and a $p75^{NTR}$ receptor that mediates apoptosis? One reason neurotrophins use a death receptor may be to prune neurons efficiently during periods of developmental cell death. In addition to competing for trophic support from the target, neurons must establish connections with the proper target. In the event of mistargeting, neurons may undergo apoptosis if the appropriate set of trophic factors is not encountered. In this case, a neurotrophin may not only fail to activate Trks but also bind to $p75^{NTR}$ and eliminate cells by an active killing process [21]. For example, BDNF causes sympathetic neuronal death by binding to $p75^{NTR}$ when TrkB is absent [3]. Likewise, NT-4 causes $p75^{NTR}$-mediated cell death in BDNF-dependent trigeminal neurons [1], due presumably to preferential $p75^{NTR}$ rather than TrkB stimulation. Therefore, Trk and $p75^{NTR}$ receptors can give opposite outcomes in the same cells. Cell death mediated by $p75^{NTR}$ may be important for the refinement of correct target innervation during development.

Signaling Specificity during Development

Specific Trk receptor expression patterns determine the development of peripheral neuron populations. In the dorsal root ganglion, small-diameter unmyelinated neurons predominantly express TrkA whereas larger sized neurons express TrkC receptors. Many of the small diameter neurons are nociceptive and frequently terminate in the epidermis (Fig. 1). NGF is important for the development of these neurons

during early postnatal periods. The large-diameter neurons are proprioceptive and are most responsive to NT-3. Consistent with the receptor expression, a lack of NGF leads to a lack of responsiveness to nociceptive stimuli, and a lack of NT-3 leads to a loss of muscle spindle afferents. Due to altered expression of specific Trk receptors, neurons change their dependency upon specific neurotrophins [27].

Trk receptors exhibit very high conservation in their intracellular domains, including the catalytic tyrosine kinase and the juxtamembrane NPXY motif that serves as the Shc binding site. However, several pronounced differences among the Trks exist. In a sympathetic neuronal background, TrkA relies predominantly upon phosphoinositide-3-kinase (PI3-K) activation for survival, whereas TrkB uses both PI3-K and ERK pathways [2]. Thus, each Trk receptor carries distinctive signaling properties. For example, TrkB may contain sequences that bind to factors that favor alternative pathways. Since there are now a number of different adaptor proteins and enzymatic functions associated with Trk receptors (Fig. 2), preferential interactions with these proteins must take place. Receptor utilization of substrates with differential association/dissociation kinetics, competition for binding among different substrates, or recruitment of unique target proteins, such as FRS-2, rAPs, and SH2-B for the Trk receptors, represent mechanisms by which each receptor may differentially utilize common substrates for signaling.

Alternatively, receptor processing or targeting into different membrane compartments may dictate function. A comparison of TrkA and TrkB receptors in neuronal cell lines has revealed a difference in turnover of each receptor. While NGF binding to TrkA does not lead to a significant downregulation of TrkA, BDNF binding to TrkB results in rapid turnover of TrkB receptors at the cell surface [28]. Additionally, the number of surface TrkB receptors is highly influenced by depolarization and levels of cAMP [22]. These observations hint at other receptor mechanisms that confer greater signaling specificities to the neurotrophins.

The Importance of Retrograde Transport

During development, neurotrophins are produced and released from the target tissues and become internalized into vesicles, which are then transported to the cell body. The biological effects of neurotrophins require that signals are conveyed over long distances from the nerve terminal to the cell body. Therefore, a central theme of the neurotrophic hypothesis is that neuronal survival and differentiation depend upon retrograde signaling of trophic factors produced at the target tissue.

Each neurotrophin binds to transmembrane receptors and undergoes internalization and transport from axon terminals to neuronal cell bodies [8,11,23]. Measurements of ^{125}I-NGF transport from distal axons to the cell body in compartment chambers indicate a rate from 3–10 mm/h. Both Trk and p75^{NTR} receptors undergo retrograde transport. The term "signaling endosome" has been coined to describe membrane vesicles that carry Trk, p75^{NTR}, and NGF [9].

A complex of NGF-TrkA has been found in clathrin-coated vesicles and endosomes, giving rise to the model that NGF and Trk are components of the retrograde signal. Several tyrosine phosphorylated proteins are associated with the TrkA receptor during transport, suggesting that signaling by neurotrophins persists following internalization of their receptors. Internalization of NGF from axon terminals is necessary for phosphorylation and activation of the

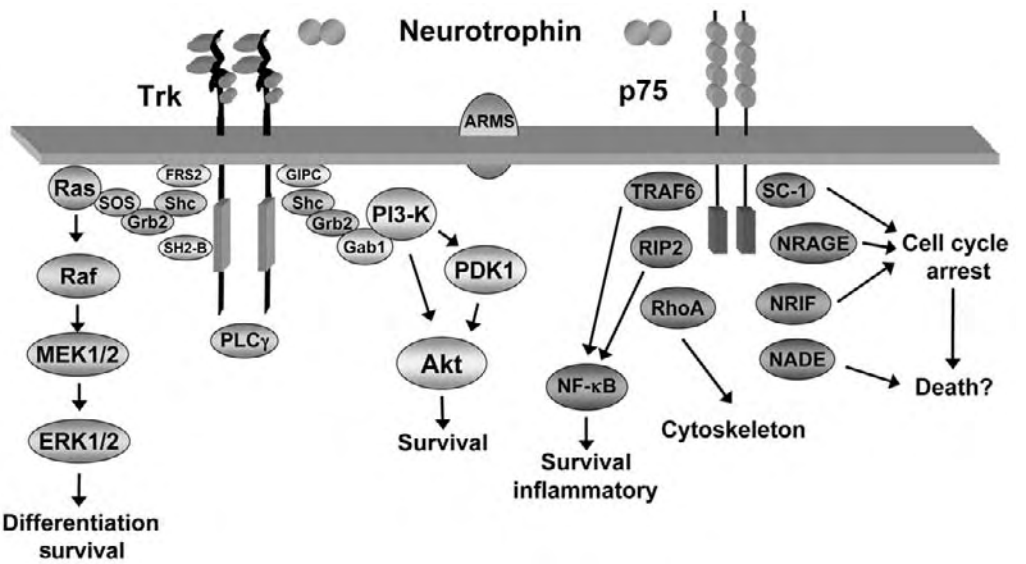


Figure 2 Neurotrophin receptors utilize multiple adaptor proteins. Each receptor undergoes ligand-induced dimerization that results in the recruitment of multiple cytoplasmic proteins, which serve to increase the activities of phospholipase Cγ, PI3-K, and MAP kinases. The p75 receptor is capable of initiating a cell death program in selected cells and signaling that leads to ceramide production and NF-κB or JNK activation.

CREB transcription factor, which leads to changes in gene expression and increased neuronal cell survival [25,30]. In addition, stimulation of Erk5 occurs in the cell body of DRG neurons after retrograde signaling by neurotrophins [29], as well as activation of PI3-K and Akt [14]. These events likely require internalization and transport of activated Trk receptors and result in a survival response. An alternative mechanism suggests that survival signals can be transmitted in the absence of NGF internalization and transport [20]. This explanation would account for the rapid appearance of phosphorylated TrkA in the cell body [26] and raises the possibility that a molecule other than endocytosed NGF may propagate a signal from distal axons to TrkA receptors at the cell body.

Interacting Proteins

The existence of neurotrophin-receptor complexes during their axonal transport suggests that other proteins may be associated with this complex. Neurotrophin receptors undergo ligand-induced dimerization that activates multiple signal transduction pathways. Neurotrophin binding to Trk family members produces biological responses through rapid increases in the phosphorylation of phospholipase C-γ and PI3-K. Increased *Ras* activity, a common signal from all tyrosine kinase receptors, results from the stimulation of guanine nucleotide exchange factors coupled to adaptor proteins which directly interact with Trk after ligand binding. These adaptor proteins include Shc, Grb2, SH2-B, and FRS-2 (Fig. 2).

A number of adaptor proteins also bind to $p75^{NTR}$ (Fig. 2). Three different proteins, NRIF, NADE, and NRAGE contribute to apoptosis in immortalized cell lines or are correlated with neurotrophin-dependent cell death. Each protein binds to a separate sequence in the cytoplasmic domain of the $p75^{NTR}$ [12]. Another protein that interacts with both $p75^{NTR}$ and Trk receptors is ARMS, an ankyrin-rich transmembrane protein [13]. ARMS is rapidly tyrosine phosphorylated after binding of neurotrophins to Trk receptors. This protein may act as a scaffold to cluster proteins essential to neurotrophin signaling. Other proteins, including RhoA GTPase, SC-1, and NRAGE, exert nonapoptotic activities, such as neurite elongation and growth arrest. These proteins expand the functional scope of neurotrophins [15]. Still other proteins, such as cytoplasmic dynein and the PDZ-domain-containing GIPC protein may serve to target neurotrophin receptors intracellularly during important cellular processes such as internalization, retrograde transport, axonal and dendritic localization, and synapse formation.

Given the wide number of activities of neurotrophins and the small number of neurotrophins and neurotrophin receptor genes, it is likely other signaling systems are used. This includes ion channels such as TRP and glutamate receptors. How these signals are integrated to yield higher level neuronal functions, such as behavior, is unknown. But there are hints from knockout animals that lowering the levels of BDNF or its receptors TrkB and $p75^{NTR}$ give rise to prominent aggressive behavior and abnormalities in eating and memory [19,24]. These findings bolster the notion that neurotrophins, in addition to their potent properties on the cellular level, possess abilities to influence cognitive functions.

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CHAPTER 261

PDGF Receptor Signaling in Mouse Development

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Introduction

Highly defined characterization of the biochemical and cellular functions of platelet-derived growth factors (PDGFs) and their receptors (PDGFRs), set the stage to use this system as a genetic model to study receptor tyrosine kinase (RTK) signaling in mammalian development. Following binding to PDGF, PDGFRs autophosphorylate, enabling them to bind and then activate intracellular proteins that relay the receptor's signals throughout the cell. PDGF stimulates cellular functions including mitogenesis, survival, and chemotaxis (for a detailed review of PDGFR signal transduction see Volume 1, Chapter 70 and the references therein). Several features of the PDGFR system make it an appropriate model to investigate whether specific signals transmitted by RTKs translate into unique physiological functions *in vivo*. First, genetic analysis is facilitated as signaling is mediated by just two highly related yet distinct RTKs, the PDGFRs α and β (PDGFαR and PDGFβR) [1]. Second, the two PDGFRs bind a highly overlapping but nonidentical repertoire of signaling proteins [2]. Third, autophosphorylation site mutant PDGFRs, which lack the ability to activate specific intracellular signaling pathways, have been well characterized [3–5]. Finally, the phenotypes of the PDGFαR −/− mice and the PDGFβR −/− mice are easily distinguishable, demonstrating that each PDGFR mediates specific functions during embryonic development [6,7]. Since the phenotypes of PDGF and PDGFR-null mutants have recently been reviewed in detail [8], only the basics will be given here. This chapter focuses on what has been learned by analyzing mice bearing specific alterations in the intracellular signaling domains of the two PDGFRs.

PDGFβR Signaling *In Vivo*

PDGFβR signaling is required at late gestation (E16.5–E18.5), as deletion of the PDGFβR results in lost integrity of the developing microvasculature leading to capillary microaneurysm and perinatal lethality [7]. Abnormal capillary development is observed in multiple organs but is prominent at the kidney glomerulus, which completely lacks a well-formed capillary tuft. This is due to the absence of mesangial cells, matrix-secreting cells related to vascular smooth muscle cells (vSMCs), which coat and maintain the integrity of glomerular capillaries. The defects observed are strikingly similar to those displayed by mice harboring a null mutation of PDGFB [9]. This is not surprising, as dimeric PDGFBB is a predominant ligand for the PDGFβR [10]. Combined analysis of the two mutant lines revealed that PDGFB/PDGFβR signaling is required for proper communication between the two major vascular cell types: endothelial cells, which express PDGFB, and vSMCs/pericytes (vSMC/PC), which express the PDGFβR [11,12]. Consistent with identified roles of PDGFBB as a mitogen and chemoattractant for vSMCs, disruption of this signaling axis results in a failure of preexisting vSMC/PC to migrate to, and proliferate along angiogenic sprouts of the developing microvasculature [11,12].

PDGFαR Signaling *In Vivo*

While loss of PDGFβR signaling results in defects restricted primarily to vSMCs and related cells, loss of PDGFαR signaling affects multiple cell types. These include

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derivatives of sclerotome and non-neuronal neural crest cells [6]. Consequently, PDGFαR-null embryos exhibit a complex and variable phenotype. The hallmarks of this phenotype include a cleft face, defects of the axial and proximal appendicular skeleton, subepidermal blebbing, and hemorrhaging. Mice lacking the PDGFαR typically die at midgestation starting at E8.5 and very few embryos survive past E15.5. Consistent with a role for PDGFαR signaling in cell survival, the cranial defects appear to be due to increased apoptosis of migrating neural crest cells. The trunk defects, including skeletal malformations, appear to arise from a deficiency in myotome formation suggesting that PDGFαR signaling participates in somite patterning. In contrast to the high phenotypic similarity between PDGFB and PDGFβR-null mice, a substantial population of PDGFA-null mice (~20%) survives well beyond gestation, a stage never achieved by PDGFαR-null animals [13]. This is explained by the fact that the PDGFαR binds multiple PDGFs including homodimers of PDGFA, PDGFB, and PDGFC in addition to heterodimers of PDGFA and PDGFB [10,14]. Studies on postnatal PDGFA–/– mutants have revealed roles for PDGFαR signaling in development of oligodendrocytes, lung alveolar myofibroblasts, intestinal mesenchyme, and Leydig cells [8].

Specificity of PDGFR Signaling *In Vivo*

One important concern in signal transduction is whether individual RTKs transmit distinct biochemical signals that translate into the ability to direct unique functions *in vivo*. Different ligand-binding affinities, patterns of gene expression, and/or mechanisms of signal transduction could all account for the functional specificity displayed by the two PDGFRs *in vivo*. Although both PDGFRs bind to many of the same signaling proteins including PI3 kinase, PLCγ, SHP-2, and Src family kinases, each receptor also binds to some proteins in an exclusive fashion. Prominent examples include binding of RasGAP to the PDGFβR but not the PDGFαR, and binding of Crk family adaptor proteins to the PDGFαR but not the PDGFβR [15–17]. Whether such differences convey an intrinsic ability upon RTKs to transmit functionally distinct signals *in vivo* is a highly debated issue. This issue was addressed in part by analysis of two complementary lines of PDGFR knockin mice [18]. In each line, the intracellular signaling domains of one PDGFR were removed and replaced with those of the other PDGFR. Since the novel chimeric PDGFR retains the ligand binding capacity and the spatiotemporal expression of the replaced PDGFR, only differences in intracellular signaling should account for any abnormal phenotypes. Mice harboring PDGFβR intracellular domains in the place of the endogenous PDGFαR regions (called αβ) developed no overt defects as homozygotes (αβ/αβ) or hemizygotes (αβ/-). This suggests that signals transmitted by the two PDGFRs are interpreted in a highly redundant manner in cells normally expressing the PDGFαR. Furthermore, mice expressing the converse chimeric receptor at the PDGFβR locus (βα) are also viable and largely normal. However, suboptimal function of cells normally regulated by PDGFβR signaling in these animals is revealed by modest heart enlargement and an impaired mesangial cell response to induced glomerular injury. Furthermore, exacerbated systemic vascular defects are observed in βα/- hemizygotes. Consistent with loss of normal PDGFβR function, the defects are due to abnormal development of vSMC/PCs and subsequent failure to coat the microvasculature. The βα/- phenotype includes perinatal lethality (~40%), substantial heart enlargement, glomerulosclerosis, and retinopathy. The retinopathy, which results in retinal detachment and migration into the vitreous body, bears striking resemblance to retinopathy of diabetes. Diabetic retinopathy primarily affects the retinal capillaries and is characterized initially by loss of PCs. Indeed, marker analysis on βα/- animals clearly demonstrates that loss of retinal PCs is the underlying cause of the observed phenotype. Cellular analysis suggests that inability of PDGFαR-type signaling to sustain MAPK activation may contribute to the decreased function of βα-expressing vSMC/PCs. Taken together, these results indicate that while the functional specificity displayed by the two PDGFRs is largely due to differences in ligand affinities and/or patterns of gene expression, PDGFβR-specific signals are required for optimal function of vSMC/PCs. Further lines of knockin mice have since been generated in which the intracellular domains of the PDGFRs have been replaced with those of further divergent RTKs. Analysis of these lines clearly shows that increased divergence from PDGFR-type signaling reduces the ability to rescue the embryological functions of the PDGFRs [19].

Another major focus of signal transduction studies is to define the contributions of distinct intracellular signaling pathways to RTK function. Many RTK-associated signaling proteins contain phosphotyrosine-binding modules such as SH2 or PTB domains [20]. Mutation of receptor tyrosine residues can be used to uncouple RTK activation from activation of specific effector proteins. Knockin mice harboring such point mutations in the PDGFRs provided a means to test contributions of distinct signaling pathways to PDGFR function *in vivo*. Studies on cultured cells expressing PDGFβR mutants identified PI3 kinase and PLCγ as the major effectors of PDGFβR-triggered mitogenesis and chemotaxis [21,22]. These analyses also demonstrated that the functions promoted by these two effectors are highly redundant. Consistent with maintenance of this redundancy *in vivo*, homozygous mutant mice expressing a PDGFβR that cannot activate PI3K still develop normally [23]. More surprising is the finding that elimination of both PI3-K and PLCγ binding sites on the PDGFβR is still compatible with normal viability [24]. *In vivo* challenge assays and chimeric analysis does reveal that loss of these signals decreases vSMC/PC function. However, the relatively normal phenotype indicates that compensating mechanisms exist, which promote a threshold level of signaling permitting vascular integrity. Microarray experiments suggest that activation of

other PDGFβR-associated signaling proteins compensate for loss of PI3-K and PLCγ signals [25]. However, PDGFβR signaling mutant mice have recently been generated that lack binding sites for SHP-2, RasGAP, Grb2, and Src family kinases, in addition to PI3-K and PLCγ, without loss of viability [26]. It is possible that a threshold of signaling required for viability is met by activating PDGFβR signaling pathways through a surrogate receptor. An obvious candidate is the PDGFαR, as heterodimerization between the two PDGFRs occurs in response to certain PDGF ligands. Thus far, PDGFαR signaling has not been shown to compensate for loss of PDGFβR signaling in cell types dependent upon PDGFβR function [24]. Perhaps interaction with less obvious cell surface receptors compensates for loss of direct PDGFβR binding to signaling proteins. Interactions between PDGFRs and integrins have been demonstrated and both activate many of the same intracellular signals [27–29]. Examination of mice harboring compound mutations in the PDGFβR and specific integrin subunits should shed light on this possibility.

In contrast to the apparent resilience of the PDGFβR to lost signals, mutations that uncouple the PDGFαR from distinct pathways cause developmental defects of varying severity. An allelic series of three PDGFαR signaling mutants has been analyzed [30]. Included in this series were mutants that prevented the PDGFαR from activating Src family kinases (α^{Src}), or PI3-K ($\alpha^{PI3\text{-}K}$), or multiple pathways including the two previously mentioned in combination with PLCγ and SHP-2 (α^{F7}). α^{Src} animals are viable past birth but suffer from neurological-related defects including shaking, seizures, and decreased limb mobility. This is due to a specific defect in oligodendrocyte development and consequent hypomyelination of the central nervous system. Consistent with the interpretation that multiple PDGFαR signals are required for oligodendrocyte development, $\alpha^{PI3\text{-}K}$ mutants also displayed this phenotype. However, while the α^{Src} phenotype is restricted to oligodendrocytes, the $\alpha^{PI3\text{-}K}$ mutation affects multiple cell types. Most $\alpha^{PI3\text{-}K}$ mutants die perinatally and exhibit defects that are similar to, albeit less severe than, PDGFαR-null embryos. While $\alpha^{PI3\text{-}K}$ mutants do not exhibit an overt cleft face, they typically display a cleft palate. This suggests that PDGFαR signaling through PI3-K is required for optimal function of non-neuronal neural crest cells. Other skeletal abnormalities resulting from loss of PI3K signaling include spina bifida, misshapen cervical vertebrae, and malformations of the shoulder girdle. Furthermore, $\alpha^{PI3\text{-}K}$ mutants exhibit abnormal placental vascularization demonstrating a requirement for PDGFαR-initiated PI3-K signaling in extra-embryonic development [19]. While signaling through PI3-K is clearly essential for PDGFαR function *in vivo*, the receptor is still able to drive development to late embryogenesis. This is not likely due to compensatory signaling by other PDGFαR-associated proteins as the α^{F7} mutation essentially phenocopies $\alpha^{PI3\text{-}K}$. Instead, it is likely that a low level of PI3-K signal, activated via heterodimer formation between mutant PDGFαRs and wild type PDGFβRs, is the compensating factor. This is based on the observation that double homozygous embryos harboring mutations in the PI3-K binding sites of both PDGFRs recapitulate the severity and the hallmark phenotypes of the PDGFαR-null mutants. These results indicate that PI3-K is the major effector of PDGFαR function *in vivo*, and that PDGFαR-initiated signals direct both specific and overlapping functions during mammalian development.

Building on work that established how PDGFRs transmit intracellular signals to carry out functions at the cellular level, genetic analysis in mice is beginning to demonstrate how these signals are utilized in the context of a living mammal. Studies are now beginning to reveal that most RTKs do not work alone, but instead participate as parts of a complex signaling matrix. Given that receptors such as integrins have been shown in cell culture systems to cooperate with PDGFRs for signal transmission, exploration of these interactions in genetic systems should be rewarding. Furthermore, while studies on mice have focused on signaling events that occur immediately following PDGFR activation, the significance of later events is largely unknown. To address this issue, gene trap screens in embryonic stem cells designed to find genes that exacerbate or ameliorate the PDGFR knockin mutant phenotypes are now underway. Such screens should provide a powerful means to identify novel players in PDGFR signal transduction *in vivo*.

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CHAPTER 262

VEGF and the Angiopoietins Activate Numerous Signaling Pathways that Govern Angiogenesis

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Angiogenesis is critical for both the growth and repair of tissues as well as for the advancement of many diseases. While there is great therapeutic potential in regulating angiogenesis, much is still to be learned about the processes that govern it. In order to design appropriate therapies to promote or inhibit blood vessel growth, we must identify factors that act selectively on the vasculature and define the mechanisms by which they act. Here we explore the signaling pathways regulated by members of two families of growth factors that act largely specifically on blood vessels: Vascular endothelial growth factor (VEGF) and the angiopoietins. These factors are involved in multiple steps of blood vessel growth where they play overlapping yet very distinct roles.

Introduction

While the great majority of endothelial cells (ECs) in the adult vasculature are quiescent, certain conditions can induce preexisting blood vessels to give rise to new vessels, a process termed angiogenesis. In some circumstances, such as wound healing, angiogenesis supports tissue repair, while in others, such as in tumors or diabetic retinopathy, angiogenesis is associated with the destruction of normal tissue. Although there are a number of processes that may contribute to angiogenesis, including vessel sprouting and branching, intussusceptive vessel growth and the recruitment of circulating endothelial cells into preexisting blood vessels, for this chapter we will focus on the expansion of the vascular tree that occurs as a consequence of the sprouting and branching of preexisting vessels.

Several events must occur in order for a vasculature to expand via sprouting angiogenesis. Endothelial cells must divide, migrate, and survive. Once a new vessel is formed, perivascular cells must be recruited to the vessel wall, and their interactions with ECs must be stabilized. Multiple factors regulate each of these events, and in some instances several factors appear to act redundantly; thus, there are numerous signaling events and interactive pathways that are associated with the growth of blood vessels. We have chosen to focus on the signaling pathways activated by VEGF and the Angiopoietins, Ang-1 and Ang-2, since these factors act far more specifically on the vasculature than other factors that regulate angiogenesis [l]. VEGF, for which both VEGFR-1 and VEGFR-2 act as receptors, appears to signal predominantly through VEGFR-2, and is required for both embryonic and adult angiogenesis [2]. Angiopoietins, which are ligands for the Tie 2 receptor, also appear to be involved in both embryonic and adult angiogenesis, although their functions are distinct from those of VEGF. Ang-1, in contrast to VEGF, is not required for the earliest stages of vascular formation in the embryo, but rather is necessary for the remodeling and stabilization of the primary vasculature [3–4]. Ang-2, which in some instances can antagonize the activation of Tie 2 by Ang-1[5], appears to play an important role in sprouting angiogenesis, possibly by destabilizing existing vessels [5–7]. Despite the cooperation between VEGF and the angiopoietins in vessel formation, these factors can also have opposing effects on vascular processes, for example, on permeability [8,9].

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Endothelial Cell Proliferation

VEGF is highly expressed in angiogenic settings and drives EC proliferation [10]. Similar to other growth factors, induction of proliferation by VEGF requires activation of the ERK (MAP kinase) cascade. In cells that are normally adherent, such as endothelial cells, the ability of receptor tyrosine kinases (RTKs) to activate the ERK pathway and to induce proliferation is dependent on integrin-mediated adhesion to matrix [12,13]. Activation of ERK in ECs by VEGF and other mitogens is enabled by multiple integrins, suggesting a general requirement for cytoskeletal assembly [14,15]. Recent data suggest that colocalization of RTKs and integrins at sites of cell attachment results in assembly of signaling complexes that enhance the coupling of RTKs to downstream effectors [16]. The specific integrins with which VEGFR-2 cooperates may depend on the composition of the matrix in a particular vessel.

In the classical model of ERK activation by RTKs, the adaptor protein Grb2, in a complex with Sos (a Ras guanine nucleotide exchange factor or GEF), binds to an activated receptor [17]. Sos promotes GTP loading of Ras, which then triggers the Raf-MEK-ERK cascade. Activated ERK translocates to the nucleus where it induces transcription of immediate early genes, thereby promoting cell cycle progression [18]. Epidermal growth factor (EGF) and bFGF, which are potent mitogens for HUVECs, stimulate the Ras-ERK pathway in this fashion [19,20]. However, the Ras-ERK pathway can also be activated via protein kinase C (PKC), which can induce formation of a Ras/Raf complex through a mechanism that is distinct from the Grb2/Sos-dependent mechanism [21]. Strong evidence suggests that VEGF, in contrast to EGF and bFGF, activates Ras-ERK signaling primarily via the PLCγ-Ca^{2+}-PKC pathway [19,20,22,23], although this appears to be cell-type dependent [24,26]. VEGF signaling to ERK via the less conventional pathway ensures that the Ras-ERK and PLCγ-Ca^{2+} pathways are activated in concert (Fig. 1). Although the functional significance of linking Ca^{2+} mobilization and ERK activation in a single signaling cascade is unclear, since bFGF can stimulate EC proliferation *in vitro* without activating the PLCγ-Ca^{2+} pathway [20], it is possible that Ca^{2+} signaling helps to create an environment that is permissive for angiogenesis. *In vivo*, EC proliferation may require disruption of interendothelial junctions, a process which may be critically dependent on Ca^{2+} (see the following section).

Interestingly, Ang-1 does not induce EC proliferation *in vitro* [3]. The inability of Ang-1 to drive proliferation may reflect its relatively weak (as compared to VEGF) stimulation of the ERK pathway [27] or its failure to activate the PLCγ/Ca^{2+} pathway (C. Daly and J. Holash, unpublished data).

VEGF Promotes Vascular Permeability

VEGF rapidly and potently increases the permeability of vessels to plasma proteins (e.g., fibrinogen)[28]. Although it has been proposed that extravasated plasma proteins provide a scaffold for EC migration during angiogenesis [28], the precise role of VEGF-induced permeability is unclear. It is possible that the destabilizing effects of VEGF on interendothelial junctions, which lead to plasma protein leak, are also important in allowing ECs to adopt a proliferative and/or migratory phenotype.

Plasma protein extravasation in response to VEGF appears to occur via intercellular gaps [29], although a transcellular route dependent on vesicular transport has also been reported [30]. Studies of other permeability-inducing agents such as thrombin indicate that formation of actomyosin

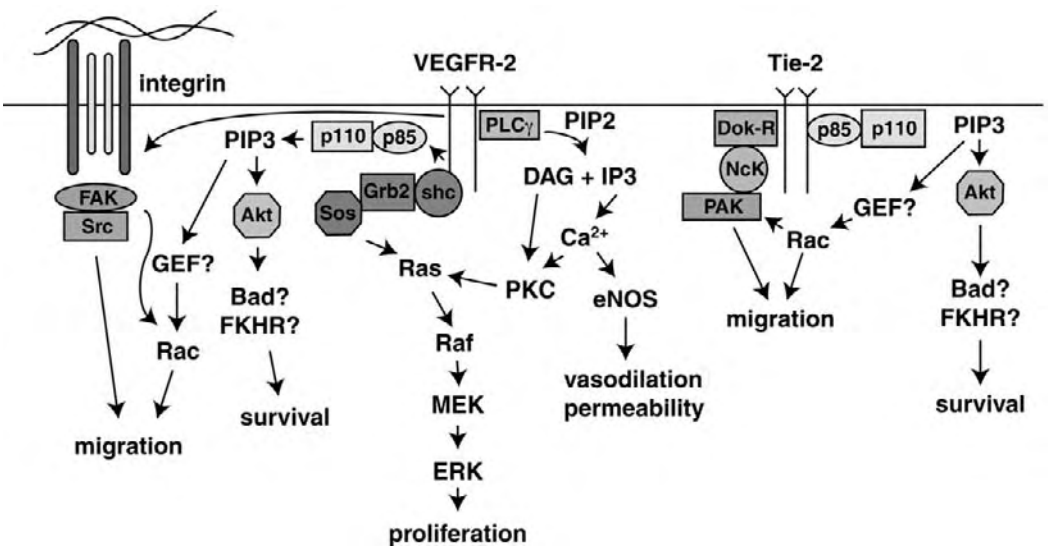


Figure 1 Major signaling pathways activated by VEGFR-2 and by Tie 2. The diagram is not meant to be comprehensive, but outlines some of the better characterized pathways employed by these two receptors. See the text for details.

stress fibers, which presumably create the tension required to form intercellular gaps, is important in controlling permeability[31]. Stress fiber formation is promoted by phosphorylation of myosin light chain, which can occur via Ca^{2+}-mediated activation of myosin light chain kinase or via activation of the small GTPase Rho [32]. Ca^{2+} mobilization is required for VEGF-induced permeability, consistent with a possible role for myosin light chain kinase [33]. In addition, VEGF appears to activate Rho and to promote stress fiber formation in HUVECs (J. Connolly and A. Hall, personal communication), although a requisite role for Rho in VEGF-induced leak remains to be demonstrated.

In addition to stress fiber formation, increases in permeability are likely to involve destabilization of the protein complexes at cell-cell junctions. VEGF has been reported to promote tyrosine phosphorylation of multiple components of EC adherens junctions, a modification which likely decreases junctional stability [34]. However, the precise mechanisms through which VEGF modulates cell-cell junctions remain to be elucidated.

eNOS, which is activated downstream of Ca^{2+} mobilization, is required for VEGF-induced permeability [35]. The generation of NO activates guanylate cyclase, resulting in cGMP production. cGMP appears to promote permeability, at least in part, by decreasing cAMP levels, although the mechanism(s) through which cAMP levels directly influence permeability are unknown [33].

Ang-1 Inhibits Vascular Permeability

Unlike VEGF, Ang-1 makes vessels resistant to leak. Transgenic overexpression of Ang-1 in the skin, or systemic administration of Ang-1, can block permeability induced by a number of inflammatory mediators including VEGF [8,9]. Intraocular injection of Ang-1 in diabetic mice reduces the edema that results from breakdown of the blood-retinal barrier, suggesting that it may be therapeutic in diabetic retinopathy [36]. The signaling mechanisms whereby Ang-1 blocks vascular permeability remain unknown, but might involve effects on the cytoskeleton and/or on cell-cell junctions.

Vessel Destabilization and EC Migration

Sprouting and migration of ECs requires the disruption of EC-perivascular cell interactions. Although signaling mechanisms controlling these interactions are not completely understood, Ang-1, as well as PDGFB [37] and TGFβ [38] appear to play important roles. The notion that Ang-1 contributes to vessel stabilization derives from the observation that Ang-1-null embryos exhibit disrupted endothelial-perivascular cell interactions [4]. Several studies have correlated Ang-2 expression in ECs with angiogenic sprouting [5,6,39], consistent with a role for Ang-2 (by antagonizing Ang-1 action [5]) in vessel destabilization [7]. However, the mechanisms through which Tie 2 signaling affects EC-perivascular cell interactions remain to be elucidated, and other factors are likely to contribute to vessel destabilization.

An essential aspect of cell migration is cytoskeletal rearrangement, a process in which Rho family GTPases play a critical role [40]. Rac, a member of the Rho family, is required for growth-factor-induced cell movement [40,41]. Through activation of a number of effector proteins, Rac promotes polymerization of actin in lamellipodia at the leading edge of motile cells [40]. VEGF has been shown to promote EC migration in a Rac-dependent fashion [42]. The pathway by which VEGF activates Rac is undefined, but may involve PI3-K-dependent stimulation of a Rac GEF [43]. Subsequent to Rac-induced lamellipodia formation, focal complexes containing clustered integrins are formed, presumably stabilizing the newly formed cell protrusion [44,46]. Focal complexes contain a variety of signaling molecules, including focal adhesion kinase (FAK), which is inducibly phosphorylated on tyrosine in response to integrin clustering and/or RTK activation [47]. VEGF has been shown to promote tyrosine phosphorylation of FAK [48], which in turn couples to multiple downstream signaling pathways, including Rac and the kinase Src [47]. Interestingly, Src appears to be required for VEGF-induced migration and angiogenesis [49,50].

Ang-1 has also been shown to induce EC migration *in vitro* [51,53], via a pathway involving Rac and the Rac effector PAK (Fig. 1)[54]. PAK, a kinase that inhibits actin depolymerization [55] and that is required for EC migration [56], is recruited to tyrosine-phosphorylated Tie 2 by the adaptors Dok-R and Nck [54,57], and subsequently becomes activated [54]. Understanding the significance of Ang-1-mediated activation of the PAK pathway will require further investigation, since the conditions under which Ang-1 is an important regulator of EC migration remain to be determined.

Regulation of EC Survival during Angiogenesis

ECs depend upon cell-cell and cell-matrix contacts for survival [58,59]. During angiogenesis, when matrix attachments and cell-cell contacts within stable vessels are disrupted in preparation for EC migration, the cells become dependent on VEGF for survival [60–63]. Once vessels mature and recruit pericytes, their dependence on VEGF is reduced [61,64], perhaps because pericytes elaborate additional survival factors or promote maturation of EC-matrix contacts.

Consistent with *in vivo* observations, VEGF promotes the survival of ECs *in vitro* under conditions of serum withdrawal [65,66]. The pro-survival effect of VEGF, like other growth factors, depends on activation of the PI3-K pathway [65,66]. Although VEGF induces tyrosine phosphorylation of the p85 subunit of PI3-K [67,68], it is unclear whether p85 interacts directly with VEGFR-2; it has been proposed that VEGF activates p85 indirectly, via a FAK dependent mechanism [69]. One critical component of the PI3-K pathway is AKT, a kinase which phosphorylates and inhibits

several pro-apoptotic proteins, including Bad and the transcription factor FKHR [70]. Whether these AKT targets are relevant to VEGF signaling is unknown. In terms of pro-survival genes, VEGF induces the expression of bcl-2, survivin, Al, and xiap [71–74], by signaling pathways that remain to be characterized.

Ang-1 also promotes survival of ECs *in vitro* via the PI3-K/AKT pathway [75–77]. Interestingly, Ang-1 is a significantly more potent activator of the AKT pathway than is VEGF [78], suggesting that Ang-1 is likely to be an important regulator of EC survival *in vivo*. The AKT targets which are important in Ang-1 signaling remain to be identified. Like VEGF, Ang-1 increases survivin expression, although the underlying mechanism is unknown [77]. Recent data from partially-rescued Tie 2 knockout mice suggest that the Ang-1/Tie 2 signaling system is important for EC survival during vascular development [79]. Interestingly, activated Tie 2 can be detected in the quiescent vasculature of several adult tissues [80], suggesting that Ang-1 has a role in vascular maintenance, possibly including a pro-survival role.

Conclusion

The development of new blood vessels is an extraordinarily complex process. Although numerous growth factors have been evaluated for their angiogenic potential, very few factors that have specificity for the vasculature have been identified. It seems that most factors that are believed to have angiogenic activity also mediate numerous other processes, limiting the therapeutic potential of manipulating these systems. Thus, in this chapter we have focused on how VEGF and the angiopoietins, which are largely specific for the vasculature, may mediate a number of steps that are required for blood vessel formation.

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CHAPTER 263

Vascular Endothelial Growth Factors and their Receptors in Vasculogenesis, Angiogenesis, and Lymphangiogenesis

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Vasculogenesis, Angiogenesis, and Lymphangiogenesis

During embryogenesis, blood and lymphatic vasculature develops through the regulated proliferation, migration, and differentiation of endothelial cells. Initially, a primary blood vascular plexus is formed in the process of vasculogenesis, where mesoderm-derived angioblasts differentiate into endothelial cells and assemble to form a network of uniformly sized primitive vessels [1]. The subsequent remodeling of the primary plexus to form a more complex treelike hierarchy of large and small vessels is called angiogenesis. Angiogenesis occurs by sprouting and branching of new vessels from the preexisting ones, followed by progressive pruning and remodeling of the vessels into a mature vascular system [1]. Periendothelial support cells, such as vascular pericytes and smooth muscle cells, are then recruited to the vessel wall and the extracellular matrix is reconstituted [2]. Vasculogenesis is probably restricted to early development, but physiological angiogenesis occurs in adult organisms during the female reproductive cycle and in wound healing. Pathological angiogenesis occurs in several diseases such as rheumatoid arthritis and diabetic retinopathy and during tumor growth [3]. The observation that solid tumors are dependent on neovascularization has brought endothelial cell signaling mechanisms and molecules into focus in the field of cancer research.

The lymphatic vessels arise from the embryonic veins through a process termed lymphangiogenesis. The lymph vessels become organized parallel to the blood vascular system and function to transport interstitial fluid, extravasated plasma proteins, and cells back into the blood circulation [4]. The lymphatic vessels also form a part of the immune system together with the lymphoid organs. Lymphatic capillaries consist of a thin, permeable layer of endothelial cells that are anchored to the surrounding connective tissue by elastic filaments called anchoring fibrils. The capillaries have a discontinuous basement membrane and only a few scattered support cells. The lymphatic capillaries transport the fluid into progressively larger collecting vessels consisting of endothelial, muscular, and adventitial layers, which ultimately drain into the venous circulation via the thoracic duct. Movement of lymph is brought about by the intrinsic contractility of the smooth muscle cells surrounding the larger vessels, and backflow is prevented by luminal valves [4]. Lymphedema, the accumulation of protein-rich fluid in interstitial tissues, occurs in humans either as hereditary disease or due to lymph vessel damage or removal. Because of

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the metastatic spread of tumor cells via the lymphatic system and tumor lymphangiogenesis, the lymphatic endothelial cell signaling is also of interest in cancer biology.

The Vascular Endothelial Growth Factors and their Receptors

Signaling by the vascular endothelial growth factor (VEGF) family members and their receptors (VEGFRs) is essential in vasculogenesis and angiogenesis, and has more recently been shown to be critical in lymphangiogenesis as well. The VEGF family consists of five members thus far, namely VEGF, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF). All family members are secreted glycoproteins that possess a VEGF homology domain belonging to the cysteine knot growth factor family, and they function as disulfide-linked anti-parallel dimers [5,6]. Out of the VEGF family, only VEGF itself is induced by hypoxia, via an intricate signal transduction mechanism involving prolyl hydroxylation and specific ubiquitin ligase complexes [7].

VEGFs transduce signals mainly via three known VEGFRs, VEGFR-1/Flt-1, VEGFR-2/Flk-1/KDR, and VEGFR-3/Flt-4. VEGFRs are high-affinity receptor tyrosine kinases, restricted largely to endothelial cells, and they are structurally and functionally related to the PlGF receptors [8].

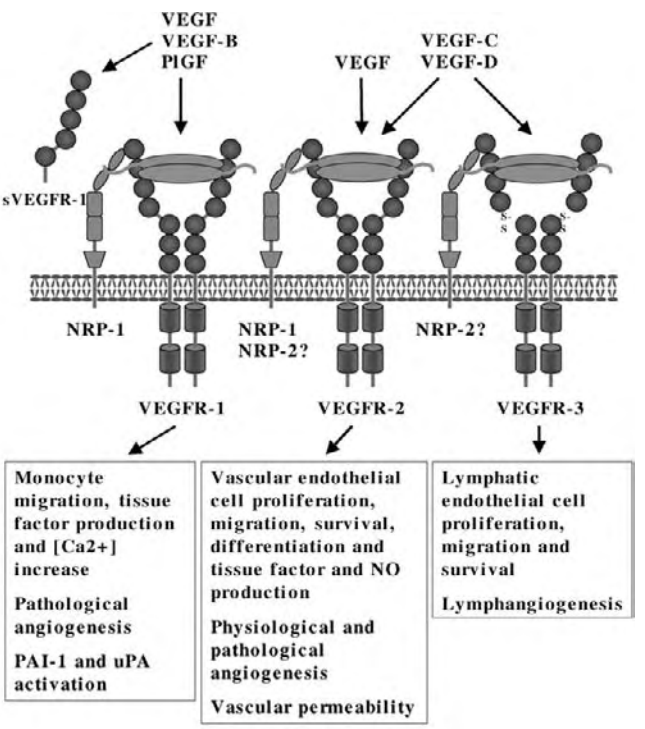


Figure 1 The VEGF receptors and their ligands. VEGFR-1 and VEGFR-2 have seven extracellular immunoglobulin homology domains (circles); in VEGFR-3 the fifth immunoglobulin domain is cleaved on receptor processing into two disulfide-linked subdomains. sVEGFR-1, soluble VEGFR-1; NRP, neuropilin; PAI-1, plasminogen activator inhibitor-1; uPA, urokinase-type plasminogen activator; NO, nitric oxide.

The VEGFRs have seven immunoglobulin homology domains in their extracellular part, and an intracellular tyrosine kinase domain split by a kinase insert sequence. VEGFR-1 exists also in a soluble form produced by alternative splicing. The VEGF family members have different binding specificities for the VEGFRs, schematically shown in Fig. 1. Neuropilins-1 and -2, which were originally characterized as receptors for semaphorins in the nervous system, also selectively bind members of the VEGF family in an isoform-specific manner and seem to act as co-receptors with VEGFRs, possibly acting by clustering of receptors (Fig. 1) [9,10]. Several excellent reviews have appeared that detail the structures of VEGFs and their receptors and their function in angiogenesis [5,8,11,12].

VEGF and VEGFR-1 and -2 are Essential for Vasculogenesis and Angiogenesis

Hemangioblasts, as yet ill-defined precursor cells for both the vascular endothelial and hematopoietic lineage [13,14], are thought to differentiate from pluripotent epiplastic precursor cells under the inductive influence of several factors, including the fibroblast growth factor [15]. VEGFR-2 is the earliest marker for hemangioblasts [16], and the importance of both VEGF and its receptors VEGFR-1 and VEGFR-2 in vasculogenesis and hematopoiesis is clear from the knockout mouse phenotypes. Inactivation of a single VEGF allele resulted in embryonic lethality between embryonic days 11 and 12 (E11–12), indicating that the effect of VEGF on vasculogenesis is dosage-dependent [17,18]. The $VEGF^{+/-}$ embryos appeared growth retarded and exhibited a number of developmental anomalies, including severe cardiovascular defects and reduced number of red blood cells. Mouse embryos lacking VEGFR-2 died at E8.5–9.5, and showed a total lack of vasculogenesis and hematopoiesis [19]. *In vitro* studies indicate that VEGFR-2 is not required for the formation of hemangioblasts, but is necessary for endothelial cell commitment [20]. Knockout studies have demonstrated the necessity of VEGFR-1 for vasculogenesis. VEGFR-1-null mice died at E8.5–9.5, and while their endothelial cells differentiated normally, they showed an increase in numbers and failed to organize into proper channels [21,22]. Disruption of VEGFR-3 also led to embryonic death due to defective remodeling of the vascular plexus and cardiovascular failure, but vasculogenesis appeared to occur normally [23].

VEGFR-2 appears to be the major receptor that conveys VEGF-induced signals in endothelial cells. In various endothelial cell types VEGF induces strong VEGFR-2 autophosphorylation, activation of the MAP kinase cascade, and the PI3-K-Akt pathway, cell survival, proliferation, and chemotaxis. The signal transduction via VEGFRs is summarized in Fig. 2; for reviews on VEGF signaling, see references [24] and [25]. Experiments with mice having the VEGFR-1 tyrosine kinase domain deleted have shown that the ligand-binding and transmembrane domains of the receptor are sufficient for normal vasculogenesis and angiogenesis [26].

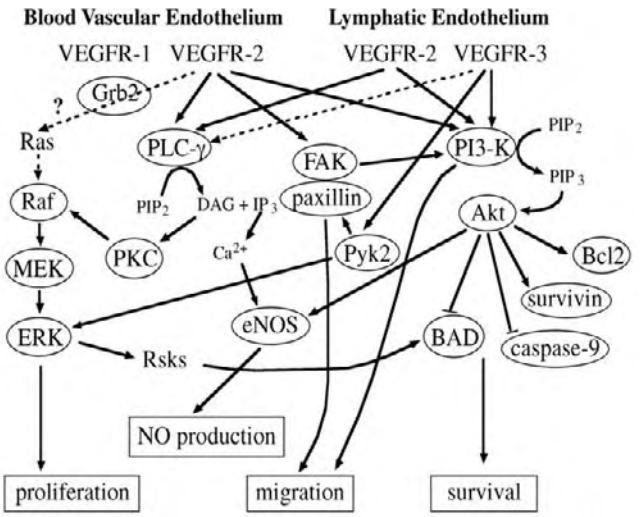


Figure 2 The major known VEGFR signaling pathways in blood, vascular, and lymphatic endothelium. PLC-γ, phospholipase C-γ; FAK, focal adhesion kinase; PI3-K, phosphatidylinositol 3′ kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PKC, protein kinase C; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; MEK, MAPK/ERK kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; Rsks, ribosomal protein S6 kinases; eNOS, endothelial nitric oxide synthase; NO, nitric oxide. (Figure modified from Karkkainen, M. J. *et al*., *Nat. Cell Biol*., **4**, E2–E5. With permission. See also references [24] and [43]).

Thus it has been postulated that VEGFR-1 does not have a signaling role in these processes, but acts as a VEGF sink to negatively regulate VEGFR-2 signaling. Yet, PlGF, which only binds VEGFR-1 is essential for all forms of pathological angiogenesis in adult tissues [27].

Lymphangiogenesis is Regulated by VEGFR-3 and its Ligands VEGF-C and -D

VEGFR-3 is initially expressed in all embryonic endothelial cells, but becomes restricted to lymphatic vessels later in development [23,28]. VEGF-C and -D bind to and activate VEGFR-3 and in their fully mature, proteolytically processed forms, are also ligands for VEGFR-2 [29,30]. Both VEGF-C and -D have been shown to be lymphangiogenic. VEGF-C can induce lymphangiogenesis when administered as a recombinant protein onto the avian chorioallantoic membrane [31] or when overexpressed as a transgene in mouse skin [32]. A VEGFR-3-specific mutant form of VEGF-C also induced lymphangiogenesis in the skin [33], indicating that the lymphangiogenic effects of VEGF-C are transduced via VEGFR-3. Furthermore, a soluble form of VEGFR-3 expressed under the control of the same skin-specific promoter caused regression of developing lymphatic vessels by inducing endothelial cell apoptosis [34]. The dermal blood vessels were not affected, confirming the specificity of VEGFR-3 function in lymphatic endothelial cells in later development. In this model, lymphatic vessels were initially lost in several internal organs, but partially regenerated in adult mice [34].

Human early-onset primary lymphedema has been shown to be linked to mutations in the *VEGFR-3* gene that inactivate the tyrosine kinase [35,36]. Chy mice that have a similar mutation developed lymphedema of limbs due to lack of subcutaneous lymphatic vessels, but new lymphatics were induced to grow by VEGF-C therapy via viral gene delivery, suggesting that human lymphedema could also be treated with VEGF-C/D therapy [37]. Interestingly, VEGF-C and -D have also been shown to promote tumor lymphangiogenesis and lymphatic metastasis is mouse tumor models [38–42], and both soluble receptors and blocking antibodies could inhibit lymphatic metastasis [38,42].

Little is known about the signaling mechanisms involved in lymphangiogenesis. VEGFR-3 signaling has been investigated in isolated lymphatic endothelial cells utilizing the VEGFR-3-specific mutant form of VEGF-C. It was found that VEGFR-3 signaling can promote the growth, survival, and migration of lymphatic endothelial cells. VEGFR-3 phosphorylation leads to PI3-kinase-dependent Akt activation and protein kinase C-dependent activation of the p42/p44 MAPK (Fig. 2) [43].

Concluding Remarks

The complicated signaling networks regulating the growth and maintenance of blood–and lymphatic vasculature are only beginning to be elucidated. In addition to the VEGF/VEGFR system, two other receptor tyrosine kinase systems have been shown to be important in endothelial cells of vasculature (reviewed in [44]). Tie 1 and Tie 2/Tek receptors and the angiopoietins, which are Tie 2 ligands, seem to be especially important in regulating the stability of the vasculature. The Eph receptors and their Ephrin ligands, were originally discovered as regulators of the nervous system. There are interesting parallels between vascular and nervous development, and also the VEGF/VEGFR system may have a function in nervous development. The intricate interactions between the different signal transduction systems and the similarities and differences between the regulation of endothelial cell signaling in different parts of blood and lymphatic vasculature pose additional challenges in this field of research.

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CHAPTER 264

Signaling from FGF Receptors in Development and Disease

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Introduction

Fibroblast growth factor receptors (FGFRs) are members of the receptor tyrosine kinase family which consists of four structurally related members: FGFR 1, 2, 3, 4, and their alternatively spliced variants. Each receptor contains two to three immunoglobulin (Ig)-like domains in the extracellular region, an acidic box between Ig-1 and Ig-2, a transmembrane domain, and a split intracellular tyrosine kinase domain (Fig. 1). Normal activation of FGFRs relies on interaction with their ligands: fibroblast growth factors (FGFs). At least 22 FGFs are known to date, and each FGFR member interacts with a specific subset of FGFs, as summarized in Fig. 1 (reviewed in [38] and reviewed in [11]). Heparan sulfate proteoglycans facilitate FGF binding with FGFR to induce FGFR dimerization and activation [44]. Crystal structure analyses have indicated that heparin binds to two FGFs such that the FGFs are not directly interacting with each other, and that each FGF is binding to an FGFR (reviewed in [19]). Another study has shown, however, that the heparin-FGF monomer alone can activate FGFR dimerization [39]. After ligand stimulation, FGFRs undergo dimerization and autotyrosine phosphorylation, resulting in kinase activation. The phosphorylated tyrosine residues may then serve as binding sites for secondary signaling molecules.

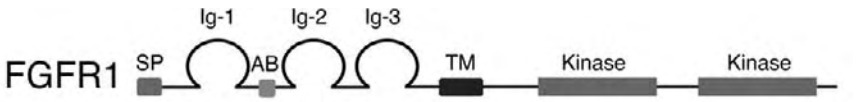
Expression of FGFR during Development

FGFRs are widely expressed during development and in adult tissues [18]. FGFR1 is expressed in the central nervous system and developing limbs during development. During early neurogenesis, FGFR1 expression is upregulated in the ventricular zone of the neural tube and is expressed in the mesenchyme of developing limbs [35,52]. At later stages of development, FGFR1 can be found in maturing neurons in the brain and in motor neurons in the spinal cord [17,35]. FGFR2 is also expressed in the brain, particularly in the developing mid- and hindbrain [35,47,52]. In addition, FGFR2 and FGFR3 expression occurs highly in the trophectoderm and to some degree in the mesenchyme [30,35,36]. Importantly, FGFR3 is expressed in the hypertrophic and proliferative zones of the bone growth plate, cochlea, brain, and spinal cord [6]. Expression levels of FGFR4 have not been examined as thoroughly as other FGFR family members, but FGFR4 has been shown to be expressed in definitive endoderm and skeletal muscle lineages, as well as the ventricular zone of developing spinal cord and dorsal root ganglia [27,34,45].

Role of FGFR in Development

Genetic alterations in mice reveal the importance of FGFR during development. In FGFR1 and FGFR2, null mutations result in embryonic lethality due to defects of mesodermal patterning or trophoblast cell proliferation [2,7,53,55]. FGFR1 chimeric mice further demonstrate that FGFR1 is necessary for neural tube and limb development [9], while FGFR2 chimeras indicate that FGFR2 is required for both limb outgrowth and branching morphogenesis of the lungs [2]. Additionally, FGFR2 has been shown to be important for keratinocyte differentiation [50]. FGFR3 null mice exhibit

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Member	Syndrome	FGF Interaction
FGFR1	Pfeiffer	FGF 1-6, 8, 10, 17
FGFR2	Apert Beare-Stevenson Crouzon Jackson-Weiss Pfeiffer	FGF 1-10,17
FGFR3	Achondroplasia Hydpochondroplasia SADDAN TDI TDII CAN	FGF 1, 2, 4, 8, 9,17
FGFR4	No known syndromes	FGF 1, 2, 4, 6, 8, 9,17, 18, 19

Figure 1 Structure of FGFR1 is shown as representative of FGFR1, FGFR2, FGFR3, and FGFR4. SP, signal peptide; Ig, immunoglobulin-like domain; AB, acidic box; TM, transmembrane domain. Developmental syndromes associated with different FGFRs are summarized. SADDAN, severe achondroplasia with delayed development and acanthosis nigricans; TD, thanatophoric dysplasia; CAN, Crouzon syndrome with acanthosis nigricans.

bone defects and deafness, demonstrating that FGFR3 is important for normal skeletal and ear development [6,8]. The FGFR4 null mutant mice appear to be normal, but have depleted gallbladders and elevated liver bile acids [56].

Syndromes Associated with FGFRs

Activating mutations of FGFR1, FGFR2, and FGFR3 can lead to craniosynostosis syndromes of various degrees of severity as listed in Fig. 1. Craniosynostosis is characterized by the premature fusion of the cranial sutures between the developing flat bones of the skull, resulting in abnormal head shape, midface hypoplasia, and other skeletal defects. Related syndromes can be distinguished by examining the extremities [16,49,51]. For example, a mutation in FGFR1 which causes Pfeiffer syndrome, a classic form of craniosynostosis, is characterized by broad, sometimes medially deviated thumbs and big toes. Mutations in FGFR2 result in a broad group of craniosynostosis syndromes, including Pfeiffer, Apert, Beare-Stevenson, Crouzon, and Jackson-Weiss syndromes. Mutations in FGFR3 are generally responsible for skeletal dysplasia syndromes, including thanatophoric dysplasia (TD) types I and II, hypochondroplasia, achondroplasia, severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN), and Crouzon syndrome with acanthosis nigricans (CAN). These FGFR3 syndromes range from a mild form of dwarfism (hypochondroplasia) to neonatal lethal dwarfisms (TD I and II). There are no known craniosynostoses or skeletal syndromes associated with mutations of FGFR4.

Signaling Pathways Mediated by FGFRs

FGFRs play key roles in cell proliferation, differentiation, migration, wound healing, survival, and angiogenesis. Ligand activation of FGFR results in kinase activity and autophosphorylation of tyrosine residues, thereby creating specific binding sites for downstream effector molecules that coordinate FGFR-mediated signaling. For example, phospholipase C-γ (PLC-γ) binds to activated FGFR1 and is phosphorylated by FGFR1, resulting in protein kinase C (PKC) activation (reviewed in [4]).

FGF receptor substrate 2 (FRS2), a membrane-associated docking protein, binds to the juxtamembrane of FGFR1 via its phosphotyrosine binding (PTB) domain [53]. The Grb2/Sos complex is recruited to the plasma membrane via FRS2 and activates Ras to trigger the mitogen-activated protein kinase (MAPK) pathway and expression of nuclear proteins including Fos and Myc [12,13,22,32]. In addition, activated FGFR1 results in FRS2 binding to Grb2 and leads to tyrosine

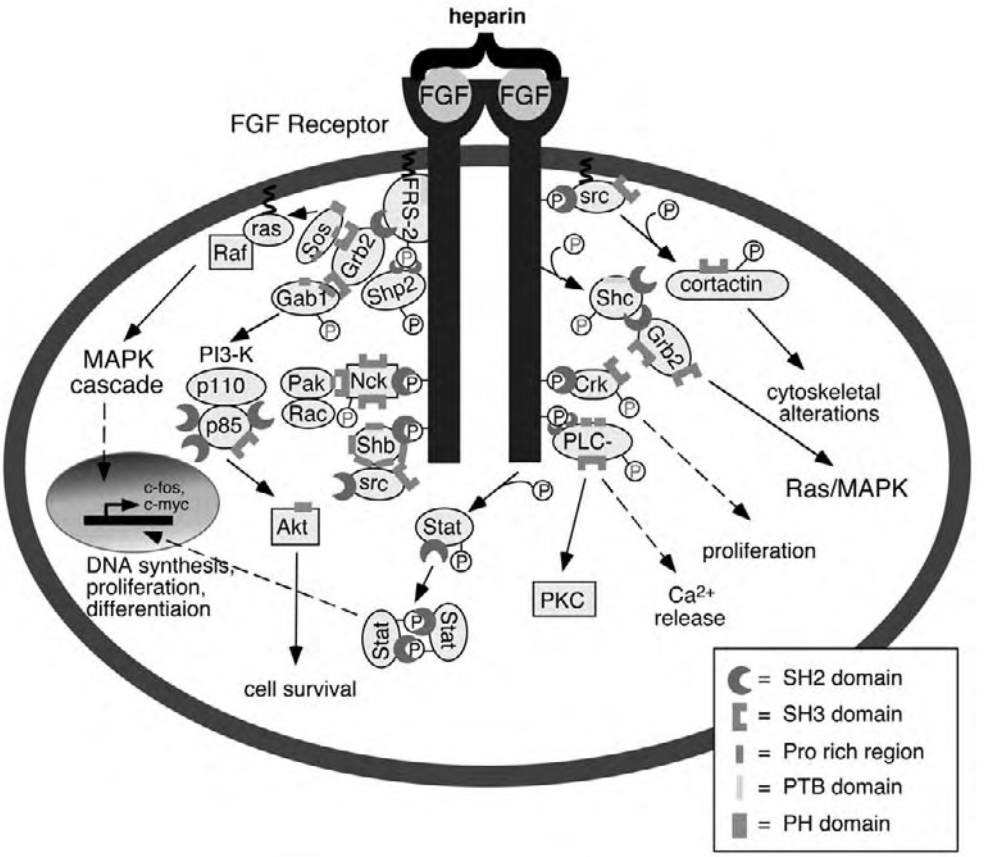


Figure 2 Intracellular signaling pathways activated by FGFRs are summarized. Tyrosine phosphorylation of receptor subunits facilitates recruitment and assembly of activated signaling complexes.

phosphorylation of a Grb2-associated docking protein named Gab1, which recruits and activates the phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway for cell survival [33]. Interestingly, Grb2 can interact with Sos and Gab1 simultaneously, allowing activation of both Ras/MAPK and PI3-kinase/Akt pathways [33]. Furthermore, the tyrosine phosphatase Shp2 becomes phosphorylated and interacts with the SH2 domain of Grb2 to form a complex with tyrosine phosphorylated FRS2, providing another link of activated FGFR to the Ras/MAPK signaling cascade [14,31,32,22]. Shp2 can also interact with Gab1 [31].

Adapter proteins play significant roles in FGFR-mediated signaling. Activated FGFR1 results in tyrosine phosphorylation of Shc. Shc binds to Grb2 and may link FGFR1, but not FGFR4, to the Ras signaling pathway [3,41,48]. The adapter protein Crk also interacts with phosphorylated FGFR1, becomes tyrosine phosphorylated, and results in cell proliferation [24]. In addition, Nck binds to activated FGFR1 and becomes tyrosine phosphorylated, potentially linking FGFR1 to the actin cytoskeleton since Nck facilitates the interaction between Pak and Rac [42], and reviewed in [26]. The adapter protein Shb also binds to FGFR1 and is tyrosine phosphorylated; Shb thus appears to be an adaptor protein linking SH3 domain proteins to tyrosine kinases or other tyrosine phosphorylated proteins [5,20]. Chimeric receptors composed of the extracellular domain of PDGFR-β and the transmembrane and intracellular domains of FGFR1, FGFR3, and FGFR4 were able to activate phospholipase Cγ, Shc, FRS2, and the mitogen-activated protein kinases, ERK1 and 2 [40]; in this study, however, these chimeric receptors did not appear to couple through Shc.

FGFRs are also implicated in other signaling pathways. Src is a non-receptor tyrosine kinase recruited by FGFRs and phosphorylates cortactin to affect cell migration; it remains unclear whether Src directly binds to FGFR1 [23,25,57]. Furthermore, activated FGFR 1, 3, and 4 promote Stat1 and Stat3 activation [15,46]. In the case of FGFR3, the adapter protein SH2-B has been found to activate Stat5 [21]. Most of these interactions and signaling pathways activated by FGFR mentioned above are summarized in Fig. 2, using FGFR1 as the model.

Summary

FGFRs play vital roles in growth, development, differentiation, and migration. Genetic analyses of FGFRs indicate their importance in organ and neuronal development. In addition, FGFs activate FGFRs to trigger multiple signaling pathways in the cell. Unfortunately, space has not permitted a discussion of the role of somatic FGFR mutations in human cancer. Many new pathways of FGFR signaling undoubtedly

remain to be discovered that will be important in understanding mammalian development and also the control of abnormal cell proliferation in cancer.

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CHAPTER 265

The Role of Receptor Protein Tyrosine Phosphatases in Axonal Pathfinding

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Introduction

One of the most impressive processes that occurs during development is the establishment of countless connections between neurons and their targets. Such precise connectivity requires long-distance growth and pathfinding by axons and short-range detection of target cells. This chapter reviews one family of molecules, the receptor-like protein tyrosine phosphatases (RPTPs), which direct axons in this astonishing feat. In the human genome there are around 22 RPTP genes, most of which have either orthologs or homologs in other species. Most RPTPs are strongly expressed, some times exclusively, in developing nervous systems, in particular within axons and their motile, pathfinding growth cones [1,2]. Evidence is reviewed here for RPTP roles in axon growth and guidance. Their potential signaling mechanisms are also briefly discussed. Due to space limitations, readers will, in most cases, be referred to two reviews and the references therein [1,2]. Figure 1 summarizes the axon growth and guidance events discussed in the following sections, and the RPTPs implicated in each. Other axonal receptor types are reviewed elsewhere in this volume.

RPTPs and the Visual System

Drosophila

The compound eye of the fly contains about 800 ommatidia, each with photoreceptor neurons R1 through R8. Axons of photoreceptors project to the optic lobe where they terminate either in the lamina (R1–R6) or in proximal layers (R8) or distal layers (R7) of the medulla. DPTP69D and DLAR control these axonal termination events [1,3,4]. If axons of R1–R6 are made DPTP69D-deficient, they will overshoot their target and terminate in the medulla. Loss of DPTP69D in R7 causes its axon to stop short in the R8 termination zone. DPTP69D thus appears to control the ability of growth cones to de-adhere (defasciculate) from R8 at correct navigational decision points. Interestingly, R7 axons that lack DLAR can reach and recognize medulla targets, but they later retract [3,4]. DLAR-deficient axons from R1–R6, however, terminate normally. DLAR may therefore be involved in specifically stabilising adhesion of R7 to its targets. DLAR mutants and cadherin mutants have similar phenotypes, suggesting that they may regulate similar adhesive signaling pathways [3]. The collective data also indicate that DPTP69D and DLAR function cell autonomously, although DLAR also shows evidence of nonautonomous function in R8. In contrast to their guidance roles, DRPTPs do not appear to be necessary for axon elongation in the visual system, unlike their vertebrate counterparts.

Vertebrate Retinotectal System

In vertebrate eyes, retinal ganglion cell (rgc) axons relay visual signals from the eye to the brain. Neighboring rgc axons establish precise topographic connectivity with neighboring neurons in the optic tectum. Several studies in cell culture demonstrate a role for RPTPs in rgc neurite growth.

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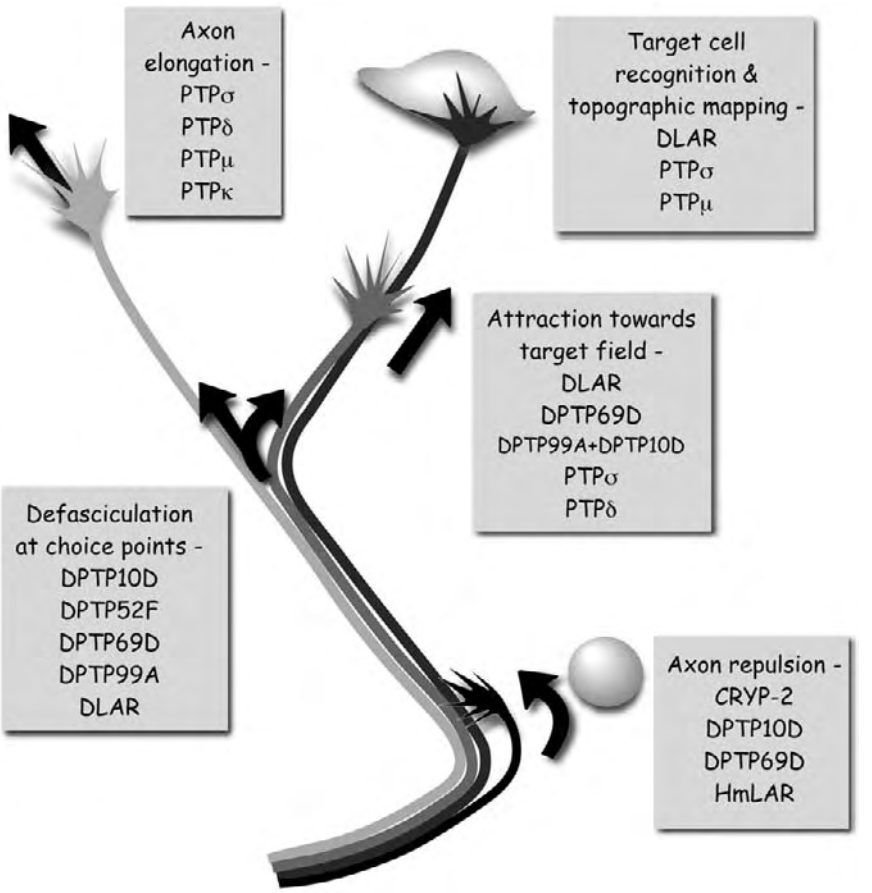


Figure 1 Schematic diagram showing the RPTPs implicated at different stages of axon growth and guidance. See text for details.

Signaling from chick PTPμ enhances cadherin-dependent retinal axon outgrowth [1]. Furthermore, PTPμ expression varies topographically across the retina and tectum and PTPμ has a selective, growth cone-collapsing function [5]. PTPμ may therefore differentially influence axon growth within the retinotectal projection. *Xenopus* PTPδ promotes both rgc axon growth along the optic tract *in vivo* and neurite growth on basement membranes in culture [6], while soluble ectodomains of human PTPδ can attract growth cones of cerebellar neurites [1]. Since both PTPδ and PTPμ bind homophilically, they may trigger signals directly between axons. Another retinal RPTP, CRYP-2 (encoded by avian *Ptpro*), is anti-adhesive in rgc cultures [7]. CRYP-2 may have an axon navigation role, since its ectodomain induces growth cone collapse and ectodomain gradients cause repulsive growth cone turning [7]. The interaction between chick PTPσ and a ligand(s) on basement membranes and glial endfeet maintains optimal retinal neurite outgrowth [1]. Perhaps counterintuitively, interference with intracellular signaling of *Xenopus* PTPσ causes faster neurite outgrowth in culture [6], suggesting a possible signaling model (see the section on ligands). The first evidence for RPTP function in axon targeting *in vivo* has come from chick PTPσ. Perturbation of the interactions between PTPσ and its ligands in the optic tectum causes retinal axon stalling and rostral mistargeting [8]. PTPσ may therefore function by maintaining retinal axon growth over the tectum and facilitating the recognition of correct target sites.

Neuromuscular System

Drosophila genetics has highlighted key RPTP functions during motor axon guidance. The segmental and intersegmental motor nerves ISN, ISNb, and SNa of the fly larva innervate body wall muscles in a highly stereotypical manner. Nerve defects arise after loss of function in *DLAR*, *DPTP69D, DPTP99A, DPTP10D,* and *DPTP52F* [2,9]. DPTP69D and DPTP99A are required for ISNb axons to defasciculate from the ISN at the correct choice point. Gene deficiency causes a "bypass" phenotype where axons fail to leave the ISN and thus travel past their targets. DLAR influences not only this defasciculation step, but also both the entry of axons into the muscle target field and synapse formation [10]. DPTP10D collaborates with other DRPTPs in guiding SNa, but antagonizes them during navigation of the ISN. Similarly, DLAR and DPTP99A antagonize each other within SNb axons. There is therefore a complex pattern of interaction between these RPTPs with "partial redundancy, competition, and collaboration" as described by Sun and co-workers [11].

Further Axon Growth and Guidance Roles

In the leech, LAR homolog HmLAR2 is expressed in growth cones of neurite-like processes of comb cells, where it controls the orderly outgrowth of these processes [1]. Evidence supports a homophilic interaction between HmLAR2 molecules, signaling a mutual repulsion between growth cones and neighboring processes.

In the ventral nerve cord of *Drosophila*, axon guidance across the midline is influenced by DLAR, DPTP99A, DPTP69D, and DPTP10D. The latter two in particular cooperate with Robo receptors to transduce repulsive signals from midline Slit protein [1]. How these receptors cooperate biochemically is unclear at present.

Several RPTP gene-deficiency models have been developed in mice. Loss of PTPσ function causes motor function deficits and hyposmia, as well as quite severe defects in sciatic nerve myelination and maturation [1]. Deficiency in PTPδ also causes milder motor defects as well as memory alterations, while loss of LAR causes a reduction in forebrain cholinergic neuron numbers and some mild defects in hippocampal innervation [1]. The developmental bases for all these neuronal and axonal defects have yet to be characterized.

Axonal Signaling by RPTPs

Instructive or Permissive?

Do axonal RPTPs send permissive or instructive signals during axon guidance? With DLAR, the fact that R7 growth cones reach targets, but then retract, supports an instructive role in securing adhesion to targets. DLAR may also control the instructive process of muscle cell recognition by motor axons [11]. For DPTP69D, the consensus is more in favor of a permissive role in controlling defasciculation rather than target recognition, although this remains under discussion [1]. In fact, the many complex interactions between *Drosophila* RPTPs may ultimately make simple instructive/permissive distinctions untenable. Vertebrate PTPδ and CRYP-2 may have instructive signaling roles given that they can force growth cone turning on otherwise permissive substrates [1,7]. In contrast, chick PTPσ may control a permissive event during axonal targeting in the tectum, given that its known ligands are uniformly distributed [8].

Ligands

PTPμ and PTPδ bind homophilically, with no evidence to date of heterophilic ligands. Ectodomains of these RPTPs can act as neurite growth-promoting substrates in culture, suggesting that the growth of fasciculated axons *in vivo* may be promoted by their homophilic action. Paradoxically, PTPμ ectodomains also have negative effects on growth cones [5], although we do not know yet if this signal is transduced by PTPμ receptors. The heparin-binding chemokine pleiotropin is a ligand for PTPζ, an RPTP expressed in both glia and some neurons. Pleiotropin can inhibit the PTPζ phosphatase and this leads to increased tyrosine phosphorylation of potential targets [12]. The heparan sulfate proteoglycans agrin and collagen XVIII are strong binding partners for chick PTPσ, although we await direct evidence for their roles in controlling PTPσ signaling in axons [13]. One of several models proposed also suggests that chick PTPσ ligands may inactivate the phosphatase, thereby facilitating neurite growth [6]. The effects of RPTP ligands are discussed further in part B of this Handbook. Although RPTP ectodomains have adhesive capacities, it is generally believed that their signaling roles require intact catalytic functions. For example, enzymatically active PTPμ is required for neurite outgrowth on cadherins [1]. Furthermore, genetic rescue studies with *Drosophila* RPTPs indicate that the rescuing genes must encode active phosphatases [1,3,4,11].

Downstream Signals

Figure 2 contains a summary of some of the known substrates and binding partners of neuronal RPTPs. Most of these impinge ultimately on the actin cytoskeleton, providing a logical handle on growth cone dynamics. DLAR interacts with several molecules including the tyrosine kinase Abl and its substrate Enabled (Ena, a VASP family member). Ena can be dephosphorylated by DLAR [1]. Dephosphorylation of Ena activates downstream signals that pass through profilin and on to actin. DLAR also interacts genetically with Trio, a large protein with two exchange factor domains for Rho family GTPases. *Drosophila* Trio also signals through the SH2–SH3 adaptor Dock and the p21-activated kinase Pak,

RPTPs	Interacting protein	Downstream effector	Refs.
LAR, PTPσ	> Trio >	Rac, Rho	1, 2
LAR, PTPδ, PTPσ	liprins		2, 10
DLAR	< Abl* <	ena*	1, 2
DLAR, DPTP69D	> ena* >	profilin	1
PTPα	> grb2 >		2
PTPα	> c-src* >		2
PTPμ	> RACK1 >	PKCδ	16
PTPμ, PTPκ, LAR family, PTPζ	< cadherins & catenins* <		15
DPTP10D, DPTP99A	gp150*		2

Figure 2 Table showing some of the proteins that interact with axonal RPTPs, and some of the predicted downstream effectors where known. Symbols > and < indicate either stimulation or repression of function by the RPTP, respectively. Asterisks indicate that the protein is a phosphatase substrate.

again impinging on the cytoskeleton. Mammalian Trio binds directly to LAR family members and signals through Rho family GTPases. Human Trio promotes neurite outgrowth in PC12 neurons, although it is not known yet if LAR RPTPs are involved in this event [14]. Catenins are a key target of several RPTPs, including PTPμ, PTPκ, PTPζ and LAR members [12,15]. These RPTPs may well antagonize cadherin/catenin-regulated cell adhesion by dephosphorylating β-catenin and p120(ctn), thereby directly influencing growth cone adhesion. PTPμ also binds to the adaptor protein RACK and in turn requires PKCδ to promote neurite outgrowth [16]. Other RPTP targets include the cell adhesion molecule-like gp150 and the tyrosine kinase c-src, but it is not clear yet if these are involved in RPTP signaling within axons. Finally, adaptor proteins of the liprin family bind to LAR family RPTPs and may be important for localizing these RPTPs in membranes and in forming complexes with roles in signaling, adhesion, and synapse function [2,10].

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CHAPTER 266

Attractive and Repulsive Signaling in Nerve Growth Cone Navigation

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Introduction

The function of the nervous system depends on complex and precise connections between nerve cells [1]. The formation of specific connections during development often requires the growing axon to navigate over considerable distances to reach their final target cells. This long-range navigation is achieved by guidance factors within the developing tissue that regulate the motility or directionality of the growing tip of the axon, the growth cone [2]. During the last decade several families of guidance factors have been identified, including netrins, semaphorins, ephrins, and slits [3,4]. In addition, inhibitory factors associated with the myelin that exert repulsive actions on the navigation of regenerating axons have also been discovered. Different classes of membrane receptors for these factors have been identified and their intracellular signal transduction mechanisms are beginning to be elucidated.

How does a guidance factor affect the navigation of the growing axon? A general scheme of signal transduction cascades from the receptor activation to cytoskeletal rearrangements is shown in Fig. 1 [4]. It starts with the binding of the guidance factor with the receptor protein or protein complexes at the cell surface. Ligand-receptor binding in general stimulates the activities of the cytoplasmic domain of the receptor, which in turn interacts specifically with cytoplasmic adaptor proteins. These adaptors may then recruit or activate their downstream effectors to further mediate the guidance signal. The effectors (or mediators) can be enzymes or second messengers that activate or inhibit cytoskeleton-associated proteins, leading to polymerization or depolymerization of cytoskeletal structures and steering of the growth cone.

Two types of guidance signals may be distinguished: signals that convey a "stop or go" command regulating growth cone motility and signals that provide directional instructions to the growing axon, triggering turning responses of the growth cone. For nondirectional signals, mediators may simply alter the global cytoskeletal activity at the growth cone. For directional guidance signals, however, a gradient of cytoskeletal rearrangements must be created in order to induce directional motility. In the latter case, mediators must be activated or distributed in a gradient across the growth cone, and such a gradient may also need to be amplified in the cytoplasm in order to achieve a reliable directional response [4]. Although a number of cytoplasmic components have been implicated in such a scheme of signal transduction, definitive identification of signaling pathways are yet to be established for any one of the major families of guidance factors. This chapter summarizes some of the putative signaling pathways that have been shown to participate in growth cone navigation.

Netrin Signaling

Netrins are a family of secreted proteins and their receptors were identified to be DCC (deleted in colorectal cancer) and UNC-5 [5], two interacting transmembrane proteins that set the polarity of growth cone responses. Ectopic expression of UNC-5 in neurons converted netrin (UNC-6)-dependent

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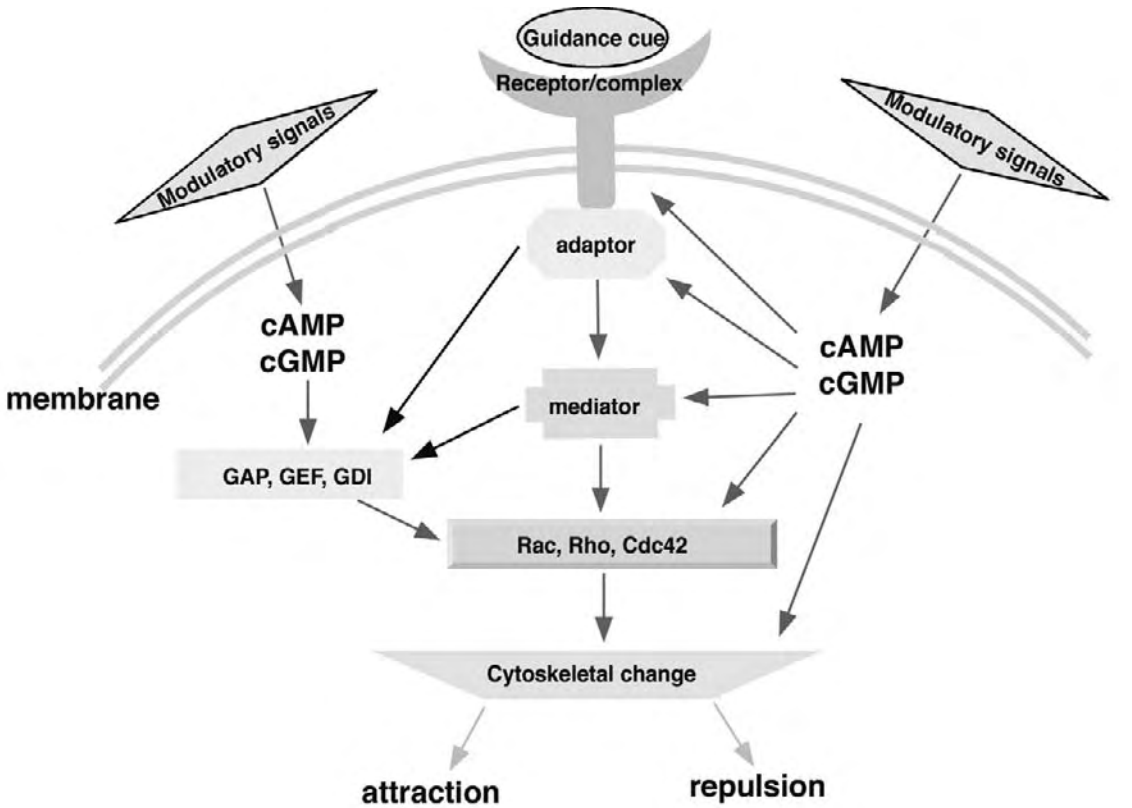


Figure 1 Signal transduction mechanism for nerve growth cone guidance.

chemoattraction to chemorepulsion in both *Caenorhabditis elegans* [6] and in dissociated *Xenopus* spinal neurons [7], a conversion that involves a netrin-dependent interaction between the cytoplasmic domain of DCC and UNC-5 [7]. The level of DCC can be actively regulated through degradation mediated by Sina/Siah protein [8] or through metalloprotease-mediated shedding [9], resulting in changes in the growth cone's sensitivity to netrin-1. Extracellular signal-regulated kinase1/2 (Erk 1/2) was found to be recruited to DCC receptor complex in rat commissural neurons [10] and activation of phosphoinositide 3-kinase (PI3-K) and phospholipase Cγ appear to mediate attractive turning of *Xenopus* spinal neurons induced by a gradient of netrin-1 [11]. More recently, it has been shown that activation of MAPK, local protein synthesis, and protein degradation are involved in the netrin-induced chemoattraction of *Xenopus* spinal and retinal neurons [13,14]. These downstream events were shown to be critical for adaptive changes of growth cone sensitivity to netrin-1 as the extracellular concentration of netrin-1 is increased [13]. How these cytoplasmic factors are linked to the cytoskeleton changes remains largely unclear. Two members of the Rho GTPase family, Rac1 and Cdc42, appear to be involved in netrin signaling [15,16], thus providing potential links to cytoskeletal regulation [17]. To serve for directional guidance signals, a mediator is not only required for the guidance responses, but must also be activated in a gradient across the growth cone. Furthermore, such a gradient should be sufficient to induce a turning response of the growth cone. None of the putative signaling components described above fulfill these criteria. Interestingly, the well-known second messenger Ca^{2+} appears to satisfy these criteria for netrin-1 signaling. Elevation of cytoplasmic Ca^{2+}, through both Ca^{2+} influx and release from internal stores, is required for netrin-1-induced turning responses and a netrin-1 gradient can trigger Ca^{2+} elevation and transient Ca^{2+} gradients across the growth cone [7,12]. Experimentally creating a gradient of Ca^{2+} across the growth cone in the absence of netrin-1 signals is sufficient to induce the turning of the growth cone [18]. However, it remains unclear how Ca^{2+} signals are linked to receptor activation upstream and cytoskeletal rearrangements downstream.

Semaphorin Signaling

The semaphorin family includes both membrane-bound and secreted molecules, thus it may work for both short- and long-range guidance [19,20]. Neuropilins were identified as semaphorin receptors and the plexin family of receptors was shown to be a co-receptor that transduces the signal. Several proteins have been shown to bind to neuropilins or plexins. These include a transmembrane protein OTK (off-track) [21], cytoplasmic protein NIP—a PSD-95/Dlg/ZO-1 domain—containing protein that may be involved in membrane trafficking [22], and MICAL, a flavoprotein oxidoreductase [23]. There is also evidence for the involvement of heterotrimeric G proteins [24]. The precise role of these receptor-interacting proteins and whether they mediate or modulate the signaling

process remain to be determined. More is known about the downstream cascades that mediate cytoskeleton changes induced by semaphorins. Of particular interest is Rac1, a small Rho family GTPase. Introduction of dominant-negative Rac1 [25] or an inhibitory peptide for Rac1 or C3 transferase, a Rho GTPase inhibitor, blocks Sema3A-induced growth cone collapse in sensory neurons [26,27]. A major downstream mediator of Rac1 and Cdc42 is P21-associated kinase (PAK), and LIM-domain-containing kinase (LIM-kinase), a direct substrate of PAK, are necessary for Sema3A-induced growth cone collapse [28]. LIM-kinase is a serine-threonine kinase that inhibits cofilin's actin-severing function. Thus at least one of the mechanisms for semaphorin-mediated cytoskeletal changes is mediated through the activation of small GTPases and their targets PAK, which then regulates actin dynamics through a LIM-kinase- and cofilin-dependent pathway. Other small GTPases such as Rho and Rnd1 [29,30] and kinases such as GSK-3 (glycogen synthase kinase) [31] and Fes/Fps tyrosine kinase [32] have also been implicated in semaphorin signaling.

Slit Signaling

Slits are a family of secreted proteins that can exert short- and long-range guidance functions by activating their receptors, the roundabout (Robo) family of proteins [33]. Slits appear to act not only as directional guidance factors but also as stop signals through activation in a combinatorial manner of different Robo receptors expressed on the growth cone surface [34–36]. Both Abelson tyrosine kinase (Abl) and its substrate Enabled (Ena) can bind directly to the cytoplasmic domain of Robo and modulate its function [37]. Interfering with the binding between Ena and Robo partially impairs the Robo function, while a mutation in a conserved tyrosine residue that can be phosphorylated by Abl generates a hyperactive Robo. Small GTPases and their regulators also affect slit-Robo signaling. A slit-Robo-GTPase activating protein 1 (srGAP1) can bind to Robo and inactivate Cdc42, resulting in repulsion of growth cones [38], while GEF64C, a Dbl family guanine nucleotide exchange factor (GEF), can activate Rho and block Robo-induced repulsion [39]. Thus, Robo-mediated cytoskeleton changes also appear to be mediated by activation of GAP or GEF of small GTPases.

Ephrin Signaling

Ephrins and the Eph family of tyrosine kinase receptors are membrane-bound molecules that mediate short-range axon guidance via cell-cell contacts [40]. Ephrin-A ligands are attached to the plasma membrane via a glycophosphatidylinositol (GPI) linkage, whereas the ephrin-B ligand contains a transmembrane domain and a cytoplasmic tail [41]. Similar to slits, ephrins can function as either directional or nondirectional guidance factors. In addition, the signaling activated by ephrin-Eph binding is bidirectional [41], so that cytoplasmic activities are triggered in both interacting cells. A GEF, ephexin, binds to the kinase domain of EphA constitutively through its Dbl homology-pleckstrin homology (DH/PH) domain and activates both RhoA and Cdc42, thus regulating cytoskeletal structures [42]. In addition, focal adhesion kinase (FAK) and its downstream factor P130(cas) which are two proteins involved in actin reorganization, are also implicated in EphA-induced cytoskeletal changes [43].

Nogo and Myelin-Associated Glycoprotein Signaling

Two proteins associated with myelin, Nogo, and myelin-associated glycoprotein (MAG), have been identified as the major inhibitory factors that prevent axon regeneration after CNS injury [44]. Although the full length of these proteins are membrane-anchored, they can be released in a truncated form and function in repelling and inhibiting axon growth [45,46]. The receptors for Nogo (NogoR) [46] and for MAG (GD1a and GT1b) [47] have been identified. Interestingly, MAG also binds to NogoR [48]. Since NogoR is a GPI-anchored protein at the cell surface [46], and an as yet unidentified co-receptor(s) is required for transducing the cytoplasmic signal. The downstream signal cascade for NogoR signaling is largely unknown, although Ca^{2+} and PI3-K are required for MAG-induced repulsion of *Xenopus* spinal neurons [11,49]. Rho is activated by MAG through receptor GD1a and GT1b [47]. Inhibition of Rho activity can promote CNS axon regeneration, suggesting that Rho may also be involved in MAG-induced cytoskeletal rearrangement [50].

Critical Roles of Modulatory Signals

For a growth cone to make its navigational decisions, it must integrate information provided not only by the guidance factors, but also by other modulatory signals. *In vitro* studies have shown that cytoplasmic cyclic nucleotides play key roles in modulating signal transduction events triggered by most guidance factors identified thus far [4]. For example, the growth cone responses to netrin and MAG are modulated by a cAMP-dependent pathway, whereas Sema3A and slit signaling is modulated by a cGMP-dependent pathway. Elevating the cytoplasmic level of cyclic nucleotides favors attraction/growth, while lowering their level favors repulsion/collapse [4]. Many extracellular ligands, including neuromodulators, adhesion molecules, and extracellular matrix (ECM) components, may change the level of cyclic nucleotides within the cell, thus altering the growth cone behavior when they are present concurrently with the guidance signal [51]. For example, laminin, an abundant ECM protein, reduces the cAMP level in *Xenopus* retinal ganglion neurons and converts the growth cone response to a netrin-1 gradient from attraction to repulsion both *in vitro* and *in vivo* [52]. Conversely, the repulsive response of DRG and cortical axons induced by Sema3A can be converted to attraction by exposure to soluble LI-Fc chimeric molecules; activation of

guanylate cyclase activity is required for the conversion [53]. The targets of PKA/PKG that are involved in regulating the polarity of growth cone turning responses remain to be identified.

Concluding Remarks

An emerging view of axon guidance factors is that they are multifunctional molecules capable of conferring attractive, repulsive, or stop signals. The precise behavior of a growth cone is determined by the nature of specific receptors and the status of cytoplasmic signal cascades, which are under the influence of a variety of extrinsic and intrinsic factors (Fig. 1). The combinatorial expression pattern of various receptors at the surface of a growth cone may trigger a differential downstream event [54]. In addition, the efficacy of receptor signaling across the plasma membrane can be modulated by various factors both extra- and intracellularly [4]. Recruitment of different adaptors and mediators in the cytoplasm can result in different growth cone behaviors. An area of interest for future studies is determining the mechanisms that control or modulate the recruitment and activation of these cytoplasmic factors and that amplify the signals conveyed by the receptors. Signal cascades triggered by all known guidance factors appear to eventually converge upon different members of the Rho family GTPases or their activators/inhibitors. It is of interest now to determine how spatiotemporal patterns of GTPase activation account for distinct navigational behaviors of the growth cone.

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CHAPTER 267

Semaphorins and their Receptors in Vertebrates and Invertebrates

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The Semaphorin Family

Semaphorins are a large family of proteins originally identified as axon guidance factors of the developing nervous system. Over 30 family members are grouped into 8 classes based on structural and phylogenetic relationships (reviewed in [1]). Classes 1 and 2 are expressed in invertebrates, classes 3 through 7 are vertebrate semaphorins, and Class V is expressed in non-neurotropic DNA viruses. All semaphorins share a highly conserved 500 amino acid "Sema" domain at their amino terminus, but different classes possess divergent sequences in their carboxyl regions. Classes 1, 4, 5, and 6 are transmembrane proteins, class 7 has a GPI-anchor, and classes 2, 3, and V are secreted proteins. The presence of both membrane-bound and secreted semaphorins suggests that semaphorins act as both short- and long-range cues. In addition, the diversity of structural properties between the classes implies roles in a diversity of biological processes.

The best-documented function of semaphorins is their role in central nervous system (CNS) development. Semaphorins act as both repellents and attractants for growing axons. The first identified vertebrate semaphorin, Sema3A, causes retraction of axons and the collapse of the growth cone, a specialization at the tip of growing axons [2]. While repellents for certain neurons, Sema3C and 3F also serve as attractants for cortical and olfactory neurons, respectively. Within the large semaphorin family, certain semaphorins can exert antagonistic activity by competitively blocking the activity of other family members at certain receptors [3]. Semaphorins may guide growing dendrites as well as axons. Specifically, Sema3A attracts the apical dendrite of pyramidal neurons in the cerebral cortex toward the pial surface [4]. Many types of neurons are responsive to semaphorins, including dorsal root ganglion, sensory, motor, hippocampal, cortical, cerebellar, and olfactory. In addition to guiding axons and dendrites, semaphorins appear to play a role in fasciculation of nerve bundles, neuronal cell migration, axoplasmic transport, and apoptosis [5]. Like developing neurons, adult neurons of the regenerating CNS are responsive to semaphorins and semaphorin expression is upregulated after nerve injury [6].

Semaphorin signaling is not restricted to the CNS as evidenced by widespread expression throughout the embryo and adult tissue. Migrating non-neuronal cells are responsive to semaphorins, and cardiovascular abnormalities are observed when semaphorin signaling is disrupted [7]. In the immune system, expression of Sema4D (CD100) is regulated upon B- and T-lymphocyte activation and migrating monocytes are responsive to Sema3A and Sema4D [8]. Malignant lung cells show reduced levels and a cytoplasmic localization of semaphorins [9]. Taken together, it can be concluded that semaphorins act as guidance cues for many types of migrating cells in developmental, adult, and pathological tissue.

Receptors for Semaphorins

Neuropilins

Neuropilins are high-affinity transmembrane receptors for the secreted class 3 semaphorins in the CNS, but play no role in the activity of other semaphorins. A neuropilin family is composed of neuropilin-1 and several splice variants

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of neuropilin-2 [1]. Neuropilin-1 and -2 contain a number of conserved motifs on their extracellular domain including two CUB domains, FV/FVIII, and a MAM domain. The CUB domains are required for ligand binding and the MAM domain mediates neuropilin oligomerization [10]. Neuropilins have a short intracellular domain containing a PDZ binding motif that targets receptor localization to signaling components in the membrane of the cell [11]; however, the intracellular domain is not required to transduce the semaphorin signal [10]. The neuropilin isoforms bind differentially to various class 3 semaphorins [12], and the specificity of binding is determined by the CUB domains [10]. Although neuropilins are sufficient to bind class 3 semaphorins, the fact that the intracellular domain is not required for signaling suggests that a co-receptor transmits the semaphorin signal into the cell.

Outside of the CNS, neuropilins are found in the mesenchyme surrounding blood vessels and act as co-receptors for vascular endothelial growth factor (VEGF). Upon binding VEGF, neuropilins potentiate the kinase activity of the VEGF receptors flt-1 and KDR, resulting in endothelial cell migration [13]. There is some evidence to suggest that semaphorins and VEGF compete for neuropilin binding and a dysregulation of the competition may lead to pathological conditions [9].

Plexins

Plexins are the predominant receptors for membrane-bound, GPI-linked and viral semaphorins, and they bind to neuropilins to act as signaling co-receptors for the secreted class 3 semaphorins [14]. The initial discovery of Plexins as semaphorin receptors occurred with the identification of virus-encoded semaphorin protein receptor (VESPR; plexinC1) as a binding site for a class V semaphorin [15]. Currently, at least ten Plexins have been identified and are classified into four groups, Plexin A–D, which have different specificities for different semaphorins [1]. Plexins are distantly related to semaphorins since they possess the conserved Sema domain on their extracellular surface [16] and also share some sequence homology with the HGF receptor Met on their extracellular surface [17]. The intracellular domain of Plexin is highly conserved among family members, but is not significantly homologous to any known signaling motif. In their native state, Plexins are autoinhibited by their Sema domain and binding to semaphorin-neuropilin complexes or cleavage of the sema domain leads to activation of the protein and growth cone collapse in sensory neurons [18].

Intracellular Signaling Pathways

Actin Cytoskeleton and Monomeric GTPases

The actin cytoskeleton in growth cones undergoes dramatic rearrangement upon exposure to Sema3A. There is a relative decrease in F-actin within the lamellipodia [19] and actin colocalizes with neuropilin-1/PlexinA1 receptor complexes [20]. The actin reorganization is linked to increased endocytosis [20]. It was thought that semaphorins might regulate the actin cytoskeleton through monomeric G proteins due to the weak similarity of the conserved intracellular domain of Plexins to an R-Ras-GAP. However, no Plexin protein has been shown to possess GAP activity and semaphorin responses are not dependent on R-Ras. Instead, Rho family G proteins, namely Rac and Rho, seem to mediate the semaphorin response [21] (also reviewed in [22]). Active Rac binds directly to the intracellular domain of vertebrate and invertebrate PlexinB1, and this interaction is enhanced by the presence of ligand binding [23]. Activation of PlexinB1 appears to sequester active Rac from its endogenous substrate, p21-associated kinase, PAK [24–26]. RhoA is also activated as a result of PlexinB1, although it is not clear whether this is due to a direct or indirect action of PlexinB1 on RhoA [26], or is downstream of Rac-Plexin interactions [24]. Together, Rac sequestration and RhoA activation appear to mediate axon repulsion by PlexinB receptors.

Although the intracellular domain is highly conserved among all Plexin family members, it is not clear whether PlexinA functions in a similar fashion as PlexinB. PlexinA1 binds to both RhoD and Rnd1, and Rnd1 binding has been suggested to induce growth cone collapse [27,28], perhaps due to Rnd1-dependent inhibition of Rac [29]. Direct Rac-PlexinA interactions have not been demonstrated. It is possible that Plexins regulate monomeric GTPases indirectly by regulating Rho family GEFs and GAPs, factors that activate or inactivate monomeric GTPases, respectively.

A direct link between Sema3A and actin dynamics was recently demonstrated. Activated complexes of NP1 and PlexinA2 lead to the phosphorylation and deactivation of cofilin by LIM kinase [30]. Cofilin leads to F-actin turnover and plays a role in protrusion of lamellipodia and filopodia [31]. Further, LIM kinase is a substrate for both PAK and Rho kinase, which is consistent with the requirement for Rac and Rho, respectively, for Sema3A-induced collapse [31].

CRMP

Collapsin-response-mediator protein (CRMP) was identified in a *Xenopus* oocyte expression screen of mRNAs required for Sema3A responses [32]. The protein sequence of CRMP shares sequence homology with the *Caenorhabditis elegans* unc-33, a protein required for proper axonal pathfinding [33]. At least five isoforms of CRMP have been identified and they form heterotetramers *in vivo* [34]. Function blocking antibodies to CRMP block Sema3A-mediated growth cone collapse in chick DRG neurons [32], and CRMP is upregulated after axotomy of the sciatic [35] and olfactory [36] nerves. The mechanism of CRMP action is still unclear. Studies have shown that it is phosphorylated

by Rho kinase, but this is not required for Sema3A-induced collapse [37]. The microtubule abnormalities of *unc-33* mutants and the observation that CRMP colocalizes with microtubules at certain stages of the cell cycle [38] suggest that Plexin/CRMP signaling may regulate microtubule dynamics. There is also evidence demonstrating CRMP binds to and inactivates phospholipase D2, an enzyme implicated in a variety of cell processes including actin dynamics, vesicle trafficking, and mitogenesis [39]. Finally, CRMP may act to mediate the cytoskeleton through Rho GTPases. Neuroblastoma cells overexpressing CRMP and constitutively active RhoA showed a Rac1-like morphology, whereas cells co-expressing CRMP and active Rac1 showed a RhoA-like morphology [40]. Thus, it seems likely that CRMP enhances the function of a Plexin-Rho family G-protein axis in axon repulsion.

Protein Phosphorylation

Receptors for several other axon guidance molecules, such as ephrins and neurotrophins, act via kinase cascades. Although semaphorin receptors themselves show no kinase activity, indirect evidence suggests that protein phosphorylation occurs and is required [41]. The involvement of PAK and LIM kinase downstream of Plexins is mentioned above. A recent study has shown that *Drosophila* PlexinA associates with the membrane-bound receptor tyrosine kinase-related protein Off-Track (Otk; [42]). In addition, two proteins with kinase activity have been co-purified with CRMP [38,43]. The serine/threonine kinase, glycogen synthase kinase (GSK)-3 is activated as a result of Sema3A in both neuronal cells and human breast cancer cells, and GSK-3 inhibitors prevent Sema3A-induced growth cone collapse [44].

Other Signaling Mechanisms

Another pathway that has been implicated in semaphorin signaling may utilize heterotrimeric G proteins. Much of the evidence for this has come from experiments with pertussis toxin (PTX), which blocks G-protein function [21,45]. Indeed, neuropilins were found to bind to a Gα-interacting protein (GIPC, SEMPCAP-1) that associates with a regulator of G-protein signaling (RGS) protein via its PDZ domains [11]. Interestingly, some transmembrane semaphorins interact with SEMCAP-1 as well, suggesting that semaphorins may act as receptors to transduce signals into the cell [46]. Semaphorin reverse signaling is further supported by the fact that Sema6B binds to Src both *in vitro* and *in vivo* [47], Sema4D interacts with a serine kinase [48], and Sema6A binds to the actin binding protein EVL [49].

Semaphorin-mediated signaling can be modulated by other pathways. Cyclic nucleotides can alter the response of growth cones to various signaling molecules [50,51]. Increasing levels of cGMP switches Sema3A responses from repulsion to attraction and decreasing cGMP potentiates the repulsive activity of Sema3A. Apical dendrites of cerebral cortical neurons are attracted to Sema3A while the axons of the same cells are repelled [4]. Remarkably, soluble guanylate cyclase (SGC) is asymmetrically localized to the dendrites of these cells, implicating an endogenous regulation of cGMP *in vivo*. The cell adhesion molecule, L1, is also able to modulate growth cone responses to Sema3A [52]. DRG neurons from L1-deficient mice show no response to Sema3A and soluble L1 protein switched repulsion to attraction. Finally, one or more of these pathways may impinge on protein synthesis and degradation within axons. Evidence indicates that local regulation of protein levels participates in multiple growth cone responses [53].

Semaphorin Signaling in the Immune System

Semaphorin signaling in activated lymphocytes does not rely on neuropilins and Plexins, but utilizes a different receptor called CD72 [54]. Under normal conditions, CD72 is phosphorylated on its intracellular domain by a Src tyrosine kinase. This phosphorylation leads to the recruitment of an SH2-domain-containing tyrosine phosphatase SHP-1, which then dephosphorylates and inactivates signaling proteins involved in lymphocyte activation [8]. Sema4D binding prevents the phosphorylation of CD72 therefore potentiating lymphocyte activation [54]. The migration of monocytes is inhibited by Sema4D and Sema3A; this effect is likely to be mediated via Plexins and neuropilins since monocytes do not express CD72 [55].

Summary and Future Directions

Many biological systems in the developing embryo and adult animal are dependent on semaphorin signaling. Although the importance of semaphorins in the developing CNS is well documented, their involvement in the immune response, the cardiovascular system, and in pathology is still being clarified. Most of the work to date has focused on the identification and classification of the various semaphorin families and their receptors, with less clarification of downstream signaling mechanisms. Regulation of the cytoskeleton is the most obvious effect of semaphorin signaling, and a number of studies have demonstrated a signaling connection of semaphorin receptors with actin filaments and microtubules. In particular, Rho family G proteins and CRMP appear to play major roles in this connection (see Fig. 1).

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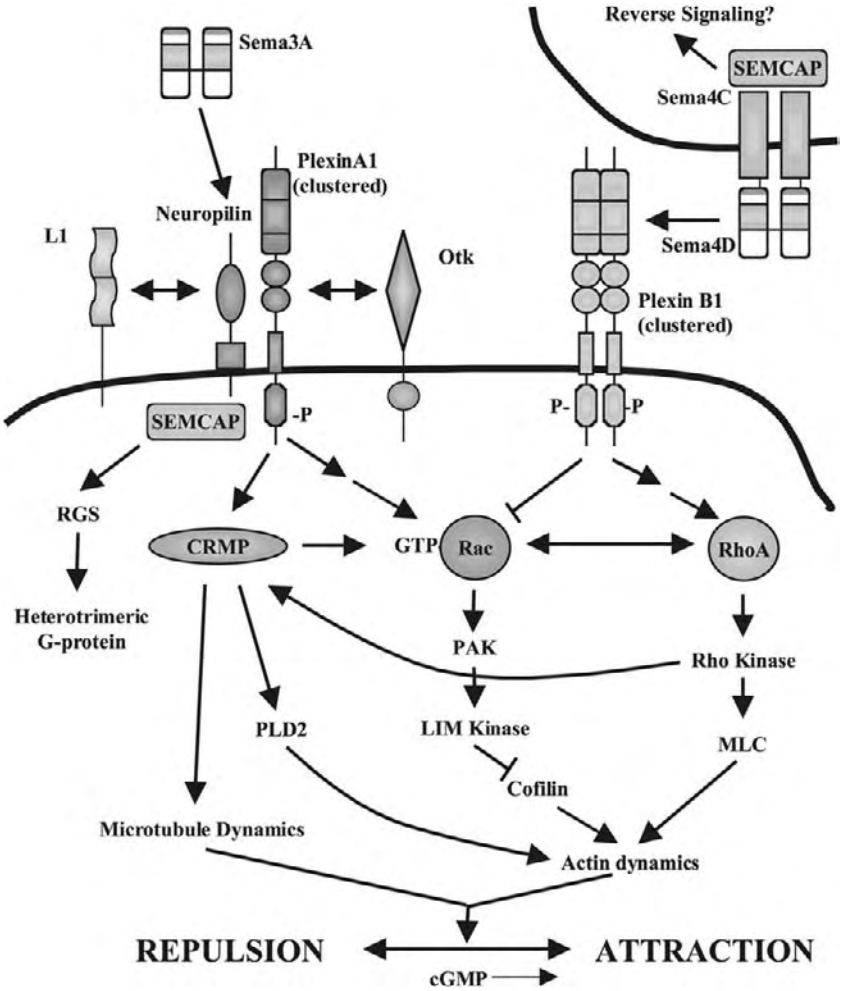


Figure 1 A schematic representation of semaphorin signaling in neuronal development. See text for details.

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CHAPTER 268

Signaling Pathways that Regulate Neuronal Specification in the Spinal Cord

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A cascade of signaling events triggers the differentiation of specific neuronal and glial cell populations that comprise the central nervous system (CNS). Epidermal ectoderm deprived of bone morphogenic protein (BMP) signaling differentiates into "neural" ectoderm [1], the precursor of the CNS. These neural cells are multipotential and respond to signals in their environment in order to generate the appropriate types of neurons and glia at the correct positions. In this chapter we focus on the spinal cord, the most caudal region of the CNS, since it has served as a useful model in which to investigate signaling events that give rise to neuronal and glial populations within the developing neural tube.

Emerging from a combination of modern molecular studies and classical cellular studies, a central theme in spinal cord development is one in which inductive factors signal along the dorsoventral and rostrocaudal axes of the developing spinal cord to specify cell fate in a Cartesian-coordinate-like manner [2]. This signaling leads to the generation of dorsal spinal cord interneurons that process sensory information and relay it to the brain, while the ventral spinal cord forms interneurons and motor neurons involved in locomotor control (Fig. 1A). Along the rostrocaudal axis, discontinuous subclasses of motor neurons are generated in register with the peripheral targets that they innervate. In addition, numerous glial cell types are formed including the roof plate and floor plate, which act as organizing centers within the spinal cord, and astrocytes and oligodendrocytes, which support neuronal function and myelinate neurons, respectively.

Patterning along the Dorsoventral Axis

Two classes of factors play prominent roles in specifying distinct cell types along the dorsoventral axis of the spinal cord: members of the transforming growth factor β (TGFβ) superfamily acting dorsally, and Sonic hedgehog (Shh) ventrally (Fig. 1A) [3,4]. TGFβ signaling from the epidermal ectoderm flanking the dorsal neural tube leads to the differentiation of the roof plate [5], and Shh expression from the notochord below the neural tube triggers the formation of the floor plate [6]. These two glial structures in the spinal cord then express TGFβs dorsally and Shh ventrally. In this way, signals from the periphery are propagated into the spinal cord to control cell differentiation locally.

The dividing progenitor cells within the ventricular (medial) region of the spinal cord monitor the types and concentrations of TGFβs and Shh in order to determine their position, and consequently their fate, as they become postmitotic and migrate laterally into the mantle region (Fig. 1A). The signaling pathways triggered by these inductive factors lead to the activation of transcriptional networks that first define distinct domains along the dorsoventral axis of the ventricular zone, and ultimately lead to the expression of genes involved in controlling cell function (Table I) [7–10].

Dorsal Spinal Cord Development

In the embryonic dorsal spinal cord, four classes of interneurons (INs) termed D1–D4 arise in an orderly fashion

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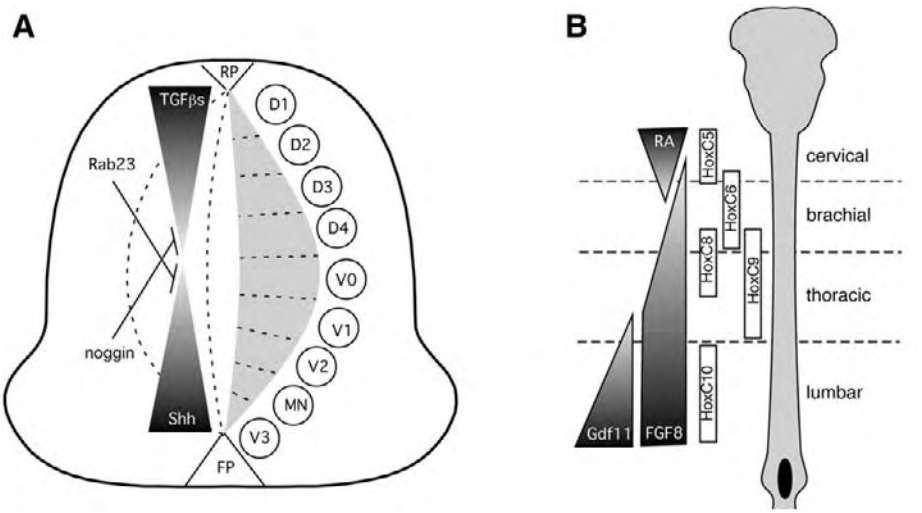


Figure 1 Patterning signals along the dorsoventral and rostrocaudal axes of the developing spinal cord. (A) Transverse view of the spinal cord in which the gray area represents the ventricular zone containing progenitor cells and the white area represents the mantle layer with mature cell types. FP, floor plate; RP, roof plate. (B) Rostrocaudal view of the spinal cord and the graded pattern of signals expressed by paraxial and axial mesoderm adjacent to the neural tube.

from specific regions of the progenitor zone (Fig. 1A). These INs, while not absolutely defined, consist of association and commissural cells that process and relay sensory information and depend upon the formation of the roof plate in order to develop [5,11]. The roof plate and adjacent neural epithelial cells express overlapping and nested combinations of several TGFβ members including BMP4/5/7, Gdf6/7, and Dsl1 [5,9]. How might the TGFβs produce different cell types in the spinal cord? Several strategies are likely to be involved including quantitative, qualitative, and timing differences in TGFβ activity. The expression pattern of the TGFβs suggests that a high-dorsal to low-ventral gradient of these proteins is present within the neural tube (Fig. 1A). Although more studies are needed to determine whether graded levels of TGFβs contribute to spinal cord patterning, *in vitro* experiments with neural explants have detected concentration-dependent activities for Activin A in the induction of D1 and D2 IN classes [5].

The clearest example of how TGFβs trigger the differentiation of specific IN types is based on the finding that individual members of this family have different qualitative activities [5]. The most convincing evidence for such a mechanism is found in the *Gdf7* mutant mice where a specific subpopulation of D1 INs fails to be generated [12]. These results suggest that the specificity of dorsal IN patterning is mediated, at least in part, by various TGFβ signaling molecules, some of which act directly to render class specificity (Table I). An additional mechanism for generating cellular diversity in the dorsal spinal cord involves a temporal switch in the way neuroepithelial cells respond to TGFβ signals. Early in development, progenitor cells produce neural crest cells when exposed to BMP4 or Activin A, but later, they give rise to dorsal INs in response to the same signals [5]. The basis for qualitative differences in TGFβ signaling and the mechanisms underlying the developmental switch in TGFβ responsiveness by neural epithelial cells remain important questions.

Shh signaling for ventral cell differentiation is attenuated by TGFβ signaling [13]. What limits the range of TGFβ activity to the appropriate areas of the developing spinal cord? Several TGFβ antagonists have been identified including noggin, chordin, and follistatin which bind directly to and sequester-specific TGFβs [14]. These antagonists are expressed by the somites and notochord near the ventral surface of the neural tube, and therefore are expected to limit the exposure of ventral cells to certain TGFβs. In *noggin* mutant mice, TGFβ signaling in the ventral neural tube is unmasked (Fig. 1A), which leads to a progressive loss of ventral cell differentiation [15].

The receptors of the TGFβs are serine/threonine kinases comprised of type I and type II dimers. These receptor complexes have not been well characterized in the spinal cord, but may select for different ligands and serve as the basis for the qualitative differences in cell differentiation induced by different TGFβ family members. The best known transducers of TGFβ signaling are the SMAD transcription factors [16], though the role of SMADs in spinal cord development also requires further characterization. Recently, a better understanding of the downstream targets of TFGβs has begun to emerge (Table I). For instance, it is now known that D1 INs, characterized postmitotically by the markers Lhx2/9, arise from progenitor cells that express the bHLH transcription factor mATH1 involved in establishing the fate of these cells [9,10]. Likewise, D3 IN progenitor cells marked by Lhx1/5 arise from progenitors that express the bHLH protein Ngn1. Thus, the identification of target genes activated by TGFβ

Table I Signaling Events in the Developing Spinal Cord

	Dorsal patterning Lateral ectoderm → Roof plate		Ventral patterning Notochord → Floor plate			Rostral/Caudal patterning Hensen's node, paraxial mesoderm		
Signal	*TGF*β *Superfamily* BMP 4, 5, 6, 7 Gdf 6, 7 Activin B; Dsl1		Sonic Hedgehog			Retinoic Acid	FGF 8	Gdf 11
Inhibitors	Follistatin, Noggin, Chordin		Hedgehog-interacting Protein (Hip) Rab23 (vesicle transporter)			Cellular Retinoic Acid Binding Protein		Follistatin, Noggin, Chordin
Receptor(s)	Type I/II TGFβ receptors (serine/threonine receptor kinases)		Patched : lignad binding Smoothened : transducing			*Nuclear Receptors* RAR RXR	FGFR (receptor tyrosine kinase)	TGFβR (receptor serine/ threonine kinase)
Signal transduction components	SMAD transcription factor family		*Gli transcription factor family* Gli 1, 2, 3			RAR RXR Cdx transcription factor family	Cdx transcription factor family	SMADs
Downstream transcription factors (ventricular zone)	*Progenitor Domain* pD1 pD3	*Transcription Factors* Math1 Ngn1	*Progenitor Domain* pV0 pV1 pV2 pMN pV3 pOlig	*Class I* Dbx1 Dbx2 Irx3 Pax6 – _	*Class II* ? Nkx6.2 Nkx6.1 Olig2 + MNR2 Nkx2.2 Nkx2.2 + Olig2	*Rostral/Caudal Level* hindbrain cervical brachial thoracic lumbar	*Transcription Factors* HoxB1/2/3/4 HoxC5 HoxC6/8 HoxC8/9 HoxC10	
Differentiated cell type (mantal zone)	*Cell Type* D1 IN D2 IN D3 IN D4 IN roof plate astrocytes	*Functional Markers* Lhx2/9 (Lh2A,B) Isl1 Lhx1/5 (Lim1, 2) Lmx1 Gdf7 GFAP	*Cell Type* V0 IN V1 IN V2 IN MN V3 IN floor plate oligodendrocytes astrocytes	*Functional Markers* Evx1/2 En1 Lhx3, Chox10 Isl1/2, HB9 Sim1 HNF3β PDGFRα GFAP		*MN Subtypes* columnar pool hindbrain visceral types	*Functional Markers* LIM transcription family ETS transcription family Phox transcription family	

signaling should help to work backward to characterize the signal transduction pathways.

Ventral Spinal Cord Development

Genetic studies as well as *in vitro* explant experiments have implicated Shh in the differentiation of ventral spinal cord cell types involved in locomotor control (V0–V3 INs and MNs), as well as oligodendrocytes (Fig. 1A) [3,8]. Unlike the nested combinations of TGFβ molecules in the dorsal spinal cord; however, only one hedgehog member appears to be involved in ventral spinal cord patterning in higher vertebrates. This raises the question of how different ventral cell types are induced by a single factor. Extensive studies with *in vitro* explants have shown that Shh concentration differences of approximately two- to threefold dramatically influence the types of cells that are triggered to differentiate. Decreasing concentrations of Shh progressively induce cell types found further from the ventral midline, recapitulating the normal organization of cells in the ventral spinal cord [17]. As with TGFβ signaling, there are also important temporal mechanisms that modify progenitor cell responses to Shh signaling during development. At early stages Shh acts on progenitor cells to trigger MN differentiation, but, later in development, oligodendrocytes are produced instead of MNs. The basis for this switch is not well understood but seems to involve the regulation of the transcription factor Nkx2.2 [18,19].

The active Shh signaling molecule is autoprocessed and cholesterol-modified, and binds to the patched/smoothened receptor complex [20]. In the absence of Shh, patched is thought to inhibit smoothened from signaling, and this inhibition is relieved when Shh binds patched. Many additional components of the Shh signaling pathway have been identified through genetic studies in *Drosophila*, including the downstream Gli family of zinc finger transcription factors [20]. Genetic studies of *Gli3* and *Gli3*/*Shh* compound mouse mutants indicate that this transcription factor is likely an intermediary in the Shh pathway, although it seems to function indirectly as a transcriptional repressor [21,22].

How might small gradations in the level of Shh signaling produce sharp progenitor cell domains that serve as the precursors for different ventral cell types? Studies of the factors regulated by Shh in the ventricular zone have uncovered a network of homeodomain proteins that mark distinct progenitor domains (Table I) [7]. The expression of these factors is controlled at two levels. First, Shh either represses (class I) or activates (class II) the expression of the homeodomain factors. If this were the only mechanism operating to control these factors, it might be expected that the interpretation of the fine Shh gradient would lead to imprecise boundaries of gene expression. However, the domains appear to be further refined by cross-repressive transcriptional interactions between factors from different domains. In this two-step manner, graded Shh leads to the activation of unique combinations of homeodomain transcription factors in precise progenitor cell domains [3,8]. The combinatorial activities of these homeodomain factors lead to the activation of downstream transcriptional regulators involved in cell specification and function (Table I) [23].

The opposing nature of the ventral Shh gradient meeting the dorsal TGFβ factors suggests that inhibitors of Shh activity might constrain its activity, much like the inhibitors of TGFβs. Hedgehog interacting protein (Hip) is a surface membrane protein that binds Shh and attenuates its activity [24]. In addition, characterization of the mouse *open brain* (*opb*) mutant, in which ventral cell types form inappropriately in the dorsal region of the spinal cord, has led to the identification of a member of the Rab family of vesicular transporters, Rab23, important in limiting the activity of Shh dorsally (Fig. 1A) [25]. Interestingly, mice deficient in both Shh and Rab23 regain many of the ventral cell types lost in Shh mutants. This, together with the observation that *Gli3*/*Shh* double mutants also regain many ventral cell types [22], suggests additional Shh-independent pathways might contribute to ventral spinal cord development. Studies to understand the basis for Shh-independent signaling have uncovered a parallel pathway involving retinoic acid (RA) expressed by paraxial mesoderm beside the neural tube [26]. It will be interesting to examine in more detail the interplay between Shh and RA signaling pathways in order to fully understand the molecular basis for neuronal specification along the dorsoventral axis of the neural tube.

Rostrocaudal Specification

The spinal cord can be subdivided into four broad, functional regions along the rostrocaudal axis: cervical, brachial, thoracic, and lumbosacral. The IN classes of the spinal cord extend continuously throughout these regions, while specific MN subclasses are found at each level [27]. Individual MN subclasses form discontinuous columns in register with their targets, such that MNs of the cervical region innervate axial muscles, brachial region MNs innervate the forelimb, MNs of the thoracic region innervate body wall muscle, and lumbar MNs innervate hindlimbs. Much like the initiation of dorsoventral patterning in the spinal cord, embryonic manipulations and *in vitro* explant studies suggest that members of several families of signaling molecules originating initially from sources outside the spinal cord contribute to the diversification process that leads to the generation of specific classes of MNs along the rostrocaudal axis [28–31].

Studies of the signals that control segmental identity along the rostrocaudal axis have used *Hox* gene expression patterns as downstream molecular correlates of the regional specification of cell identity (Table I). Furthermore, there is increasing functional data to suggest that *Hox* genes contribute to the proper development of MN subclasses [27,32,33]. As neuroepithelial cell identity is first established, it is thought to have a rostral identity which is then modified by "caudalizing" signals [4]. Hindbrain studies have found that increasing levels of RA activate more caudal-type *Hox* genes [34–36].

Likewise, the pattern of *Hox* gene expression in the cervical spinal cord is regulated by RA synthesized by the cervical paraxial mesoderm flanking the neural tube (Fig. 1B) [31].

However, at more caudal regions of the spinal cord, RA is insufficient to confer positional identity. A major source of additional regionalizing signals is detected in Hensen's node (HN), a precursor of the axial mesoderm that moves in a caudal direction below the nascent neural tube as development progresses. Interestingly, HN tissue taken from different stages (i.e., different rostrocaudal levels) is able to specify different regional values in neural explants [31]. Studies utilizing fibroblast growth factor (FGF) receptor antagonist SU5402 and expression of constitutively active FGF receptors are found to alter the Hox coding in neural cells, implicating FGF signaling as a mediator of HN activity. FGF8 is expressed by the HN and *in vitro* studies have found that this factor can act in a concentration-dependent manner to induce progressively more caudal positional values in neural explants. The Cdx family of transcription factors represents possible downstream mediators of both RA and FGF8 signaling in the regulation of Hox expression [36].

Taken together, these findings suggest that the signaling activity of FGF8 increases as the HN moves caudally (Fig. 1B) [31]. An additional mechanism that appears to contribute to the increased activity of FGF8 at more caudal positions is the involvement of accessory factors that enhance FGF signaling. One such example is the TGFβ superfamily member Gdf11. This factor is expressed in HN as it progresses through lumbosacral levels, where FGF signaling is expected to be highest (Fig. 1B). Unlike other TGFβ members, Gdf11 does not influence the dorsoventral pattern of the spinal cord, but rather acts to enhance FGF8 signaling activity. In this way, progressively more caudal regions of the spinal cord are defined by the composite functions of FGF8 and Gdf11 through the regulation of Hox codes involved in establishing regional levels of the spinal cord that will generate different MN subclasses (Table I).

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CHAPTER 269

Cadherins: Interactions and Regulation of Adhesivity

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Introduction

The ability of cells to distinguish between its neighbors and to recognize and associate with cells of specific subtypes is a prerequisite for the organization of the vertebrate and invertebrate body plan [1,2]. Cadherins are a class of transmembrane cell surface glycoproteins that regulate tissue morphogenesis and are necessary for maintaining the integrity of adult tissues [3–7]. The hallmark of cadherin molecules is their ability to confer calcium-dependent cell-to-cell adhesion, predominantly in a homophilic manner (e.g., molecules on one cell surface bind to molecules of the same molecule type on opposing cell surfaces [8]). This function plays profound roles in developing embryos as cells segregate from each other to form and distinguish specialized tissues from surrounding cells [9–12]. In the vertebrate nervous system, cadherins are implicated in the embryonic formation of specific brain subdivisions and the establishment of functional circuits [13]. Cadherins are clustered at synapses where they straddle the active zone [14,15] and regulate synaptic morphology and strength [16–18]. In epithelial tissue, cadherins are prominent components of adherens junctions [19,20]. In their junctional most adhesive configuration, they associate with the armadillo-repeat protein β-catenin (or plakoglobin) that connects the transmembrane cadherins via α-catenin to the actin-based cytoskeleton. Modulation of cadherin function is one of the central events that initiates cell spreading and migration during morphogenetic movements and cancer. Moreover, the transition from an epithelial to an invasive phenotype during growth and metastasis of malignant tumors is often associated with the loss of E-cadherin, and reintroduction of E-cadherin into metastatic cells can suppress the tumor phenotype [21,22]. Because of the recognized importance of cadherin cell adhesion molecules, major research efforts have concentrated on understanding the structural properties, dynamics, and signal transduction mechanisms by which cadherins regulate adhesive strength. Significant evidence has accumulated demonstrating that the fine-tuned balance of cellular signaling pathways that either enforce or reduce adhesion dynamically controls cadherin function. Moreover, it has become clear that cells regulate the degree of cadherin-mediated adhesion by diverse mechanisms. This chapter will summarize the mechanisms that contribute to the regulation of cadherin-mediated adhesivity.

The Members of the Family

Classical Cadherins

The vertebrate classical cadherins are transmembrane proteins composed of five tandemly arranged cadherin domains and a highly conserved cytoplasmic tail (Fig. 1). Cadherin domains (CD) are defined by a stretch of approximately 110 amino acids that contains characteristic motifs involved in calcium binding [3,23]. Classical cadherins or vertebrate type I cadherins, such as E-, P-, N-, and R-cadherin, display in the amino terminal CD1, a conserved HAV motif that has gained attention as a potential cell recognition sequence [24]. Although peptides containing this motif indeed inhibit cadherin-mediated functions [25,26], structural studies are inconsistent with this suggestion [27]. Atypical or vertebrate classical type II cadherins share the structural

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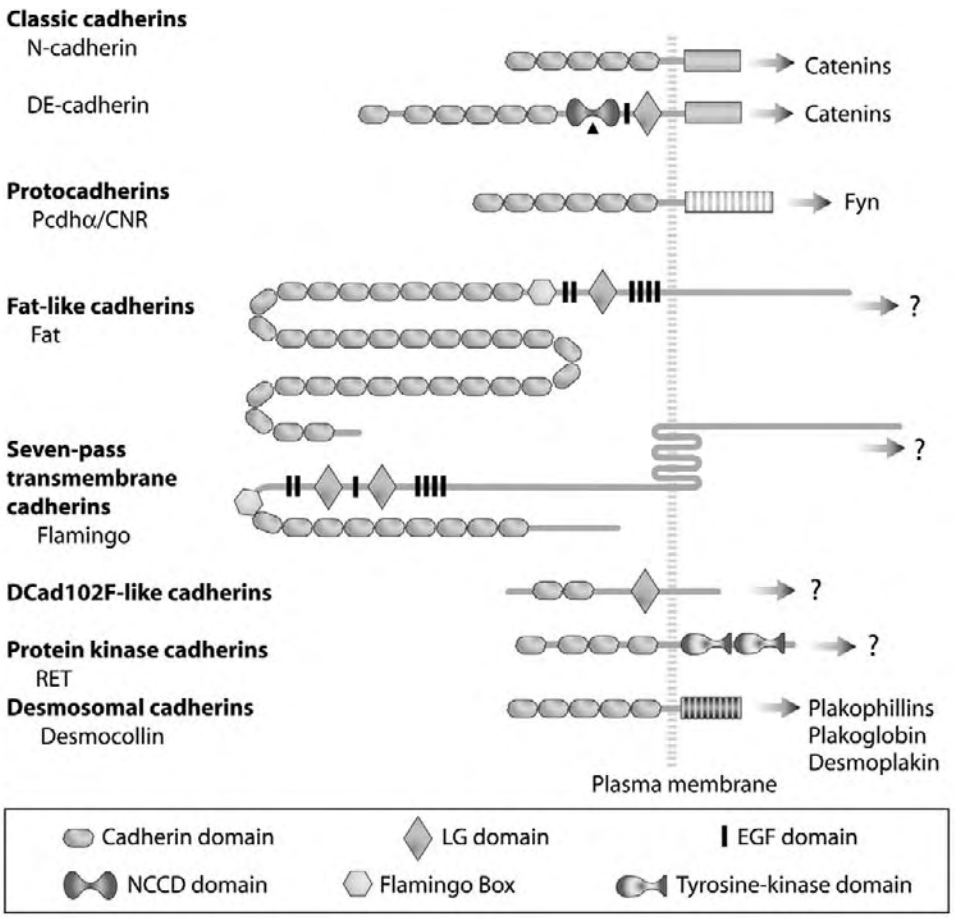


Figure 1 Molecular diversity of the cadherin family. The domain organization of representative members of each of the identified cadherin families is depicted. Arrows indicate the linkage with indicated intracellular molecules.

organization with the type I cadherins, but are less related based on sequence homology [28]. The intracellular domains of both groups are highly conserved. Transmembrane classical cadherins associate through interactions with the armadillo repeat family proteins β-catenin/plakoglobin with the actin cytoskeleton. This interaction is critical for dynamically regulating cadherin-mediated adhesivity. Classical cadherins exert profound functions in animal morphogenesis. Deletions of E- or N-cadherin gene expression in mice result in embryonic lethality respectively at the embryo preimplantation stage [29,30] and at embryonic day 10 due to defects in cardiac development [31].

Invertebrate classical cadherins including *Drosophila* DE- and DN-cadherin [32–34], *Caenorhabditis elegans* HMR-1 [35], and sea urchin LvG-cadherin [36] diverge in their extracellular domain organization from their vertebrate counterparts and contain additional CDs, cysteine-rich EGF repeats, laminin A G domains, and often a nonchordate classic cadherin domain (NCCD) [37] (Fig. 1). The cytoplasmic domain of invertebrate classical cadherins associates with *armadillo*, a molecule of the catenin family that can be linked to the actin cytoskeleton [5]. Mosaic analysis of mutations in the classical DE-cadherin-encoding *shotgun (shg)* gene provides evidence for homophilic binding activity [38]. Thus, despite structural divergence of their extracellular domains, the signaling mechanisms and principal functions of the classical cadherins are conserved between vertebrate and nonvertebrate species [39].

Cadherins with Divergent Structures

Bioinformatics has revealed CD-like structures in yeast and bacteria [40] suggesting that the CD represents an ancient structural motif. With the evolution of multicellular organisms, the diversity of proteins containing CDs has expanded enormously, and in humans more than 180 genes encoding proteins with cadherin domains have been reported (www.pfam.wustl.edu). Most, but not all of these genes are classified into distinct subgroups based on sequence homologies, domain organization, CD number, and genomic organization [5,28,39]. Representative members of the major subclasses are shown in Fig. 1.

PROTOCADHERINS

The largest subgroup of cadherin-related proteins is represented by the transmembrane protocadherins, which are composed of up to seven cadherin domains and contain

divergent and distinct cytoplasmic regions [41,42]. Protocadherins are most abundant in the nervous system. Three gene clusters Pdchα, Pdchβ and Pdchγ located on human chromosome 5q31 are predicted to encode 52 cadherin-related molecules with a variable ectodomain and a characteristic cytoplasmic region [43]. Pdchα orthologs were independently identified as cadherin-related neuronal receptors (CNR) in mouse brain by their ability to interact with Fyn [44], an Src-related intracellular kinase that supports synaptic plasticity [45]. The observation that CNRs demarcate specific synapse populations [44] raises the possibility that these proteins regulate synaptic function or plasticity. Indeed, one of the synaptic protocadherins, Arcadlin (activity-regulated cadherin-like protein), was identified as a gene product induced by synaptic activity [46]. The functions of specific protocadherins during CNS development and in synaptic function are not yet known. Outside of the nervous system, the paraxial protocadherin (PAPC) was found to control cell movements during gastrulation [47] and establish segmental boundaries during somite formation [48]. These functions together with the demonstrated homophilic binding activity indicate that PAPC function resembles that of the classical cadherins [49,50], although its divergent cytoplasmic region indicates a different intracellular signaling mechanism.

FAT-LIKE CADHERINS

Drosophila Fat, the prototype of the fat-like cadherins, acts as a tumor suppressor as indicated by recessive lethal gene mutations that cause overgrowth of larval imaginal discs and other morphogenetic defects [51–54]. The hallmark of the fat-like transmembrane cadherins is their large extracellular domain that is composed of 17–34 tandemly arranged cadherin domains, EGF repeats, laminin A G domains, and, in some cases, a flamingo box [5,52]. *Drosophila fat* and the fat-like *dachsous* gene product are predominantly expressed in epithelial cells and contribute to planar polarity, the coordinated orientation of cells, in the eye and the wing [55,56]. Fat-like cadherins with functions in tissue morphogenesis are reported for the nematode *C. elegans* [57,58] and mammalian homologs have been identified [59,60], although their functions remain to be elucidated.

SEVEN-PASS TRANSMEMBRANE CADHERINS

This group of cadherins is anchored within the membrane by a seven-pass transmembrane domain similar to G-protein-coupled receptors. The extracellular domain of *Flamingo/starry night*, a *Drosophila* seven-pass transmembrane cadherin, consists of nine amino terminal cadherin domains, a flamingo box, EGF repeats and laminin A G domains [61]. *Flamingo/starry night* acts in concert with *fat*, *dachsous* and components of the *wnt/frizzled* pathway to establish planar polarity of hair cells in the *Drosophila* wing [53,56,61,62]. Seven-pass transmembrane cadherin receptors including mouse *Celsr1* [63], mouse *Fmi* [61], and rat *MEGF2* [64] have also been isolated from the vertebrate nervous system and remain to be assessed for their functions and molecular associations.

DCAD 102F-LIKE CADHERINS

Drosophila Dcad 102F and *C. elegans* CDH-11 represent a separate subclass of cadherin molecules with yet unknown functions. These transmembrane cadherins are composed of two cadherin domains, a laminin A G domain and a Glu-Ser-rich cytoplasmic domain region unrelated to other members of the cadherin family. They share significant homology with two proteins of unknown function from human brain (KIA0911 and KIAA0726) [65].

PROTEIN KINASE CADHERINS

The protein kinase cadherins are represented by Ret, which consists of four cadherin-like domains and an intracellular kinase region [66,67]. Ret is part of a tripartite receptor complex that is activated by interactions with neurotrophic factors of the glial cell-line-derived neurotrophic factor (GDNF) family. GDNF induces or stabilizes a complex between Ret and GPI-linked alpha receptors (GFR alpha 1–4) resulting in dimerization and activation of the Ret kinase [68]. Mutagenesis studies have shown that Ret, GFR alpha 1, and GDNF affect multiple developmental events including development of the enteric nervous system affected in Hirschsprung's disease [69–71].

DESMOSOMAL CADHERINS

The desmosomal cadherins comprise two separate subfamilies, the Desmocollins (DSC) and the Desmogleins (DSG) [72,73]. Each subfamily is represented by three members (DSC-1, -2, -3 and DSG-1, -2, -3) and each of these molecules displays a cell type- or differentiation-specific expression profile [74,75]. The extracellular domain of desmosomal cadherins is composed of five cadherin domains and confers homo- or heterophilic binding interactions with other members of the desmosomal cadherin family. DSCs and DSGs contain characteristic intracellular domains that diverge from those of the classical cadherins and interact with either of the armadillo family proteins plakoglobin and plakophilin. The latter provide a link (via desmoplakin) to intermediate filaments. The complex constituted by desmosomal cadherins and associated intracellular proteins is essential for the assembly of desmosomal plaques [76–79]. Several autoimmune skin blistering diseases (such as pemphigus vulgaris or foliaceus) are caused by desmoglein autoantibodies that act on disrupting desmosomes within epidermal layers [80].

Cadherin Structure-Function Relationships

Cadherin activity is typically measured by the ability of transfected cells to confer calcium-dependent cell-to-cell adhesion and is well established for the classical cadherins. Accordingly, analyses probing cadherin structure and function have focused on classical cadherin subtypes. Earlier work has established that the active binding configuration of these cadherins is only accomplished in the presence of calcium [3]. Mutation of one of the calcium-binding sites renders E-cadherin inactive in conferring homophilic binding [81].

Calcium binds to conserved motifs (DXD, DRE, and DXNDNXPXF) at the interface between cadherin domains and provides for the structural rigidity and dimerization of the extracellular cadherin domain. In the absence of calcium, E-cadherin polypeptides appear collapsed and disorganized by rotary shadowing and electron microscopy, while the addition of calcium at low concentration (50 μM) enforces the formation of rigid rod-like structures [82,83]. An increase in the calcium concentration (500 μM) supports *cis* association of adjacent cadherin extracellular domains, and concentrations above >1mM drive *trans* interactions between opposing *cis* strand dimers [83–85]. The strongest adhesive force between cadherin molecules is exerted in the *trans* dimer configuration. These and cell biological studies have corrected the previous concept that the classical cadherins are inactive without their linkage to the actin cytoskeleton [86–90]. It is now accepted that the cadherin extracellular domain alone suffices to induce cell-to-cell adhesion and the interaction of the full-length molecules with the cytoskeleton is required for compaction. Thus, dimerization is a critical parameter in regulating the strength of cadherin-mediated adhesivity.

Initial structural studies of the specificity-determining cadherin extracellular domain CD1 [91] revealed a β-strand organization similar to the immunoglobulin fold [92,93]. The crystal structures suggest that calcium plays an important role in cadherin dimerization [92,94–96]. Dimerization can occur between identical cadherin molecules (homodimerization) and heterodimers [97]. One of the suggested major features for cadherin dimerization is the association of Trp-2 of mature cadherin polypeptides with Ala-80, which is presented within a hydrophobic acceptor pocket formed around the HAV sequence [27,94,96–98].

Several experimental approaches provide evidence for multiple binding sites along the cadherin extracellular domain. First, biophysical studies demonstrate that the distance between opposing cadherin-covered lipid bilayers (250 Å) corresponds to the length of the cadherin extracellular domain [99,100]. Force application results in the stepwise increase of the intermolecular distance between cadherin molecules and suggests additional binding sites along the molecule [99,100]. Second, analyses of the binding properties of C-cadherin domain constructs show that the highest homophilic binding activity is conferred by the entire extracellular domain, while CD1 polypeptides exhibit only low binding activity [101]. Third, the crystal structure of the full C-cadherin ectodomain displays *cis*- and *trans* associations of the CD1 domain with multiple sites along the cadherin extracellular domain [27].

These cumulative data on cadherin structure-function relationships are consistent with a model in which adhesion occurs in multiple steps (Fig. 2). First, in the presence of calcium, cadherins adopt a rigid structure that is able to undergo *cis* dimerization. Lateral cadherin clustering into strand dimers enables subsequent engagement with *cis* dimers from opposing cell surfaces. One may speculate that the initial interactions occur between opposing CD1 domains. Upon favorable conditions, the adhesive bonds tighten and the cadherin extracellular domains fully interdigitate to bring adjacent cell surfaces into tight contact. Despite these seminal insights into the structure and interactions of the cadherin extracellular domains in these models, interactions *in vivo* may be more complex as cadherins were recently shown to also communicate at adherens junctions with another adhesion system, the actin-linked nectin-afadin-ponsin complex [102,103]. Studies of the molecular and structural interactions between these combinatorially expressed protein complexes may reveal new insights into the adherens junction assembly.

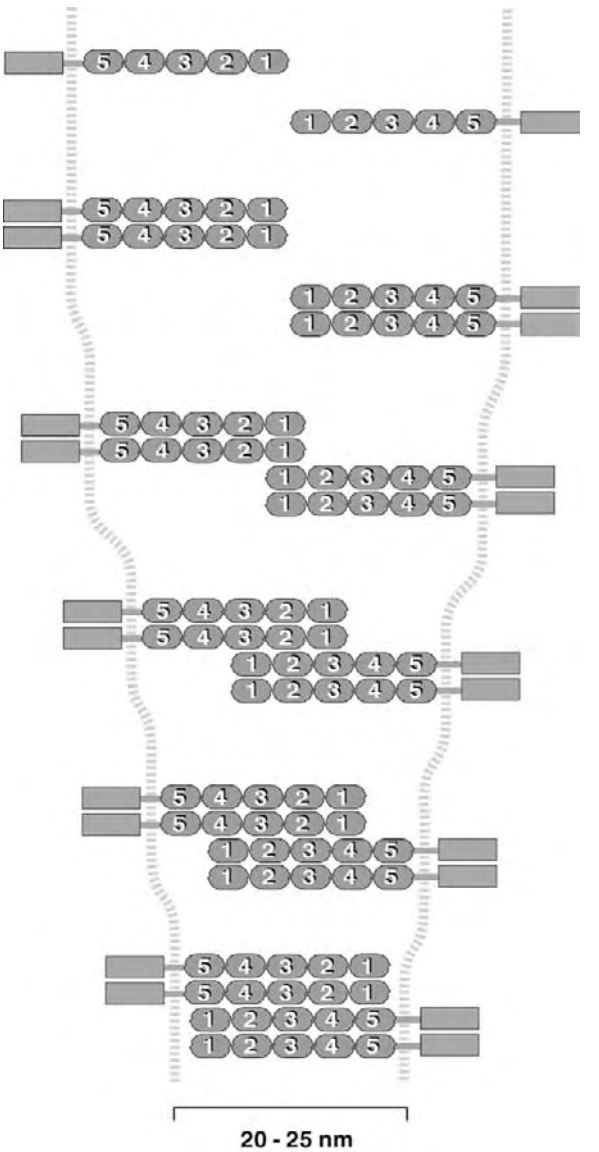


Figure 2 Stages of cadherin-mediated adhesive interactions. Cadherin monomers associate into strand dimers that subsequently engage with *cis* dimers from opposing cell surfaces. Initial interactions may occur between opposing CD1 domains. Upon favorable conditions, the adhesive bonds tighten and the cadherin extracellular domains fully interdigitate to bring adjacent cell surfaces into close contact.

Multiple Modes for Regulating Cadherin Adhesive Activity

During tissue morphogenesis, cells undergo dynamic rearrangements that require the spatial and temporal regulation of adhesive strength. The dynamic expression patterns of cadherins in developing embryos, and the functional correlation of cadherin-mediated adhesion with tissue reorganization during development suggest that regulation of cadherin function is critical for morphogenetic processes. In their most activate state, cadherins contribute to the formation and maintenance of tight adhesive contacts between adjacent cells. In this configuration, cadherin clusters at the contact site link adjacent cell surfaces to the filamentous actin cytoskeleton [104,105]. Cell migration requires the loosening of strong adhesive bonds and the downregulation of cadherin function. For example, neural crest cells modulate N-cadherin-mediated adhesion prior to migrating and ectopic cadherin expression in crest cells prevents migration [106]. The demonstrated role of N-cadherin in neurite extension *in vitro* [107,108] and axon guidance *in vivo* [109,110] requires low stringency adhesive interactions to allow cell motility. A myriad of studies have established that cadherin function is regulated at multiple levels, including gene transcription, proteolysis, endocytosis, and association with intracellular proteins. The highly conserved cadherin cytoplasmic domain provides selective target sites for regulating the cadherin-actin association that in turn influences the adhesive cell properties (Fig. 3).

Association with Intracellular Proteins

β-CATENIN/PLAKOGLOBIN

The central player in regulating the function of the classical cadherins is the armadillo repeat protein β-catenin (or the closely related plakoglobin) that binds to a conserved sequence within the cadherin carboxy-terminal cytoplasmic domain [3,81]. Deletion of the catenin-binding domain renders the mutant cadherin polypeptides inactive in promoting cell-to-cell aggregation [86,87]. The central status of β-catenin is derived from its ability to associate in a mutually exclusive manner with different cellular signaling pathways that regulate the formation of adherens junctions, gene transcription, and protein degradation [111–113]. In association with the cadherin cytoplasmic tail, β-catenin complexes with the actin-binding protein α-catenin to regulate cadherin association with actin filaments [86,114]. The small GTPases Rac, Rho, and Cdc42 are well known for regulating and specifying membrane interactions with cortical actin filaments [115], and thus it is not surprising that cadherin function is modified by such proteins. IQGAP1, an effector of the Rho family GTPases Rac and Cdc42, can modify the association of the cadherin cytoplasmic region with actin filaments [116,117]. At cell-to-cell contact sites, IQGAP1 is associated with active GTP-bound Rac and Cdc42 which prohibits binding to β-catenin. In their inactive GDP-bound form, Rac and Cdc42 do not associate with IQGAP1, which can then bind to β-catenin and dissociate the cadherin-β-catenin complex from α-catenin and the actin cytoskeleton thereby downregulating adhesivity [118]. The association of β-catenin with the cadherin cytoplasmic domain is also regulated by the Gα subunit of heterotrimeric G proteins. Binding of activated GTP-bound Gα12/13 to the cadherin cytoplasmic region dissociates β-catenin from the complex and negates adhesive interactions [119,120]. These and many other studies provide evidence that cadherin-mediated adhesive interactions are controlled to a large extent by the available pool of β-catenin in binding configuration to interact with cadherin cytoplasmic tails.

The pool of intracellular β-catenin that is not bound to cadherins can associate with a large protein complex that contains the adenomatous polyposis coli (APC) tumor suppressor gene product, axin, conductin, and the glycogen synthase kinase-3 (APC complex). In association with this complex, β-catenin becomes rapidly phosphorylated and is subsequently targeted for ubiquitination and degradation in the proteosome [121]. An effective way to counteract β-catenin phosphorylation and degradation is by activation of the Wnt signaling pathway. Wnt plays an important role in cell fate determination during embryonic development and in cancers [121,122]. Wnt-binding to a receptor of the Frizzled family antagonizes β-catenin phosphorylation and degradation. Free unphosphorylated β-catenin can then bind to unoccupied cadherin cytoplasmic domains and enforce adhesive interactions through the cytoskeletal link. When the free β-catenin pool exceeds the number of available cadherin binding sites, it accumulates in the nucleus where it can bind to DNA binding proteins of the T-cell specific factor (TCF)/lymphoid enhancer binding factor (LEF) family. β-catenin binding to TCF/LEF transcription factors enhances expression of new genes that contribute to regulating the cell cycle [121]. Thus, β-catenin serves as a molecular switch for regulating cell behavior. The pool of β-catenin, its posttranslational modifications, and its association with the cytoskeleton are central to the regulation of cadherin-mediated adhesive activity.

p120ctn

An earlier study [9] attributed functional importance to the cadherin membrane proximal cytoplasmic domain, and it has now become clear that this site is a target for a complex regulation of cadherin-mediated adhesivity. The membrane proximal cadherin cytoplasmic region contains the binding site for the armadillo repeat protein, p120ctn. Similar to β-catenin, it is dynamically regulated by phosphorylation [90,123,124]. Phosphorylated p120ctn does not interact with the binding site on cadherins, and other proteins to regulate cadherin-mediated adhesivity can occupy the binding region (see Fig. 3). P120ctn function is also affected by Rho family GTPases which act on the actin cytoskeleton to increase or decrease cell motility [118,125,126]. As p120ctn per se seems dispensable for adhesive interactions, it may

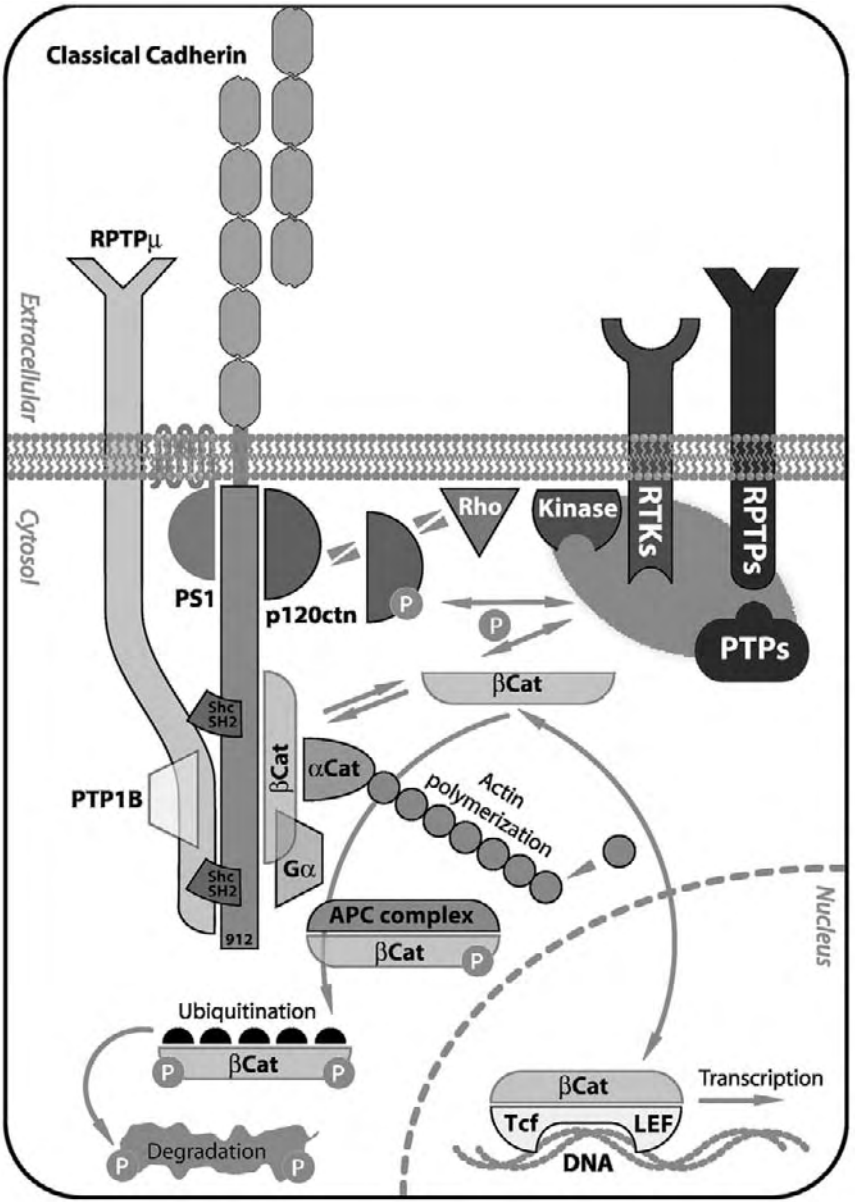


Figure 3 Regulation of cadherin function by associated intracellular proteins. The conserved cytoplasmic region of the classical cadherins interacts with multiple proteins that regulate adhesivity. Cadherin association with β-catenin and linkage of the complex to actin filaments (via α-catenin) plat a central role in this regulation. Receptor protein kinases (RTKs), receptor tyrosine phosphatases (RPTPs), ad intracellular phosphatases (PTPs) balance cadherin, β-catenin, and p120ctn phosphorylation. PS1 = Presenilin-1, βcat = β-catenin, αcat = α-catenin, and RPTPμ = receptor tyrosine phosphatase α.

serve as a molecular switch that can promote or prevent adhesion depending on its state of phosphorylation and the cellular context [90,124,127].

Protein Phosphorylation and Dephosphorylation

Structural studies of the interacting cadherin-β-catenin domains show that the cadherin domain is structured only when it is phosphorylated on serine residues [128]. The cadherin cytoplasmic region displays serine phosphorylation consensus sites for casein kinase II and glycogen synthase. Serine phosphorylation seems to strengthen the association between the cadherin cytoplasmic region and β-catenin and strengthen adhesion [129]. In contrast, tyrosine phosphorylation of the cadherin cytoplasmic tail and β-catenin dramatically decreases the interaction between these proteins and weakens adhesive bonds [130–132]. Both nonreceptor- and receptor-type kinases regulate phosphorylation of the cadherin cytoplasmic tail and β-catenin. Overexpression of the nonreceptor tyrosine kinases Src or Fer in cultured cells induces phosphorylation of tyrosine residues on cadherins and β-catenin [130,133,134] and decreases adhesive interactions

in favor of a motile phenotype. Interestingly, in cancer cells, the lipid phosphatase activity of PTEN can counteract Src-induced cell scattering and invasiveness and stabilize the E-cadherin junctional complex through an as yet unknown mechanism [135]. Similarly, E-cadherin and β-catenin tyrosine phosphorylation by receptor kinase type growth factor receptors results in cell scattering [136,137]. Tyrosine-phosphorylated cadherin can bind the adaptor protein Shc, which participates in stimulating mitogenic signaling pathways by growth factor activation of Ras [138]. An alternate pathway for tyrosine-phosphorylated E-cadherin is ubiquitination and rapid endocytosis. Recent work has identified Hakai, an E3 ubiquitin-ligase that can interact with E-cadherin in a tyrosine-phosphorylation-dependent manner and stimulate endocytosis [139]. Cell surface removal of E-cadherin through this pathway decreases adhesion and enhances cell scattering. Thus, cadherin function is controlled by multiple cell signaling pathways, which regulate the availability of cadherins on the cell surface and balance the cadherin association with β-catenin and hence with the cytoskeleton.

Formation of the cadherin-catenin complex is also fine-tuned by the balance between protein kinase and phosphatase activity. Several phosphatases associate with and stabilize the cadherin-β-catenin complex, presumably by preventing phosphorylation. The nonreceptor-type phosphatase PTP1B is targeted to the cadherin complex where it interacts with sequences partially overlapping with the binding site for β-catenin [140]. The receptor tyrosine phosphatase μ (PTPμ) can interact with a largely overlapping site [141], indicating that the contributions of these proteins are mutually exclusive. Downregulation of either PTP1B or PTPμ suppresses N-cadherin-mediated neurite extension [142,143] by an as yet unresolved mechanism. Also, the nature of the interactions between phosphatases and β-catenin during cell adhesion and the process of neurite growth has not been determined. Other phosphatases including LAR, receptor tyrosine phosphatase β/ζ, and the Meprin/A5/Mu domain receptors κ and γ do not associate directly with cadherins, but may regulate cadherin function through β-catenin modifications.

Regulation by Proteolytic Cleavage

An effective strategy for regulating cadherin activity is cleavage by extra- and intracellular proteases. Mature classical cadherins are derived from precursor proteins that are cleaved at the RKQR sequence in transit to or at the cell surface [144]. Mutation of the cleavage site renders the mutant E-cadherin protein inactive in conferring cellular aggregation [144]. The proprotein convertase furin can exert proprotein cleavage of E-cadherin *in vitro* [145]; however, the expression and activity of furin *in vivo* have not been established.

The cadherin extracellular domain is subject to cleavage by metalloproteases. A 90-kDa soluble extracellular fragment of N-cadherin is generated during retinal development and may partake in modulating retinal axon guidance [146,147]. Numerous studies have implicated the loss of E-cadherin with tumor cell growth and metastasis [21]. E-cadherin is proteolytically cleaved in noncancerous mammary epithelial cells by ectopically expressed metalloprotease stromelysin-1 [148]. E-cadherin cleavage triggers the progressive conversion of the epithelial into a mesenchymal-invasive phenotype characterized by the disappearance of E-cadherin and β-catenin from cell-cell contacts and induction and activation of growth factors and endogenous metalloproteases [148]. Stromolysin, however, could not be detected in cancer cells or in the embryo, and the tissue-endogenous metalloproteases cleaving E-cadherin [149] remain to be defined.

Cellular responses to apoptotic signals are characterized by the disruption of cell-to-cell and cell-to-extracellular matrix contacts and cytoskeletal reorganization. During programmed cell death, adherens junctions disintegrate due to the actions of both metalloproteases and caspases on cadherin and β-catenin/plakoglobin molecules [150,151]. A metalloprotease activity releases most of the E-cadherin extracellular domain, while caspase-3 cleaves at an intracellular membrane proximal site [150]. These data enforce the suggestion that the structural integrity of cadherins, their assembly within adherens junctions, and their linkage to the actin filament network are critical for cell survival [152].

Lastly, in response to apoptotic stimuli, the γ-secretase activity of presenilin-1, a protein associated with Alzheimer's disease, can cleave E-cadherin at the membrane-cytoplasm interface [153]. The cleavage releases the cadherin intracellular domain, increases the intracellular pool of β-catenin, and facilitates the disassembly of adherens junctions by disconnecting cadherins from the cytoskeleton. However, under conditions that favor cell-to-cell adhesion, presenilin-1 binding stabilizes the junctional complex [154]. Such dual functions have also been reported for p120ctn which competes for the presenilin-1 binding site on E-cadherin in a mutually exclusive manner [154]. Presenilin-1 has also been demonstrated to bind to and regulate β-catenin function and trafficking thereby providing an additional mechanism for regulating adhesivity [155–157]. As presenilin-1 can be recruited to sites of synaptic contact [158] and synaptic morphology and function are regulated by the cadherin-catenin system [16–18], the cadherin-catenin-presenilin-1 interaction may favor the loss of synaptic structures at an early stage of Alzheimer's disease and increase vulnerability to neuronal apoptosis.

Conclusions and Perspectives

Over the years, it has become clear that the adhesive function of the classical cadherins is dynamically regulated. While beginning to grasp some of the principal mechanisms of this regulation, we are faced with new challenges. First, a large number of new cadherin-like molecules with cytoplasmic sequences different from those of the classical cadherins have been revealed. Little is known about the distribution, function, and modes of signaling of these molecules. Second, recent work suggests that cadherins may be far more

promiscuous in their binding specificities than previously assumed [159]. N-cadherin-deficient mutant mice die of defects in heart development [31]. This phenotype can be rescued by the cardiac-specific ectopic expression of E-cadherin [160]. suggesting that cadherin-mediated adhesivity but not adhesive specificity is required. Moreover, recent work has demonstrated that cadherin function is required for the sorting of motor neurons into specific pools [161]. While cadherin-mediated sorting has been attributed to the binding specificity of one cadherin type, motor neuron pools are defined by the combinatorial expression of multiple cadherins. Overexpression of one of these cadherins, MN cadherin, disrupts pool sorting. Although the mechanism for MN-cadherin function remains to be determined, there is good evidence to suggest that the cadherin homophilic binding activity is not required as ectopic expression of E-cadherin in motor neurons has no effect on pool sorting [161]. The myriad of data that speak to the regulation of cadherin function have made it clear that cadherin-mediated adhesivity is a multifaceted issue. The diversity and magnitude of the cadherin family adds a new level of complexity to understanding the cellular interactions conferred by the combinatorial cadherin expression during development and in adult organisms.

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PART III

Nuclear and Cytoplasmic Events: Transcriptional and Post-Transcriptional Regulation

Michael Karin, Editor

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PART III

Introduction

Michael Karin

All living organisms, whether unicellular or multicellular, animals or plants, need to be able to constantly adapt to changing environmental conditions and be capable of successfully handling stressful and life-threatening challenges. This requirement for constant adjustment and adaptation is the major evolutionary driving force underlying the development of signal transduction systems that control and modulate the use of genetic information stored in the cell's DNA in response to extracellular conditions and stimuli. Thus the *raison d'etre* for the various receptors, channels, adaptors, GTP binding proteins, protein kinases, and protein phosphatases discussed in Parts I and II is to allow the regulation of the cellular gene expression program in response to the cell's environment, neighborhood, and history. Part III of the Handbook is therefore focused on the different mechanisms through which signal transduction systems regulate the utilization of genetic information.

The process of gene expression in both prokaryotes and eukaryotes can be divided into several mechanistically and temporally distinct steps: transcription and generation of the primary RNA transcript, processing and transport of the RNA transcript, translation of the mRNA into protein, turnover (decay) of the mRNA, and, finally, turnover of its protein product. In a eukaryotic cell, the first two steps (transcription and RNA processing) take place in the nucleus, whereas the last three steps (translation, mRNA turnover, protein turnover) take place in the cytoplasm (although nuclear proteins are turned over in the nucleus). Regardless of locale, all of these processes are subject to regulation by signal transduction pathways.

The first two sections of Part III deal with transcriptional control mechanisms that are targeted by signal transduction pathways. The most common form of regulation is based on phosphorylation of either sequence-specific transcription factors or proteins that directly interact with such transcription factors. The phosphorylation event can either affect the subcellular distribution of the transcription factors (e.g., NFAT, NFκB), their ability to bind DNA, or their ability to activate or repress transcription (e.g., CREB, c-Jun). As mentioned earlier, regulation can be achieved through phosphorylation of the transcription factor itself (e.g., CREB) or through phosphorylation of an interacting protein, for instance, an inhibitor (e.g., IκB) that regulates the activity or subcellular distribution of the transcription factor. Rather than being extensive and cover every single transcription factor known to be regulated via phosphorylation, Section B of Part III includes authoritative reviews that highlight the general aspects and principles governing this form of regulation.

In addition to posttranslational modifications, such as protein phosphorylation, sequence-specific transcription factors are also subject to allosteric regulation. In this case the binding of a small ligand has a dramatic effect on either the DNA binding or transcriptional activation properties of the transcription factor. This type of regulation is most relevant to members of the nuclear receptor family that play very important roles in signal transduction and cell regulation. Members of this family of ligand-regulated transcription factors, reviewed in Section A, form the core of signal transduction pathways that regulate gene expression in response to steroid and thyroid hormones, fatty acids, bile acids, and cholesterol metabolites as well as certain xenobiotic compounds. Unlike the multicomponent pathways that control transcription in response to activation of cell surface receptors, nuclear receptors are multifunctional proteins that incorporate signal detection, amplification, and execution in one molecule.

Although sequence-specific transcription factors represent the most common target for signal transduction pathways, as far as transcriptional regulation is concerned, new evidence is emerging that some of the coactivators, corepressors, or mediators with which these factors interact may also be subject to regulation. Coactivators, corepressors, and mediators are often large multicomponent protein complexes that are recruited to promoters or enhancers through interactions with sequence-specific transcription factors. We have much more to learn about the regulation of these protein complexes,

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which may act either through chromatin modifications or direct interactions with the RNA polymerase holoenzyme. In addition to modulation of chromatin structure via recruitment of chromatin modifiers to sequence-specific transcription factors, evidence also exists to show that signal-responsive protein kinases may directly phosphorylate histones and regulate chromatin structure via a more direct route. All of these modes of transcriptional regulation are discussed in Sections A and B. Evidence is also growing to indicate that the activity of various transcription factors and chromatin components can be regulated via additional posttranslational modifications, such as acetylation and methylation. However, it remains to be demonstrated that signal transduction pathways can directly regulate the activity of protein acetylases or methylases in a way that does not involve protein phosphorylation. Finally, another important form of regulation discussed in Section B is regulated proteolysis, which is responsible for both activation and inactivation of transcription factors.

While transcriptional regulation has received most of the attention, it is quite clear that every single step in the gene expression process, including RNA processing, RNA transport, RNA translation, and RNA and protein turnover can be subject to regulation. Therefore a section of Part III is dedicated to posttranscriptional regulation of gene expression. Because much of the available information pertains to translational control and regulation of mRNA turnover, these topics receive most of the coverage. However, regulated RNA processing (splicing) and transport are also discussed. Although the currently available information regarding posttranscriptional regulation is far from being extensive, it is our hope that this largely neglected area of gene regulation will receive more attention in the near future and that, one day, it will be as well understood as transcriptional regulation.

Given the important role of transcriptional and posttranscriptional control of gene expression in adaptation to adverse environmental conditions, an entire section of this volume is dedicated to the signaling mechanisms underlying various stress responses. Among the topics covered are the heat-shock and UV responses and the different responses that are elicited by DNA damage, which have provided valuable basic information relevant to many other aspects of cell regulation and signal transduction. In addition to metabolic control, these stress responses are evolutionarily ancient and are conserved in many eukaryotic orders.

Like all of the other parts of this Handbook, the prevailing approach was thematic in spirit, intended to provide a state-of-the-art understanding of fundamental principles rather than detailed coverage of the entire field. One remaining challenge for the future is to be able to identify all of the transcription factors and their target genes that fall under the influence of a given cell surface receptor or a signal responsive protein kinase. Such information will be essential both for the ultimate understanding of biological specificity and for being able to predict the biological and pathophysiological outcomes of activation or inhibition of a particular signaling molecule.

SECTION A

Nuclear Receptors

Michael G. Rosenfeld, Editor

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CHAPTER 270

History of Nuclear Receptors

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Introduction

Nuclear receptors comprise a group of intracellular proteins found either in the cytosol, weakly bound in the nucleus, or both, that, in combination with small molecule ligands, bind tightly in the nucleus, recruit appropriate cofactors, and regulate transcription in target genes. Included in this "superfamily" are receptors for the steroid hormones (estrogens, progestins, androgens, glucocorticoids, mineralocorticoids, ecdysteroids, vitamin D), for thyroid hormone and retinoids, and for an ever-increasing number of "orphan" receptors, which show structural similarity to the other receptors but for which ligands are only now being gradually identified.

Progress in the understanding nuclear receptors and their action is summarized in Table I. Because studies with estrogens began earlier than those for the other agents, the first six discoveries, identifying receptor proteins and establishing the basic action pattern, as well as the preparation of specific antibodies, were first made with estrogen receptors and later extended to other types of cellular regulators. For the most part, the remaining findings were first obtained with progestins or glucocorticoids and then shown to apply to estrogens. Thus, this short history of the receptor concept focuses on the earlier studies with estrogens that provide the general model, with a brief summary of more recent discoveries that have expanded our understanding. For the most part, only the initial reports, which stimulated other investigations, are cited for each phenomenon, in some cases along with relevant review articles. Detailed references to the many studies that have contributed to our current knowledge of hormone-receptor interaction can be found in comprehensive review articles or monographs [1–13].

Discovery of Receptors and Shift in Research Direction

The synthesis in 1957 of estradiol labeled with carrier-free tritium provided a means for determining what actually happens to the steroid itself as it exerts its hormone action. Knowledge about what the target cell does to the steroid, taken together with the influence of the hormone on cellular processes, has provided a better understanding than would have been possible from either approach alone [14].

The demonstration that rat uterus and other estrogen-responsive tissues contain characteristic hormone-binding components [15], with which estradiol associates to induce growth without itself undergoing chemical alteration [16], directed thinking away from earlier concepts that had linked estrogen action to participation in the enzymatic processes of steroid metabolism. It became apparent that interaction with the steroid must in some way alter the properties of macromolecules involved in RNA and protein biosynthesis, processes that were known to be enhanced by hormone action [17]. But in the 1960s the idea that steroid hormones must interact with enzymes was so firmly entrenched that many biochemists questioned whether the observed binding had any direct role in hormone action. The finding that the progressive inhibition of estradiol uptake in the rat uterus by increasing amounts of the antiestrogen, nafoxidine, closely parallels its inhibition of uterine growth [18] provided evidence that hormone binding actually is involved in uterotrophic action and that binding components could be regarded as true receptors.

Receptor Forms and Physiological Action

When tritiated estradiol of high specific activity became commercially available in late 1962, other investigators

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Table I Steps in Development of the Receptor Concept

A. Conceptual stepping stones
1. Hormone-binding components in target cells
2. Hormonal action without metabolism
3. Binding substance is true receptor
4. Two receptor forms–cytosol and nuclear bound
5. Nuclear receptor derived from cytosol receptor
6. Composition of the two receptor forms
7. Hormone-induced receptor "transformation"
8. Transcriptional enhancement by transformed receptor
B. Detailed understanding of receptor structure and function
9. Antibodies to receptor proteins
10. Cloning and sequencing
11. Domain content and function
12. Interaction of transformed receptor with target genes
a. Hormone-response elements
b. Recruitment of coactivators and corepressors

confirmed its specific uptake in target tissues of various species. Cell fractionation and autoradiographic studies from several laboratories showed that most of the hormone becomes tightly bound in the nucleus, with a smaller amount present in the cytosol fraction, whereas, before hormone treatment, the binding substance appears entirely in the cytosol fraction. Similar results were obtained with excised uteri exposed to hormone at physiological temperature *in vitro*.

The application of sucrose gradient centrifugation to characterize the cytosol receptor complex as an 8 S entity (originally called 9.5 S) was an especially valuable contribution [19], because it provided a means to show that the complex bound in the nucleus is different from that in the cytosol. After exposure of rat uteri to estradiol *in vivo* or at physiological temperature *in vitro*, extraction of their nuclei with 400 m*M* KCl was found to solubilize the bound hormone as a 5 S estradiol–receptor complex [20], readily distinguished [21] from the 4 S entity produced from the cytosol complex by exposure to similar salt concentrations. After several studies suggested that the 5 S nuclear receptor is not present as such in target cells but is derived from the 8 S cytosol receptor [22,23], direct evidence was provided by the fact that simply warming cytosol to physiological temperature in the presence, but not the absence, of estradiol converts the receptor to the 5 S form [24], a process that is accelerated by the presence of DNA [25].

Based on observations first made with progestin and glucocorticoid receptors, the 8 S cytosol complex was shown to be a conglomerate of the 4 S receptor protein associated with a variety of molecular chaperones, including heat-shock proteins and immunophilin [10], which is dissociated by interaction with the hormone, as well as by exposure to salt. The 5 S "transformed" receptor was recognized as a dimer of the 4 S protein thus produced [26,27]. When it was found that the 5 S, but not the 4 S, complex stimulates RNA synthesis in isolated nuclei from hormone-responsive cells [28], the biochemical role of the hormone became clear, namely, to produce a functional transcription factor from an inactive precursor [29].

After analogous receptor systems were identified for other classes of steroid hormones (summarized in [7]), similar hormone-induced conversion of the native receptor to a nuclear binding form was observed. Subsequent molecular biological studies demonstrated that all hormone-transformed receptors react in dimeric form with their target genes to regulate transcription [30,31], but only with the estrogen receptor is the dimer stable enough to permit its identification in the absence of target cell DNA.

Subsequent Discoveries Relevant to Receptor Structure and Function

As summarized in Table I, more recent investigations, especially those involving techniques of molecular biology, have greatly increased our understanding of the processes by which nuclear receptors serve as regulators of cellular processes. After several investigators had tried unsuccessfully to obtain specific antibodies to estrogen receptors by conventional immunochemical techniques, application of sucrose density sedimentation to identify the nonprecipitating immune complexes formed with tritiated estradiol–receptor complexes provided specific polyclonal [32] and monoclonal [33] antibodies. Subsequently, antibodies were obtained for all classes of steroid hormone receptors, permitting the cloning and sequence determination, first of the glucocorticoid receptor [34], followed by the estrogen receptor [35,36] and other members of the nuclear receptor family.

Early studies with the glucocorticoid receptor indicated that it contained at least three distinct functional domains [37]. After cloning and sequencing, it became evident that receptors for steroid and thyroid hormones, vitamin D, and retinoids represent a superfamily of regulatory agents [4], consisting of DNA-binding (C) and ligand-binding (E) domains, a small, highly conserved hinge region (D) joining these two units, and, at the amino acid terminus, a large region (A/B) that shows little homology among the different receptors. Receptors for estrogens and thyroid hormones contain a small additional region (F) at the carboxy terminus, the function of which is not entirely clear.

For the regulation of transcription, it was found that the transformed hormone–receptor complex does not interact with the site of transcription but with a *hormone response element*, an enhancer located at the promoter area in the 5′-flanking area of the target gene [38,39]. Palindromic sequences appear to make up the binding site for the hormone–receptor complex. This interaction leads to the recruitment of coactivators or corepressors that modulate the resulting transcription [40–43].

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CHAPTER 271

Regulation of Basal Transcription by RNA Polymerase II

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Introduction

Transcription of protein-encoding target genes by RNA polymerase II (Pol II) represents the end point of many signal transduction pathways. A wide variety of cell type- and gene-specific transcriptional regulators (activators and repressors), including nuclear receptors, are the conduits through which these signals are transmitted to Pol II. These regulators typically function by binding to cognate promoter or enhancer elements located distal to the transcription start sites of target genes. From there they relay their regulatory effects to Pol II, which is directly controlled by the general transcription factors (GTFs: TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) (Table I) [1,2]. A primary function of the GTFs is to position and orient Pol II on core promoter elements that determine the transcription start site and to provide the active site of Pol II with initial access to the transcribed strand of the template. Despite the potential of Pol II and GTFs to support low levels of accurately initiated transcription *in vitro* (*basal transcription*), as well as the structural complexity (>40 polypeptides) of this machinery, responsiveness to regulatory factors is dependent on additional cofactors (coactivators and corepressors, see later discussion) [3]. The most general cofactors include TATA box binding protein (TBP)-associated factors (TAF_{II}s) in TFIID [4,5], positive cofactors (PC1, PC2, PC3, PC4, and PC52) derived from the USA cofactor fraction [3,6], and a multiprotein Mediator complex [7–9]. Together, Pol II, GTFs, and these cofactors constitute an intricate piece of machinery that makes it possible for the signals borne by activators and repressors to be translated into the appropriate genetic response on specific target genes.

This chapter discusses structural and functional aspects of the basal transcription apparatus and also addresses the role of regulatory cofactors that in turn, ultimately appear to function by directly or indirectly impacting Pol II and GTFs.

The Preinitiation Complex

Structural Organization

Multiple approaches, including X-ray crystallography and protein–DNA cross-linking, have led to a detailed description of the Pol II- and GTF-containing preinitiation complex (PIC) (Fig. 1), the key protein–DNA intermediate whose efficient assembly (and subsequent function) dictates, to a large extent, the level of expression of target genes. The crystal structure of Pol II from yeast cells reveals a complex machine that can effectively carry out RNA synthesis from double-stranded DNA templates [10–12]. Like their bacterial counterparts, with which they share extensive sequence homology [13], the two largest subunits (RPB1 and RPB2) of Pol II constitute the core of the structure and are surrounded by various smaller subunits. The catalytic site contains (at least) one magnesium ion that is buried deep within the cleft of the jaw-like assembly, from where it can drive templated incorporation of nucleotides into the growing RNA chain [12].

Pol II has not yet been visualized in the context of the PIC. However, subsets of the PIC components have been analyzed by X-ray crystallography. Together with other biophysical and biochemical approaches that have probed the fully assembled PIC, it has been possible to construct a working model of this multiprotein complex. On the paradigmatic TATA-containing core promoter, the TBP subunit of TFIID

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Table I Subunit Composition of RNA Polymerase II and Associated Factors

Pol II	TFIIA	TFIIB	TFIID	TFIIE	TFIIF	TFIIH	Mediator
RPB1	α	35 kDa	TBP	α	RAP30	ERCC3	TRAP240
RPB2	β		TAF250	β	RAP74	ERCC2	TRAP230
RPB3	γ		TAF150			P62	TRAP220
RPB4			TAF135			p52	TRAP170/RGR1
RPB5			TAF100			p44	TRAP150β/hSUR2
RPB6			TAF80			cdk7	TRAP100
RPB7			TAF55			cyclinH	TRAP97
RPB8			TAF43			p34	TRAP95
RPB9			TAF31			MAT1	TRAP93
RPB10			TAF30				TRAP80
RPB11			TAF28				p78/CRSP70
							SRB10/CDK8
							p37/CRSP34
							p36
							MED7
							MED6
							SRB11/Cyc C
							TRFP
							TRAP25
							SRB7
							SOH1
							NUT2
							p12
							p10

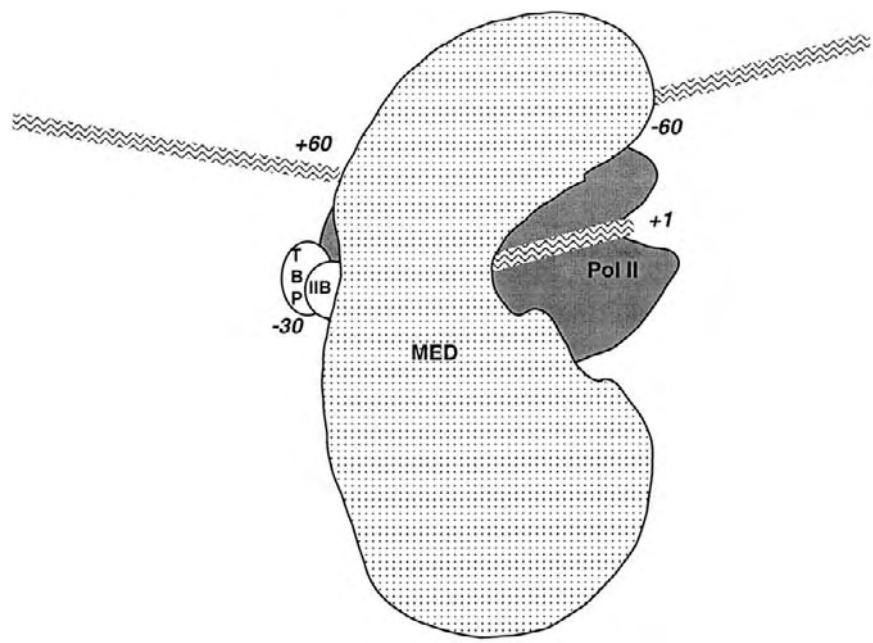


Figure 1 A schematic drawing showing a partial preinitiation complex. See text for details. For clarity, the TFIID-associated TAF_{II}s, TFIIF, TFIIE, and TFIIH are not shown. The figure is meant to emphasize the interaction of Pol II with Mediator and to highlight how the PIC likely consists of a central protein core wrapped within the template DNA (wavy lines). Although the positions of the factors are conjectural, care has been taken to ensure that they conform with previously published structural data. Activators and other cofactors, which are predicted to impinge on this machinery, have also been left out.

provides the main recognition contacts with promoter DNA. X-ray crystal structures of the TBP–promoter [14,15] and the TBP–TFIIB–promoter [16] complexes have revealed that the saddle-shaped TBP molecule interacts with the minor groove of DNA through its concave underside and induces both an unusual distortion of the central TATA element and a sharp bend in the template DNA. This arrangement leaves available large areas on the relatively convex upper surface of TBP for potential interactions with TAF_{II}s within TFIID and other factors.

The interaction of TBP with promoter DNA is stabilized by TFIIA, which binds upstream of TBP and makes contacts both with TBP and with phosphates in the DNA backbone upstream of the TATA box [17,18]. The TFIIA requirement in transcription, which appears to be variable [2], is also reflected in its ability to alleviate repression by negatively acting cofactors such as NC2 [6] (see later discussion also) and may be related to how TFIIA is positioned within the complex.

The TBP–promoter complex is also stabilized by interactions with the C-terminal core repeat domains of TFIIB, which essentially slots into the TBP–promoter complex and makes contacts both with the underside of TBP and with promoter DNA upstream and downstream of the TATA element [16]. Fluorescence anisotropy measurements suggest the presence of a conserved element (TFIIB recognition element, BRE) through which TFIIB makes specific contacts with the DNA [19]. These conclusions are directly confirmed by the crystal structure of a promoter complex containing archaeal TBP and TFB, its TFIIB ortholog [20]. Together these results establish the underlying basis for polarity in transcription start site selection. The N-terminal zinc-ribbon region of TFIIB has not been structurally analyzed in the context of the PIC, but the well-documented role of TFIIB in transcription start selection [21] suggests that it must be positioned toward the start site in order to make necessary contacts with Pol II and the RAP30 subunit of TFIIF [21–23].

Although high-resolution three-dimensional structures are available for segments of TFIIE [24] and TFIIF [25,26], currently it has only been possible to deduce the positions and function of these and other GTFs (including TFIIH) in the PIC using different approaches. Photo cross-linking analyses, in which the trajectory of the promoter DNA was mapped onto the PIC components [27,28], suggest that TFIIF plays a critical role in the fully assembled complex both in recruiting Pol II and in compacting the complex by tightly wrapping the promoter DNA around Pol II. This wrapping involves DNA contacts both upstream and downstream of the start site and may be further enhanced by TFIIE, which appears to be primarily positioned close to the transcription site [27,28]. Consistent with these results, a low-resolution three-dimensional structural analysis [29] has localized TFIIE close to the DNA binding cleft of Pol II. An important structural role for TFIIE is to recruit TFIIH to the PIC [30–32]. However, it has been suggested that, at the structural level, TFIIE also might contribute to the establishment of the PIC by a TFIIH-independent mechanism involving direct stabilization of TBP binding to the TATA element [33].

PICs on Nonstandard Core Promoters

Most of our understanding of PIC structure is based on analyses of TATA-containing promoters, but these may represent only a minority of cellular gene promoters [34]. Additional distinct elements that dictate core promoter function have been identified. These include the initiator (Inr), which spans the transcription site [35], and the downstream promoter element (DPE) [36], located approximately 30 nucleotides downstream of the start site. As expected, PIC assembly on promoters with these elements requires additional factors. Thus, transcription from Inr-containing promoters has been shown to require distinct TAF_{II}s in TFIID [37,38]; uncharacterized activities designated TIC1, TIC2, and TIC3 [39]; and possibly other transcription factors as well [40]. DPE promoter activity also appears to require TAF_{II} interactions [36] and, intriguingly, the cofactor NC2 [41], which was previously thought to possess only a negative modulatory function [6] (see later discussion also). An altogether novel strategy may be employed by core promoters that utilize the various TBP-related factors (TRFs) [42] in place of TBP-containing TFIID, and whose target sites differ considerably from the canonical TATA box [43,44].

In all of these cases of divergent core promoter usage, the precise composition of the final PIC that is competent for transcription initiation remains to be determined. Inasmuch as the function of the basal machinery is to enable selective transcription initiation by Pol II, these elements would be expected, *a priori*, to nucleate a PIC whose overall architecture would be rather similar to that described earlier for PICs formed on canonical TATA boxes. However, the primary recognition would be unlikely to be mediated by a TBP–promoter interaction. This expectation is strengthened by the observation that the TATA binding function of TBP is dispensable for transcription of an Inr-containing promoter [38] and by the presence of TBP in initiation factors required by Pol I and Pol III for transcription of promoters that contain no discernible TATA-like sequences [45].

Global Mechanisms of PIC Function

Promoter Melting

Kinetic and structural analyses have revealed a series of well-orchestrated events that precede processive RNA synthesis by Pol II [46,47]. In the broadest terms, the PIC described earlier, which is experimentally isolated in the absence of nucleotides, can be thought of as corresponding to the "closed complex" originally described for *Escherichia coli* RNA polymerase [48]. The isomerization of the eukaryotic closed complex to the open complex (in which the double-stranded promoter DNA around the start site is partially unwound) is dependent on energy derived from the hydrolysis of ATP [46,47,49,50]. Although the details are

still not clear, initial promoter melting appears to entail multiple transitions involving TFIIE and the helicase activity of the ERCC3 subunit of TFIIH [51,52]. In the first step, the promoter region between positions –9 and +2 relative to the start site is unwound. In the next transition, the downstream edge of the unwound region is extended to +4 [46]. Templated phosphodiester bond formation ensues, but, perhaps because of the intrinsic thermodynamic instability of the complex, appears to result only in abortive synthesis of short RNA products [46].

Two distinct models have been proposed for how the TFIIH helicase catalyzes promoter melting [53]. As a conventional helicase, it could simply peel the two DNA strands apart until intrinsic Pol II functions, which are also involved in this process, take over. Alternatively, coupled with ATP hydrolysis, TFIIH could twist the DNA and crank it into an unwound state by strongly gripping DNA surrounding the melted region [54]. Although the role of TFIIE in this process is, in part, to deliver TFIIH, there also are indications that under some circumstances it directly contributes to promoter melting independently of TFIIH and ATP hydrolysis [50]. Consistent with the primary roles of TFIIE and TFIIH in promoter melting, the requirement for these factors can be partially obviated either with supercoiled templates [55] or with templates containing nonpaired regions at the start site [51,56].

Promoter Clearance

As the melted region is extended (in an ATP hydrolysis-independent fashion) to +9, longer RNA chains begin to form [46,50]. Concomitantly, the upstream DNA abruptly reanneals, whereas the downstream DNA is melted in a continuous motion into a more or less stable *transcription bubble* that is further propagated as Pol II moves into an elongation mode. This process, referred to as *promoter clearance*, prepares the promoter for a second incoming Pol II molecule for additional rounds of transcription [57].

Promoter clearance involves extensive rearrangements of transcription factor interactions, including relinquishing of contacts that hold Pol II in a stable structure. Thus, in addition to the changes in the DNA template structure and the Pol II movements discussed earlier, the PIC undergoes loss of a subset of GTFs. Early studies (of basal transcription only) suggested that TFIID remains bound to the promoter [58,59], whereas other GTFs are sequentially released as Pol II advances along the template; TFIIB and TFIIE are released by register +10 and TFIIH after +30. On the other hand, TFIIF appears to travel with Pol II. A more recent study analyzing activator-dependent transcription in extracts from yeast cells points to a somewhat different mechanism [60]. In this case, a "scaffold" consisting of TFIIA, TFIID, TFIIH, TFIIE, and Mediator (see later discussion) remains behind at the promoter after the Pol II departs. In both situations, the residual PIC is thought to serve as an efficient intermediate for a second round of transcription (reinitiation), requiring, in the latter case, only TFIIB and TFIIF [60].

Phosphorylation of the Pol II CTD

Cyclical changes in the phosphorylation status of the CTD repeats of the largest subunit (RPB1) of Pol II have long been correlated with promoter escape by Pol II [61,62]. It has been proposed that hypophosphorylated Pol II (form IIA) preferentially associates with the PIC [63]. Following promoter melting, and either concomitant with or immediately subsequent to promoter escape, the CTD is phosphorylated at specific serine residues by the cdk7 kinase subunit of TFIIH [62]. Thus, in the cell, the hyperphosphorylated form of Pol II (IIO) is predominantly engaged in transcription elongation [61]. The observation that the CTD is dispensable for transcription of many promoters in purified cell-free systems [64,65] has suggested that its phosphorylation is unlikely to be a causative factor in the initiation-to-elongation transition. However, in less-purified systems and *in vivo,* other constraining factors could impose a CTD phosphorylation requirement for this transition [66]. A role for CTD phosphorylation in transcription elongation has also been suggested, consistent with the identification of an elongation factor (pTEFb) that actively phosphorylates the CTD [67]. However, new evidence strongly suggests that CTD phosphorylation also might be a device to couple transcription and RNA processing [68,69]. Importantly, CTD phosphorylation does not constitute a unidirectional pathway because Pol IIO may be converted back to the IIA form by a variety of phosphatases, such as the TFIIF-regulated FCP [70], thereby allowing it to reinitiate.

Gene-Specific Regulation of PIC Function by Transcriptional Activators

Each of the steps in PIC assembly and function is a potential target for transcriptional regulators, especially activators. Early work emphasized direct interactions of activators with GTFs. Beginning with the original observations that TFIID could be directly targeted by activators [71], interactions of various regulators with each of the GTFs have now been reported [72–76]. However, GTFs do not suffice to support activator function *in vitro* [77] and mutations in some GTF residues that are critical for activator interactions *in vitro* do not affect transcriptional activation in functional tests [78].

Identification of the Mediator as a Key Coactivator

As mentioned, initial biochemical analyses in metazoan systems strongly suggested that TAF_{II} subunits of TFIID [4] and various USA-derived PCs [3,6] are essential coactivators for activated transcription from DNA templates. In yeast, concurrent genetic studies aimed at delineating the role of the Pol II CTD and various regulatory pathways, as well as biochemical studies aimed at eliciting activator function *in vitro*, led to the identification of SRB/Mediator (or Mediator) as the key coactivator [8,9,79]. Although the yeast

Mediator was isolated as a holoenzyme complex with Pol II, this association is reversible [80]. Variable levels of subsets of GTFs were also found to be present in the Pol II holoenzyme [80,81]. A genome-wide analysis has further suggested that, like Pol II, the Mediator is required for essentially all genes [82]. These observations have suggested that Mediator function in transcriptional regulation might be intimately associated with basal transcriptional control.

Subsequently, in metazoan systems, parallel efforts to recapitulate thyroid hormone receptor activity *in vitro* and to purify complexes containing orthologs of yeast Mediator subunits converged with the description of the TRAP [83] and SMCC [65] coactivator complexes, which proved to be identical [84]. Similar complexes were independently identified based on different criteria [7]. These include DRIP [85], ARC [86], and hMediator [87], which were isolated on the basis of their ability to interact with distinct activators. Furthermore, USA-derived PC2 also was identified as a Mediator-like complex [88]. The variously isolated complexes may all represent the same cellular entity or its derivatives [7], which by analogy to the yeast complex is also referred to here as the Mediator.

The metazoan Mediator complex consists of about 25 polypeptides (Table I) that include RGR1, SRB10/CDK8, MED7, MED6, SRB11/cyclin C, SRB7, SOH1, and NUT2, which are phylogenetically conserved from yeast to human [7]. Other polypeptides that are specific to the metazoan complex include the various TRAP polypeptides, as well as additional less-well characterized polypeptides. As for the yeast Mediator, which the metazoan complex resembles in its overall three-dimensional structure [89], the metazoan Mediator subunits are predicted to be organized in a modular fashion. There appears to be a relatively stable core (likely represented by the subunits found in PC2) surrounded by somewhat more reversibly associating polypeptides such as the kinase-cyclin pair that consists of SRB10/CDK8 and SRB11/cyclin C [7,88].

Potential Mechanisms of Mediator Function

Although no stable, stoichiometric Mediator–Pol II complex akin to the yeast holoenzyme has been reported in metazoans, there is evidence for direct interactions of metazoan Mediator with Pol II [7]. Furthermore, activators appear to contact distinct subunits within the Mediator. Thus, nuclear receptors primarily target TRAP220 [90], whereas activation domains of the viral activator VP16 and the tumor suppressor p53 interact with TRAP80 [84]. Based in part on these data, as well as similar data from yeast [80,81], a simple model for the mechanism of action of the Mediator postulates that it functions as an adaptor that links activators with the basal transcription machinery. Nonetheless, it remains to be established whether the initial PIC-forming entity is the Pol II holoenzyme or whether, as some recent reports suggest, PIC formation involves temporally separable recruitment of Mediator and Pol II [91,92].

It is possible that Mediator effects are exerted both at the level of recruitment (that is, by enhancing PIC formation) and by alterations in the function of the PIC (Fig. 1). In this regard, note that both the yeast and metazoan Mediators directly enhance basal (activator-independent) transcription [80,93,94], especially in the context of a complement of other nuclear factors. Direct interactions with the basal transcription machinery that could stabilize the PIC may be involved. Thus, in yeast, SOH1 displays genetic interactions with Pol II and TFIIB [95]. (Note, however, that although SOH1 appears to be an integral subunit of the human Mediator [7,65], it has not yet been directly implicated as such in yeast.) Similarly, the GAL11 subunit of the yeast Mediator (which has no apparent ortholog in the metazoan complex) interacts both physically and genetically with TFIIE [96]. Related, *in vitro* recruitment experiments have revealed that the Mediator-containing yeast holoenzyme is dependent on both TBP and TFIIB for PIC formation in some assays [97]. Functionally, the Mediator can also modulate the TFIIH CTD kinase activity and, thus, potentially also intervene at a postinitiation stage [80]. On the other hand, it has been proposed that the SRB10/CDK8 kinase subunit down-regulates TFIIH activity by phosphorylating its cyclin H subunit [98]. Effects at the level of promoter opening have not been reported. However, a role for the Mediator in efficient multiple-round transcription is likely because, in a yeast transcription system, the scaffold that remains after Pol II clears the promoter contains Mediator [60].

It is intriguing that multiple subunits in the Mediator have been identified as targets of activators [7,84,85,87, 90,99]. Given that many activator signals emanate from enhanceosome-type assemblies that are composed of various activators [100], and that distinct activators may simultaneously interact with the Mediator [84], this provides a basis for synergistic activation mechanisms. Furthermore, these observations are consistent with coactivators (especially the Mediator) being devices for processing diverse signals into an integrated output for the Pol II machinery.

Mediator in Relation to Other Coactivators

An unresolved issue related to the mechanisms by which the various coactivators affect the basal machinery is whether they act synergistically or whether they are functionally redundant and thus reflect alternative activation pathways. In the case of USA-derived positive cofactors, we have previously found that Mediator-like PC2 functionally synergizes with PC4 and PC3/topoisomerase I [88]. Here, PC4 and PC3/topoisomerase I are believed to fulfill a structural role in stabilizing the PIC. In the case of the TAF_{II} subunits of TFIID, the relative contributions of these components and the Mediator in activated transcription have been harder to dissect. Earlier biochemical studies in yeast systems demonstrated Mediator function, including transcriptional activation, in reactions that contained TBP rather than the intact TFIID [80,81]. Moreover, genetic analysis revealed that at least a subset of TFIID-specific TAF_{II}s was dispensable for

general activator function in yeast cells [101,102]. In the small number of cases in which a strong TAF_{II} dependence was demonstrable, the TAF_{II} requirement appeared to be dictated by the core-promoter sequences [103]. However, more recent genome-wide analyses in yeast have identified a subset of genes whose expression is critically dependent on histone-like TAF_{II}s [104] (including $yTAF_{II}$ 17, a component shared by both the TFIID and SAGA complexes [105]). These genes display a requirement of their upstream activating sequences for this dependence [104,106].

In metazoan systems, differential requirements for TAF_{II}s have been observed depending on the experimental system employed [107,108]. Nonetheless, under some conditions in unfractionated extracts, TAF_{II}s within TFIID and Mediator appear to be jointly required both for activator-dependent and activator-independent transcription [94]. An interesting possibility is also suggested from a recent study in which the relative contributions of TAF_{II}s and Mediator in activation of several *Drosophila* promoters were examined [109]. It was observed that TBP sufficed to support activation from a TATA-box containing promoter (albeit still in a Mediator-dependent manner). However, activation through another promoter, which lacks a TATA box but contains a DPE, required intact TFIID (and simultaneously also displayed a Mediator dependence). These results are consistent with the idea that TAF_{II}s are required primarily for core promoter recognition, whereas Mediator is required for both activator-dependent and activator-independent effects. Therefore, it may be that, ultimately, both the TAF_{II} and Mediator requirements are intertwined, and reflect, at least in part, how each of these complexes interacts with the basal transcription machinery.

Although factors and mechanisms involved in transcription from DNA templates have been stressed in the foregoing discussion, it is important to keep in mind that in the cell transcription takes place within the constraints of a chromatin template. This necessitates other coactivator functions such as those associated with histone acetyl transferases like CBP/p300 and the p160 family of nuclear receptor coactivators [105]. A sequential mode of action of the factors, entailing an initial activator-mediated chromatin remodeling step followed by the recruitment of Pol II, GTFs, and Mediator, has been proposed [3,7]. Thus the mechanisms discussed earlier are likely to be relevant regardless of the nature of the template (chromatin or DNA). Indeed, in a sense, the PIC, with promoter DNA wrapped around a central protein core, can be viewed as being in competition with the structurally similar nucleosomes (Fig. 1) [110].

Negative Control of GTF Function

In addition to constraints imposed by chromatin structure, the basal machinery is subject also to other kinds of negative control. This introduces antirepression as an additional mode of gene activation [3]. It is noteworthy that some of the USA-derived PCs contain intrinsic repressive capabilities that are not only reversed during transcriptional activation but also are exploited to effect net transcription levels that exceed the basal (unregulated) levels [3]. Similarly, TFIID-associated TAF_{II}s, especially TAF_{II} 250, can also be repressive in the absence of an activator [111]. Part of the mechanism by which TAF_{II} 250 represses basal transcription involves molecular mimicry. Thus, the TAF_{II} 250 N-terminal domain resembles the unwound DNA structure of the TBP–TATA complex and makes repressive contacts with the underside of the TBP saddle that normally interacts with the TATA sequences [112]. In the presence of an activator, this interaction can be eliminated, thus allowing PIC formation to proceed. NC2 also is a negative cofactor that interacts directly with the basal transcription machinery [6]. A recent crystal structure of a ternary complex containing TBP, NC2, and promoter DNA nicely illustrates how a potential steric clash with TFIIB would preclude productive PIC assembly [113]. While the precise mechanisms are unknown, antagonism of this repression could be an important regulatory step in the cell.

Other negatively acting cofactors that have functional links to TBP and that have been identified both in yeast and metazoans include MOT1 [114,115] and the various NOT proteins [116,117]. In yeast, MOT1 may function to "remodel" TBP complexes in an ATPase-dependent fashion. Although regarded as a negative cofactor, MOT1 also has been localized to active genes [118]. This situation is somewhat reminiscent of the apparent functional duality associated with NC2 [119], and it is not immediately apparent how the various observations can be reconciled. The NOT proteins form heterogeneous complexes that include the CCR4 protein [120]. Their mechanism of action also is unclear, although one recent report has identified an ubiquitin ligase activity associated with the metazoan ortholog of one of the NOT proteins [121].

GTF Regulation by Covalent Modification

GTFs also undergo covalent modifications that might have regulatory consequences, but which at present are largely uncharacterized. The effect of the SRB10/CDK8 kinase on TFIIH was mentioned earlier. Other modifications include TAF_{II} 250-mediated phosphorylation of the RAP74 subunit of TFIIF [122] and acetylation of the large subunit of TFIIE by p300 [123]. In some cases, such as for the extensive phosphorylation of TFIID subunits (TBP and TAF_{II}s) during mitosis [124], these modifications may not reflect gene-specific controls in the context of the PIC but, instead, global control mechanisms that operate, for example, during progression through the cell cycle.

Conclusion

As summarized here, strong evidence linking activator function to the basal transcription machinery has been obtained. At the same time, structural, mechanistic and kinetic details of how this machinery carries out its basic functions have begun to emerge. Therefore, a main task for the future

is to understand more completely how the various subprocesses that constitute PIC function are modulated by activators and coactivators. The description in recent years of the Mediator as a key transcriptional regulator has opened up the possibility of understanding these mechanisms in greater depth.

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CHAPTER 272

Structural Mechanisms of Ligand-Mediated Signaling by Nuclear Receptors

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Introduction

Nuclear receptors (NRs) contain three major domains: an N-terminal domain, a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD). The N-terminal domain contains, in some NRs, an "activation function 1" (AF-1) but little is known about its structure. The DBD, consisting of two highly conserved zinc-finger motifs, recognizes specific DNA sequences in the target promoters, and is responsible for the specificity of gene regulation. The LBD, as its name suggests, is responsible for ligand recognition and subsequent signaling cascades. In most NRs, the LBD also contains dimerization functions and a C-terminal "activation function 2" (AF-2) that promotes ligand-dependent transcription. The LBD is thus the key to the ligand-dependent regulation of NR signaling and, as such, has been the focus of intense structural studies. In this brief review, we focus on structural insights into the molecular basis of ligand binding and signaling by nuclear receptors.

Overall Structure of the LBD

All of the NR LBD structures determined to date share a similar fold, where about a dozen α-helices are packed into a three-layer sandwich with a cavity for binding ligands (Fig. 1). The C-terminal AF-2 region also adopts an α-helical conformation, but can pack onto alternative sites on the core structure depending on the presence and nature of the ligand. Aside from the AF-2 helix, the core helical structure is relatively stable and well preserved among different receptors, particularly in the top half of the LBD. The conservation of the overall structure suggests that the LBD fold is selected through evolution for the binding of small molecule ligands despite a wide divergence in their amino acid sequences.

Ligand-Binding Pockets

Helices 3, 5, 6, 7 and 10 enclose a ligand-binding pocket in the bottom half of the LBD. This pocket is covered by a β-sheet on one side, and it can be covered by the C-terminal AF-2 helix on the other side. Although different NR LBDs have similar overall structures, their ligand-binding pockets are remarkably diverse, with pocket volumes ranging from 390 to 1440 $Å^3$ (Table I). The receptors with smaller pocket volumes, such as the retinoid and steroid receptors, seem to recognize specific ligands, whereas the receptors with larger pockets, such as the pregnane X receptor (PXR) and the peroxisome proliferator-activated receptors (PPARs), are able to bind a broader range of ligands. The ligand promiscuity in the PXR and the PPARs may be essential for their physiological functions as sensors of diverse xenobiotic substances and diverse fatty acids and eicosanoids, respectively.

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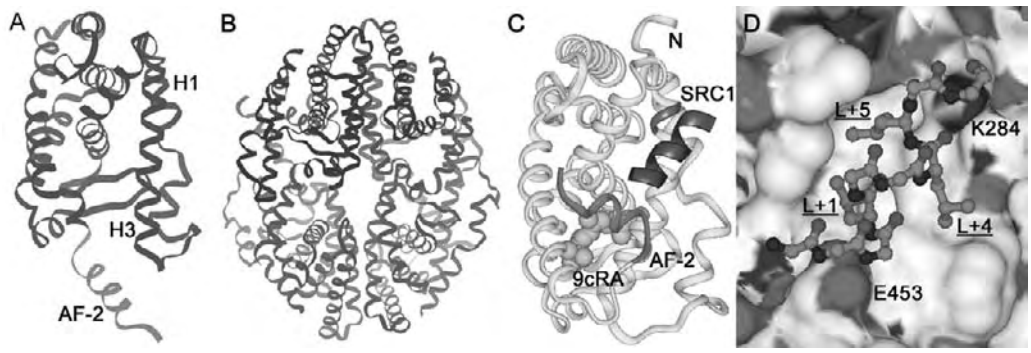


Figure 1 Structures of the RXR LBD in different conformational states. A, an overview of the apo-RXR LBD where the key helices are noted. B, the self-repressed tetrameric form consisting of two symmetric dimers packed together (dark and gray ribbons). C, RXR bound to 9 cis-RA (gray worm) with the SRC-1 LxxLL motif (dark) and D, a close-up view of the RXR charge-clamp groove where the leucine residues of the LXXLL motif are underlined.

Table I Volumes (Å^3) of NR Ligand Binding Pockets

Receptor	Ligand	Pocket vol	Ligand vol	PDB ID
ERβ	Genistein	390	236	1QKM
AR	DHT	422*	319*	1I37
RARγ	Retinoic acid	429	335*	2LBD
ERα	Estradiol	450	245	1ERE
RXRα	9-cis RA	470	350	1FM6
TRα	T3	600	530	–
PR	Progesterone	603	349*	1A28
VDR	Vitamin D	697	390	1DB1
RORβ	Stearic acid	766	–	1K4W
PXR	SR12813	1150	552*	1ILH
USP	Phospholipid	1256	878*	1G2N
PPARδ	EPA	1300	300	3GWX
PPARα	GW409544	1400	524*	1K7L
PPARγ	GI262570	1440	580	1FM6

Volumes indicated with an asterisk were calculated using the methodology of Gampe *et al.* [1]. Other values were compiled from the literature.

The shape of the ligand-binding pocket also varies greatly, allowing different NRs to accommodate ligands with different shapes. An interesting example of ligand selectivity between different subfamilies of receptors is provided by the retinoid X receptor (RXR) and the retinoic acid receptor (RAR), where 9-*cis* retinoic acid can bind to both receptors, but the all-*trans* retinoic acid binds only RAR. RXR has an L-shaped pocket, allowing it to accommodate the bent shape of the 9-*cis* isomer but not that of the extended all-*trans* isomer, [1]. By contrast, the RAR pocket is relatively straighter, and can accommodate both isomers [2]. The role of the pocket shape in ligand selectivity can also be found in receptors from the same subfamily. The three PPARs (α, γ, and δ) show different ligand preferences for fibrates, a class of lipid-lowering drugs, or glitazones, a class of diabetes drugs. A single residue variation that alters the shape of the PPAR headgroup pockets has been shown to be responsible for the differential binding of these ligands [3]. These examples show that the pocket shape is a key factor in determining ligand specificity.

Ligand-Mediated Activation: Mouse Trap versus Charged Clamp

Activation of nuclear receptors depends on the precise positioning of the AF-2 helix, which in turn depends on the nature of the bound ligand. In the apo-RXR structure, the AF-2 helix was extended away from the main body of the domain, leaving an opening into the ligand-binding pocket [4,5]. In contrast, the AF-2 helix is packed over this opening in the agonist-bound structures. This observation led to a "mouse-trap" model for activation, in which the ligand enters the pocket through this opening, causing the AF-2 helix to close over the ligand like a mouse trap closes over a mouse. The mouse-trap model was confirmed, for RXR, by the subsequent PPARγ/RXR heterodimer structure, in which the RXR monomer was bound to 9-*cis* retinoic acid [1]. However, the activation process has proven to be more complex than originally proposed because RXR activation requires the dissociation from its self-repressed tetrameric form (Fig. 1B) [4]. The apo structures of PPARγ, PPARδ, and PXR have shown that the AF-2 helix can be packed against the receptor in the active conformation even in the absence of ligand [6–8]. With the AF-2 opening blocked, the ligand instead enters the PPAR pocket through a large channel between helix 3 and the β-sheet. Ultraspiracle (the insect homolog of RXR) also has an alternate channel for ligand entry [9,10].

In contrast to multiple pathways of ligand entry, a conserved mechanism is employed by nuclear receptors for ligand-dependent activation. In the apo state, the lower half of the LBD is structurally unstable due to a large unfilled cavity inside the protein. Ligand binding, which partly fills this cavity, stabilizes the protein and allows the AF-2 helix to dock into the active conformation. In PPARs, the AF-2 helix is further reinforced in the active conformation by a direct

hydrogen bond with the acidic headgroup of the bound ligand. In this conformation, the AF-2 helix positions a conserved glutamate so as to form a charge clamp with a lysine from helix 3. Coactivators such as SRC-1 contain an LxxLL motif that forms two turns of α-helix, orienting the leucine side chains into a hydrophobic cleft between the two charge-clamp residues (Fig. 1). The charge-clamp residues make a network of hydrogen bonds with the ends of the LxxLL helical motif, further stabilizing the docking of the coactivator helix. This mode of coactivator interactions appears to be common for ligand-mediated activation because residues that contact coactivator are highly conserved among NRs.

Ligand-Mediated Repression

Some nuclear receptors can actively repress the transcription of target genes by recruiting corepressors such as *nuclear corepressor* (N-CoR) and *silencing mediator for retinoid and thyroid hormone receptors* (SMRT) [11,12]. The recent structure of a ternary complex of the PPARα LBD bound to an antagonist and a segment from SMRT reveals that the corepressor interacts with the receptor through an α-helical LxxxIxxxL/I consensus sequence that packs into the same general site as the LxxLL motif of the coactivator [13]. However, the LxxxIxxxI/L helix is longer than the LxxLL helix, with three turns of α-helix instead of two (Fig. 2). Whereas the coactivator interacts with a glutamate residue in the AF-2 helix, the corepressor helix protrudes into volume normally occupied by the AF-2 helix, effectively displacing the AF-2 helix from its active position. The net effect is that coactivators recognize and bind to NRs when the AF-2 helix is in its "active" conformation, whereas corepressors recognize and bind to NRs when the AF-2 helix is in other, "inactive" conformations.

The antagonist in the PPARα/SMRT structure was specifically designed, based on an earlier agonist-bound PPAR structure, to protrude into volume normally occupied by the AF-2 helix. The structure confirms that the designed antagonist displaces the AF-2 helix out of the active position. However, different from the antagonist-bound estrogen receptor structures, the AF-2 helix does not occupy the coactivator-binding groove but is loosely packed against helix 3 (Fig. 2). The displacement of the AF-2 helix out of its active position by the antagonist opens up a larger groove composed by helices 3, 4 and 5 and further reinforces the binding of the three-turn corepressor helix. These structural observations highlight the conformational flexibility of the AF-2 helix, which functions as a molecular switch to turn on or turn off NR transcriptional activities in response to the binding of an agonist or an antagonist.

Dimerization

As DNA-binding transcriptional factors, most NRs function as dimers. X-ray structures of the estrogen receptor (ER)

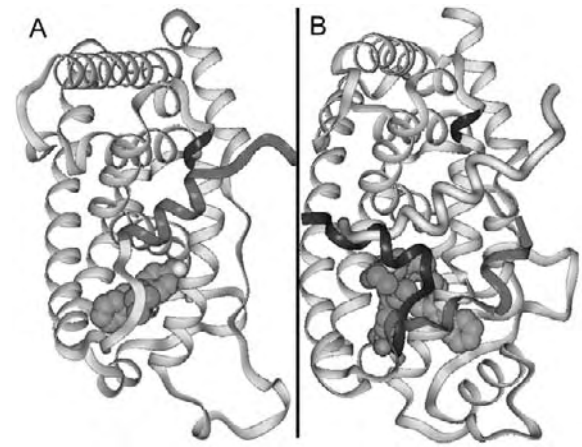


Figure 2 Antagonist-bound LBD structures. A, ERα bound to tamoxifen (spheres), with the AF-2 helix shown in a darker shade of gray; and B, the ternary complex of PPARα with GW6471 (spheres), with the AF-2 helix and SMRT peptide in darker and lighter shades of gray, respectively. The active position of the AF-2 is shown with a very dark gray to illustrate how it would bump the SMRT corepressor helix.

homodimer and two different RXR heterodimers reveal a common mode of dimerization, in which the helix 10 from one monomer packs against the helix 10 from the other monomer in a parallel manner [4,5]. The dimerization involves only the N-terminal (upper) half of helix 10, with the lower half of the helix curving away from the partner monomer. Although the homodimers have perfect twofold symmetry, the PPARγ/RXR heterodimer displays significant asymmetry. This can be seen in the contacts made by the AF-2 helix. In the homodimers, both AF-2 helices fail to contact the partner, whereas in the heterodimer, the PPARγ AF-2 helix contacts RXR, but the RXR AF-2 helix fails to contact PPARγ [1]. The RXR/RAR complex has a similar asymmetry, although the RAR AF-2 helix cannot make analogous interactions with RXR because the C-terminal end of its AF-2 helix is not conserved, and because the AF-2 helix lies in the coactivator pocket. Interestingly, the RXR/PPARγ heterodimer is "permissive" in that it can be activated by RXR ligand alone, whereas the RXR/RAR heterodimer is nonpermissive, and cannot be activated by the RXR ligand alone. The asymmetric interactions between RXR and its partner's AF-2 helix may provide a structural basis for the permissive activation by the RXR ligand.

The steroid receptors of progesterone (PR) and androgen (AR) reveal a distinct mode of dimerization involving the AF-2 helix (in its active conformation) and the C-terminal (lower) half of helix 10 [14,15]. The dimer interface in these steroid receptors is significantly smaller than that seen in the RXR heterodimer and ER homodimer, and its physiological relevance remains to be determined.

Summary

X-ray structures have now been solved for more than a dozen NR LBDs, bound to agonists and antagonists,

coactivators and corepressors, and as monomers, homodimers, heterodimers, and tetramers. These structures have illustrated the details of ligand binding, the conformational changes induced by agonists and antagonists, the basis of NR dimerization, and the mechanism of coactivator and corepressor binding. All of the NRs studied to date have broadly similar structures and mechanisms of activation, but functionally significant differences have arisen in different NRs over the course of evolution. We can expect more surprises as structural work continues on the remaining NRs, and as crystallographers tackle higher order complexes involving the LBD with the AF-1 domain, the DBD, and other proteins and nucleic acids involved in gene transcription.

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CHAPTER 273

Nuclear Receptor Coactivators

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Introduction

Nuclear receptors (NRs) comprise a family of DNA-binding transcription factors that regulate programs of gene expression related to cellular growth, differentiation, and homeostasis in a ligand-dependent manner [1]. In addition to receptors for ligands such as steroid hormones, retinoids, and metabolites of fatty acids, the NR family also includes several so-called "orphan receptors," representing members for which regulatory ligands are not known [2]. NRs activate transcription by binding as monomers, dimers, or heterodimers to specific hormone response elements (HREs) within target genes [3]. The central highly conserved DNA-binding domain (DBD) mediates sequence-specific recognition of HREs. Two distinct domains contribute to transcriptional activation functions, referred to as AF-1 and AF-2 [1]. The AF-1 domain is located in the N-terminal region and is poorly conserved among NR family members. In some NRs, particularly steroid hormone receptors, AF-1 plays a quantitatively important role in transcriptional activation. AF-2 resides in the C-terminal ligand-binding domain (LBD), which in addition to conferring the specific ligand-binding properties of each receptor, also contains a dimerization interface and ligand-regulated transcriptional activation and repression functions. Several lines of evidence have emerged in recent years indicating that ligand-dependent transcriptional activation involves the recruitment of a series of coactivator proteins to the NR LBD [1,4]. Several of these proteins are components of complexes that remodel chromatin, modify histone tails, or act to recruit core transcription factors (Fig. 1). In this chapter, we discuss general molecular mechanisms responsible for ligand-dependent interactions among NRs and coactivator proteins and provide examples of different classes of coactivators that have been linked to NR function.

Mechanism of Coactivator Recruitment

The ligand-dependent transactivation function of NRs has been demonstrated to depend on a short conserved sequence within the C terminus of the LBD [5,6]. Mutations within this region have been identified that have little or no effect on ligand binding, but abolish ligand-dependent transcription. Crystallographic analysis of several nuclear receptor LBDs has revealed a conserved structure in which a three-layered antiparallel α-helical sandwich encloses a central, hydrophobic ligand-binding pocket [7,8]. In the unliganded RXR structure, the AF-2 helix extends away from the ligand-binding domain [9]. In contrast, in the agonist-bound RAR, TR, and ER LBD structures, the AF2 helix is tightly packed against the body of the LBD and makes direct contact with ligand [10–13]. In concert, these studies are consistent with the idea that ligand-dependent changes in the conformation of the AF-2 helix result in the formation of a surface that facilitates coactivator interactions. Intriguingly, the structures of the estrogen receptor LBD bound to the antagonists raloxifene or dihydroxytamoxifen (OHT) demonstrate a distortion in the position of the AF-2 helix that prevents the binding of coactivators [12,13].

Extensive analysis of the amino acid sequences of several nuclear receptor coactivators revealed that a short helical sequence LXXLL (L: leucine and X: any amino acid) within the nuclear receptor interaction domain was necessary for ligand-dependent recruitment to the NR LBD [14–16]. LXXLL motifs have been identified within nearly all of the putative coactivators that interact with NRs in a ligand-dependent manner, including members of the p160/SRC family (Fig. 2A), CBP/p300, TRAP220/DRIP205/PBP, and PGC-1. Structural studies of the PPARγ LBD complexed to a fragment of the SRC-1 nuclear receptor interaction domain containing two LXXLL motifs revealed that these motifs

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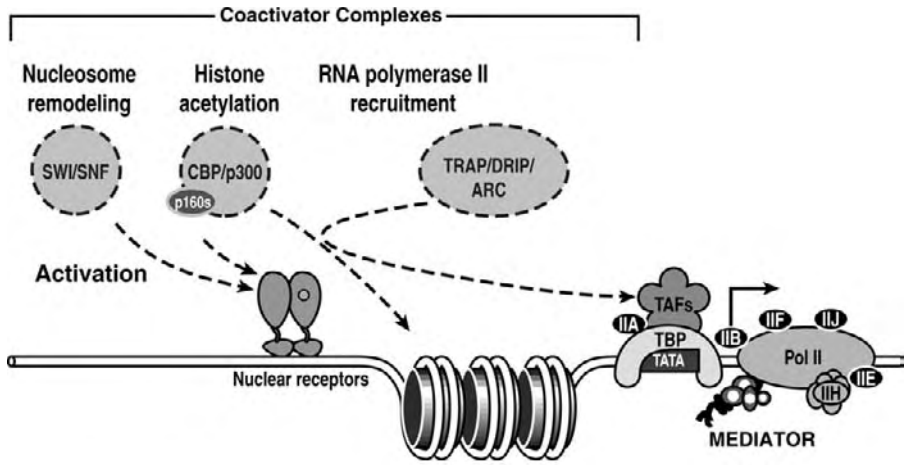


Figure 1 Ligand-dependent transcriptional activation of nuclear receptor target genes. Nuclear receptors bind to hormone response elements in promoter or enhancer elements and recruit one or more coactivator complexes in response to activating ligands. Many coactivator proteins have been identified that are components of multiprotein complexes, such as Swi/Snf complexes involved in nucleosome remodeling, p160/CBP complexes containing histone acetyltransferase activities, and TRAP/DRIP/ARC complexes involved in recruitment of basal transcription factors.

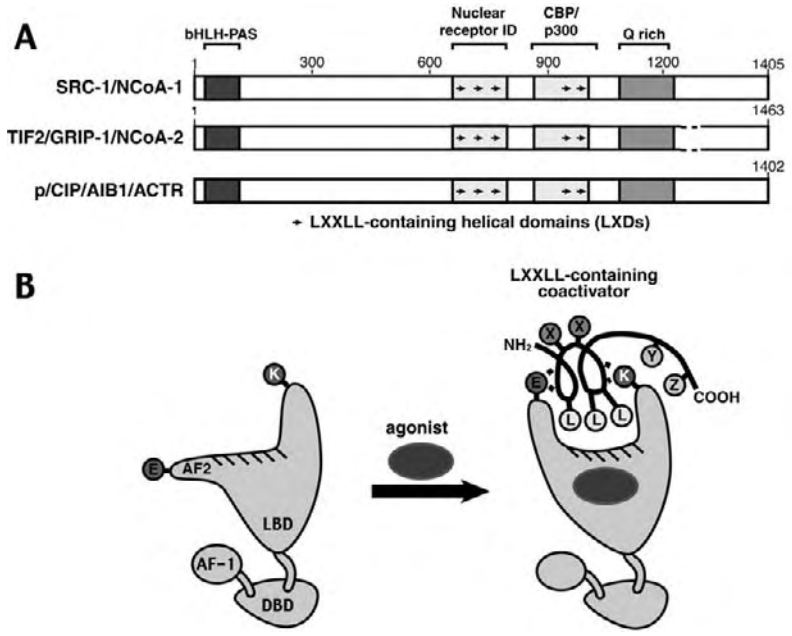


Figure 2 Mechanism of coactivator recruitment. (A) Alignment of members of the p160/SRC family of nuclear receptor coactivator illustrating the positions of LXXLL motifs mediating NR interaction and the CBP/p300 interaction domain. (B) Binding of agonist to the NR LBD induces a conformational change in the C-terminal AF-2 helix. This results in the formation of a charge-clamp that grips the ends of the LXXLL helix and allows the leucine residues to pack into an intervening hydrophobic cavity.

form short α-helices [17]. The two LXXLL motifs within this fragment of SRC-1 interacted with each subunit of a LBD dimer, suggesting a mechanism for cooperative interactions among coactivators and nuclear receptor dimers or heterodimers. The LXXLL helix was gripped at each end by a charge-clamp consisting of a conserved lysine in helix 3 of the LBD and a conserved glutamate in the AF-2 helix (Fig. 2B). This positioned the LXXLL helix so that the leucine residues could pack into a hydrophobic pocket between the end of helix 3 and the AF-2 helix, stabilizing the interaction. Structures of the thyroid hormone receptor and estrogen receptor LBDs complexed to LXXLL peptides from other p160 family members exhibit the same structural basis for binding [13,18,19]. Although the LXXLL helix is necessary for ligand-dependent interactions, additional residues N and C terminal to the helix contribute to binding specificity [14,20].

General Classes of Coactivator Complexes

Since the initial biochemical characterization of p160 and p140 proteins as ligand-dependent estrogen receptor-associated proteins [21,22], dozens of additional proteins have been identified that interact with nuclear receptors in a ligand-dependent manner [1]. Functional assignment of these proteins as NR coactivators has generally been based on co-immunoprecipitation experiments demonstrating that they interact with liganded nuclear receptors in cells and that forced expression results in increased ligand-dependent transcriptional responses. The development of chromatinized *in vitro* transcription assays has allowed biochemical approaches to functional analysis of coactivator function [23]. More recently, demonstration of ligand-dependent recruitment of putative coactivators to NR target genes by chromatin immunoprecipitation assays has been considered as an additional line of evidence for physiological relevance [24]. Relatively few coactivators have been demonstrated to be required for NR action by loss of function (e.g., gene knock-out) experiments. Gene knock-out experiments are in some cases difficult to interpret because of the potential for coactivators to serve redundant functions. In many cases, proteins initially identified to function as nuclear receptor coactivators have proven to function as coactivators for other classes of transcription factors.

Coactivator proteins are generally considered to function by modifying chromatin architecture or by serving as adapters that recruit core transcription factors to the promoter [1,4]. Many coactivator proteins have been found to be components of stable, multiprotein complexes. Modifications of histone tails are becoming increasingly recognized to constitute a "code" that specifies overall chromatin architecture and the potential of genes to be silenced or transcribed [25]. Several proteins shown to function as nuclear receptor coactivators harbor histone acetyltransferase activity, including CBP and p300. Conversely, the nuclear receptor corepressors N-CoR and SMRT are components of protein complexes that contain histone deacetylase activities [4]. Histone methylation has also emerged as a modification that is associated with either transcriptional activation or gene silencing, depending on the specific histone and amino acid residue that is modified [26]. The nuclear receptor coactivator CARM-1 methylates histone H3 at arginine 3, and this modification is correlated with transcriptional activation [27]. Histone phosphorylation has been linked to transcriptional activation, but NR coactivators with histone kinase activities have not yet been described. A distinct type of chromatin modification is provided by ATP-dependent chromatin remodeling complexes, exemplified by the BRG/BAF complexes, which are highly related to SWI/SNF complexes originally identified genetically in yeast and *Drosophila.* SWI/SNF complexes mediate the repositioning of nucleosomes, presumably facilitating access to DNA-binding transcription factors. Although these proteins may not interact directly with NRs, there is evidence that they are recruited to promoters in a ligand-dependent manner and cooperate with other classes of coactivators [28].

The p160/SRC and TRAP220/DRIP205/PBP proteins are examples of nuclear receptor coactivators that appear to function as adapter proteins. SRC-1 and related proteins interact with liganded NRs via LXXLL motifs, as described earlier, and with CBP and p300 via a C-terminal domain (Fig. 2A). The ability of SRC-1 to function as a coactivator is based in part on its ability to recruit CBP/p300 [15,29,30]. TRAP220/DRIP205/PBP is a component of the TRAP/DRIP/ARC complex identified biochemically as a coactivator complex required for *in vitro* transcriptional activities of the thyroid hormone receptor, vitamin D receptor, and SREBP [31–33]. The TRAP220/DRIP205/PBP subunit contains two LXXLL motifs, which allow the entire complex consisting of more than a dozen proteins to be recruited to nuclear receptors in a ligand-dependent manner [34]. The TRAP/DRIP/ARC complex is proposed to function by recruiting RNA polymerase II holoenzyme to the promoter in a receptor- and ligand-dependent manner [35].

Coactivators as Targets of Signal Transduction Pathways

Components of coactivator complexes are emerging as targets for various signal transduction pathways, providing an additional level at which these pathways may be integrated. An example is provided by PGC-1, initially reported as a fat cell-specific coactivator for PPARγ [36]. Recently, PGC-1 has been found to enhance the expression of key gluconeogenic enzymes, including phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase in the livers of fasting mice, leading to increased glucose synthesis [37]. These genes are under combinatorial control of several sequence-specific transcription factors, including the glucocorticoid receptor and the orphan nuclear receptor HNF-4, which collectively utilize PGC-1 as a coactivator. The expression of PGC-1 is stimulated by glucagon and suppressed by insulin, suggesting that it may be a key target of these hormones in the maintenance of blood glucose homeostasis. These observations are of particular interest because they suggest that the expression of a specific coactivator is required for a specific program of gene expression that is regulated by transcription factors that have multiple physiologic roles.

Conclusion

The identification and functional characterization of nuclear receptor coactivators represent major current efforts being undertaken in the nuclear receptor field. Many fundamental questions remain to be answered, including why so many of these proteins exist, and how they exert their transcriptional effects. The study of these proteins could have practical benefits in that it may be possible to selectively regulate patterns of gene expression by developing synthetic NR ligands that differentially recruit coactivators to the LBD.

It is likely that differential recruitment of coactivators and corepressors underlies the tissue-specific actions of selective estrogen receptor modulators, and that ligands with similar properties can be developed for other members of the NR family. Such ligands would have potential therapeutic applications in a variety of human diseases, including metabolic syndromes, atherosclerosis, diabetes, and chronic inflammatory diseases.

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CHAPTER 274

Corepressors in Mediating Repression by Nuclear Receptors

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Introduction

Activation of gene transcription has long been recognized as an essential component of regulation of gene expression by nuclear receptors, but repression of transcription plays an equally important role in developmental and homeostatic gene regulation. Transcriptional repression by nuclear receptors requires the actions of the closely related receptor-associated corepressors, nuclear receptor corepressor (N-CoR) and silencing mediator of retinoic acid and thyroid hormone receptors (SMRT), which were identified based on their ability to associate with and mediate transcriptional repression by nuclear receptors. These corepressors recruit many classes of transcription factors as components of multiprotein complexes, some containing specific histone deacetylases (Fig. 1A). This association with histone deacetylase activity provides one component of the mechanism that allows DNA-binding proteins interacting with N-CoR or SMRT to repress transcription of specific target genes. However, both N-CoR and SMRT are components of additional complexes that can result in permanent repression. The biological importance of these corepressors has emerged from genetic studies both of humans and mice. In turn, various signaling pathways regulate levels of expression, subcellular localization, and function of these corepressors.

N-CoR and SMRT in Repression by Nuclear Receptors

Thyroid hormone and retinoic acid receptors (T_3R and RAR) actively repress transcription in the absence of their cognate ligands via transferable repression domains [1] that can associate with N-CoR [2] and SMRT [3–5]. These corepressors contain a conserved bipartite nuclear receptor interaction domain [6–8] and three independent repressor domains that are capable of transferring active repression to a heterologous DNA-binding domain (DBD) [2–5] (Fig. 1B). The interaction domains contain related putative helical motifs LXXXIXXXI/L [9–11], one helical turn longer than the LXXLL recognition motif present in nuclear receptor coactivators [12,13] that binds in a hydrophobic pocket in the hormone binding domain, similar to coactivator binding. Specific sequences in the nuclear receptor interaction motif are suggested to account for the preference of RAR for SMRT and T_3R for N-CoR [14].

The mechanism by which N-CoR and SMRT function proved, in part, to reflect their association with mRpd3 and mSin3A and B, mammalian homologs of the yeast proteins Rpd3p/histone deacetylase 1 and Sin3p [15,16]. Previous observations that acetylation of specific lysine residues in the N termini of histones correlates with increased transcription, and that heterochromatic regions are generally hypoacetylated [17,18], led to studies of the role of HDAC proteins in transcriptional repression. There are now 10 HDACs, classified on the basis of their homology to two closely related yeast HDACs, RPD3 and Hda-1 [19,20], as class I or class II HDACs, respectively [21]. In some assays, specific HDAC proteins appear to require association with N-CoR or SMRT for full enzymatic activity [22,23]. Additionally, there is evidence of a combinatorial code based on distinct histone modifications, indicating a complex relationship between various recruited enzymatically active complexes [24].

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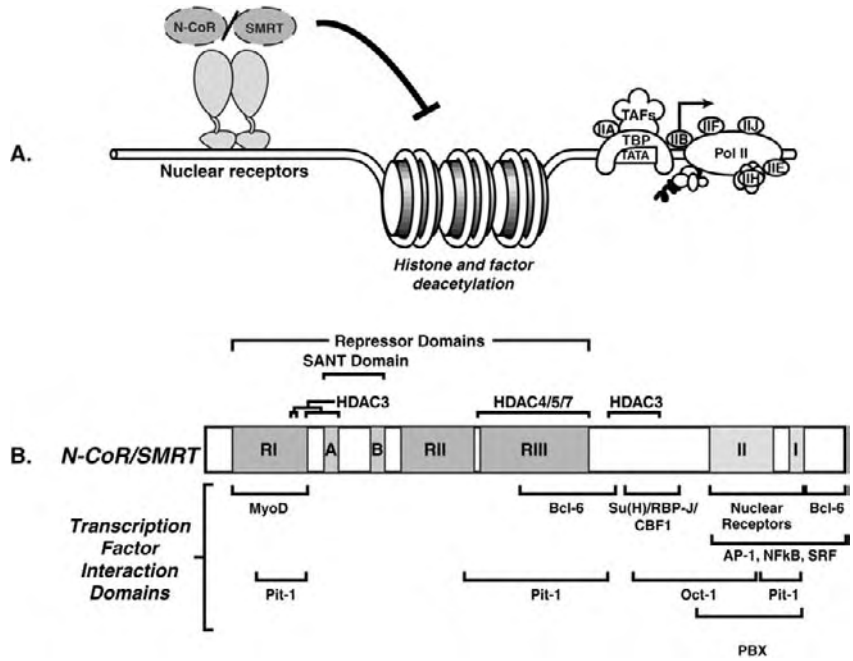


Figure 1 (A) Transcriptional repression by nuclear receptors is regulated by recruitment of the corepressors N-CoR and/or SMRT. (B) Schematic of domains of N-CoR/SMRT. Repression domains (RI, RII, RIII) and SANT domains (A and B) are indicated, as are interaction domains for HDACs, nuclear receptors (I and II), and other transcription factors.

Purification of Corepressor Complexes

Biochemical purification of complexes using anti-N-CoR or anti-HDAC3 antibodies revealed several corepressor-associated complexes, one which contains HDAC3, N-CoR, or SMRT, and transducin (beta)-like protein 1 (TBL1) [23,25–27]. TBL-1 has six WD-40 repeats [28], a motif also present in the Tup1 and Groucho corepressors, and is homologous to the *Drosophila* protein ebi, which is involved in epidermal growth factor receptor signaling pathways [29]. Under different conditions, an N-CoR–SMRT–HDAC3 complex can also contain Krab associated protein 1 (KAP-1), a TSA-sensitive corepressor that interacts with members of the heterochromatin protein 1 (HP1) family, and several members of the Swi/Snf ATP-dependent chromatin-remodeling complex family, which is reminiscent of the ATP-dependent chromatin-remodeling proteins found in the NURD complex (Fig. 1B) [27]. A third N-CoR/SMRT complex, which appears to be among the weakest, shares common components (HDAC1, HDAC2, and mSin3) with the Sin-associated protein (SAP) complex [30,31]. Several groups have also shown that the third repressor domain of N-CoR and SMRT can directly interact *in vitro* with class II HDACs, including HDAC4, HDAC5, and HDAC7 [32,33], suggesting that the full range of complexes has yet to be purified.

Other Nuclear Receptor and Transcription Factor Partners of N-CoR/SMRT

Although cloned based on their interactions with unliganded RAR and T_3R, N-CoR and SMRT are capable of conferring transcriptional repression to an ever increasing number of transcription factors (Fig. 1B). N-CoR or SMRT serve as corepressors for several other members of the nuclear receptor superfamily, including v-ErbA, RevErb, COUP-transcription factors, PPARα and DAX1 [34]. Although steroid hormone receptors do not appear to interact with N-CoR or SMRT in the absence of ligand [2,3], both the estrogen receptor (ER) and progesterone receptor (PR) can interact with these corepressors in the presence of their respective antagonists to repress transcription [35–38]. In chromatin immunoprecipitation assays (ChIP) performed in the breast tumor derived cell line MCF-7, N-CoR and SMRT were present on the estrogen-responsive cathepsin D and pS2 promoters in the presence of the antagonist tamoxifen but not estrogen [39]. These data suggest a role for N-CoR and SMRT in mediating the antagonist-associated effects of steroid hormone receptors [2,3] and provide a mechanism for the clinical application of antagonistic ligands.

In addition to their interactions with members of the nuclear receptor family, N-CoR and SMRT have been implicated as corepressors for a variety of unrelated transcription factors that regulate diverse cellular processes, including various homeodomain factors, MAD, SRF, BCL-6, MyoD, STAT5, HERP, and Su(H) [34].

Multiple Mechanisms of N-CoR/SMRT Regulation

The actions of N-CoR and SMRT are highly regulated. The N terminus of N-CoR interacts with mSiah2, the mammalian homolog of *Drosophila Seven in absentia* [40]. Consistent with evidence that mSiah2 regulates proteasomal

degradation of proteins [41], cotransfection of N-CoR and mSiah2 resulted in a dramatic decrease of N-CoR protein levels, an effect abolished by inclusion of a proteasome inhibitor [40].

Stimulation of the MAPK and PKC pathways resulted in decreased association of N-CoR and ER in the presence of the antagonist tamoxifen [38]. In one example, EGF-dependent phosphorylation of ER converted tamoxifen from an antagonist to an agonist [38]. Activation of the mitogen-activated protein kinase (MAPK) pathway by L-thyroxine (T_4) results in serine phosphorylation of TRβ1 and dissociation of SMRT in a hormone-independent manner [42], while phosphorylation of SMRT by MAPK-extracellular signal-regulated kinase 1 (MEK-1) and MEK-1 kinase (MEKK-1) is suggested to inhibit interactions between SMRT and nuclear receptors or PLZF [43]. In contrast, phosphorylation of SMRT by the protein kinase casein kinase II (CK2) is reported to stabilize the SMRT/nuclear receptor interaction [44]. Thus, different cell signaling pathways can effect different transcriptional outcomes.

Signaling pathways have also been shown to cause changes in subcellular distribution of the corepressors, as CamKIV, MEK-1, and MEKK-1 signaling are suggested to result in a redistribution of SMRT from the nucleus to the perinucleus or cytoplasm [43,45]. Indeed, specific signaling events influence the subcellular distribution of HDAC proteins [46–48].

Roles in Development and Disease

During normal development N-CoR is required for normal progression of specific developmental stages in erythrocyte and thymocyte development and in neural maturation [49] based on evidence from gene deletion studies. Expression of a dominant-negative N-CoR protein in hepatocytes of transgenic mice causes an increased proliferation of hepatocytes and derepression of T_3-regulated hepatic target genes [50], while expression of a dominant-negative N-CoR protein in lactotropes abolishes long-term repression of the growth hormone gene [51]. In *Xenopus*, expression of a dominant-negative N-CoR protein can result in embryos exhibiting phenotypes similar to those treated by RA, namely, reduction of anterior structures such as forebrain and cement gland, suggesting that RAR-mediated repression of target genes is critical for head formation [52]. Resistance to thyroid hormone (RTH), characterized by an impaired physiological response to thyroid hormone, is associated with mutations in the T_3R-β gene that fail to release N-CoR or SMRT upon hormone treatment [53–55]. Gene repression appears to be a critical component of normal thyroid hormone physiology, and deletion of all known thyroid hormone receptors results in a phenotype less severe than that of thyroid-hormone-deficient mice [56], implying the role for repression by the unliganded T_3R. Roles for N-CoR and SMRT in several types of leukemia are also well characterized [57].

Other Mediators of Nuclear Receptor Repression

The p140 factor, RIP 140, has proven to be an example of an inhibitor of ligand-occupied receptor based on displacement of other activators [58–62], and perhaps based on its interactions with other corepressors [63]. In addition, other corepressors, acting both in the presence and absence of ligand, may act through nuclear receptors to exert critical biological roles [64,65].

Conclusion

Nuclear receptors can serve as repressors of transcription, characterized by association with proteins with enzymatic functions. In addition to a ligand-dependent switch, various signal transduction pathways can modulate interactions of corepressors with nuclear receptors or mediate their activity or distribution between nuclear or cytoplasmic compartments.

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CHAPTER 275

Steroid Hormone Receptor Signaling

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Introduction

Steroid hormones are essential regulators of key physiological processes such as reproduction, glucose metabolism, and the response to stress and salt balance. The biological effects of steroid hormones are transduced by intracellular receptors that directly mediate the action of their cognate hormone [1]. Steroid hormone receptors (SHRs) were the first recognized members of the steroid/thyroid/retinoid nuclear receptor superfamily, a class of transcription factors whose activity is regulated by small lipid-soluble molecules. The SHR subgroup includes the receptors for estradiol: estrogen receptor α and β [ERα (NR3A1) and ERβ (NR3A2)]; cortisol: glucocorticoid receptor [GR (NR3C1)]; aldosterone: mineralocorticoid receptor [MR (NR3C2)]; progesterone: PR (NR3C3); and dihydrotestosterone: androgen receptor [AR (NR3C4)]. In addition, the SHR subgroup contains three orphan nuclear receptors closely related to the ERs: the estrogen-related receptors α, β, and γ [ERRα (NR3B1), β (NR3B2), and γ (NR3B3)] for which a natural ligand remains to be identified. SHRs share a common modular structure composed of independent functional domains [2]. The DNA-binding domain (DBD) is centrally located, well conserved among SHRs, and comprised of two zinc-finger motifs involved in both protein–DNA and protein–protein contacts. The ligand-binding domain (LBD), located at the carboxy terminal of the receptor, is moderately conserved and folds into a canonical α-helical sandwich generally consisting of 12 α-helices (H1 to H12) [3]. The LBD can also contain a ligand-dependent nuclear translocation signal, determinants to bind chaperone proteins, dimerization interfaces, and a potent ligand-dependent activation domain referred to as AF-2. A ligand-independent activation domain (AF-1) is encoded within the nonconserved amino-terminal region of the receptors.

Activation by the Hormone

The classic model of SHR action dictates that SHRs interact with chaperones in the cytoplasm and be dissociated in a ligand-dependent fashion, leading to the reorganization of the receptor and exposure of nuclear localization signal(s) and translocation to the nucleus. Although this model is widely accepted for SHRs, there are important exceptions [4]. The ERs are clearly localized to the nucleus despite being part of a complex with chaperone proteins. Perhaps more striking is the finding that the two forms of PR, PR-A and PR-B, that differ only in the length of their amino-terminal domains have distinct cellular localization. PR-A is found predominantly in the nucleus, whereas PR-B is mainly located in the cytoplasm in the absence of hormone [5]. Given that the nuclear localization and chaperone binding functions are identical for both forms of PR, these data strongly suggest that the interactions of SHRs with complexes containing coregulatory proteins might influence the intracellular distribution of SHRs.

Intracellular redistribution of ligand-bound SHRs is accompanied by the recognition of specific sites on chromatin, referred to as hormone response elements (HREs) [6]. The HREs are short *cis*-acting sequences located within the promoters or enhancers of target genes. SHRs bind DNA as homodimers to HREs composed of inverted repeats of AGGTCA (for the ERs and ERRs) or AGAACA (for the GR,

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MR, PR, and AR) motifs spaced by three nucleotides. SHR isoforms such as ERα and ERβ or PR-A and PR-B can also form functional heterodimeric complexes on DNA [7–9]. In the case of the ERs, the functional properties of each isoform are retained in the heterodimeric complex [10], whereas PR-A is dominant over PR-B [11].

The transcriptional activity of the SHRs is mediated by the independent AF-1 and AF-2 [12]. AF-1 is ligand independent and constitutive since it can activate transcription in the absence of the ligand when fused to a heterologous DBD. The structural determinants and mode of action of the distinct AF-1 domains found in each SHR have yet to be characterized. In contrast, the ligand-dependent AF-2 is well defined: a short amphipathic α-helix (H12) located at the carboxy-terminal end of the LBD is repositioned on hormone binding into a hydrophobic cleft formed mainly by residues from helices 3, 4, and 12. The resulting structural change provides a functional interface for coactivator recruitment by the receptor [13]. AF-1 and AF-2 recruit both common and specific cofactors. These regulatory proteins possess various enzymatic activities such as acetylase, deacetylase, methylase, kinase, and ubiquitinase functions [14]. One RNA molecule known as SRA has also been characterized as a SHR coactivator [15]. The cofactors participate in the remodeling of chromatin, the formation of a stable transcription initiation complex, the association or dissociation of other cofactors within the SHR-cofactor complex as well as recycling and degradation of the receptor [16].

Hormone-Independent Activation

SHRs are phosphoproteins and targets of kinase cascades involved in the response to growth factors and cytokines [17]. Many aspects of SHR function can be modulated in this way, including dimerization, DNA binding, and both ligand-independent and -dependent activation [18]. SHRs are the targets of protein kinase A, mitogen-activated protein kinase (MAPK), cyclin-dependent kinases, casein kinase, and glycogen-synthase kinase. The molecular mechanisms underlying modulation of SHR activity on phosphorylation have yet to be elucidated in most instances. However, in the case of ERβ, phosphorylation of two serine residues within AF-1 promotes the recruitment of steroid receptor coactivator-1 (SRC-1) both *in vivo* and *in vitro* [19]. The physiological relevance of hormone-independent activation pathways was clearly demonstrated using the uterus of the ERα mouse knock-out as a model [20]. In wild-type animals, the uterus displays growth responses to epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1). In the ERα knock-out animals, the uterus is unresponsive to the mitogenic actions of EGF and IGF-1, demonstrating an essential requirement for ERα in these biological responses [21, 22]. In cellular and *in vitro* models, EGF, IGF-1, and other agents can induce the phosphorylation of serine 118 in the ERα amino-terminal region [23]. Mutation of this residue abolishes the response of ERα to EGF and considerably reduces the ability of the receptor to respond to its cognate ligand.

Cross-Talk with Other Transcription Factors

Steroid hormone regulation of a number of target genes does not require the presence of a HRE within the transcriptional unit of those genes. Instead, SHRs can tether to a transcription factor and thus modify its activity. The formation of a SHR–transcription factor complex can lead to changes in cellular localization, DNA binding activity, and enzymatic function within the transcription initiation complex, or conversely can provide a hormone-dependent transcriptional activation function to a non-HRE site [24, 25]. The biological actions of SHRs have been linked to binding sites for more than a dozen transcription factors, as well as to non-HRE sites for unidentified factors. The *in vivo* relevance of the HRE-independent pathways was highlighted by the observation that, in contrast to GR-deficient mice, genetically engineered mice expressing a non-DNA binding form of the GR are viable [26].

Nongenomic Action of Steroid Hormones

Steroid hormones have been shown to elicit biological responses too rapid to involve gene transcription and subsequent synthesis of new proteins. Rapid effects of steroid hormones have been reported in blood vessels, bone, breast cancer cells, nervous system, sperm, and maturating oocytes [27–31]. These effects involve changes in the activities of enzymes such as phospholipase C, PI3 kinase, and adenylate cyclase, leading to increases in intracellular calcium levels, second messengers, and activation of kinase cascades [32,33]. It is not clear whether the membrane SHRs are identical to the nuclear isoforms, but responses to pharmacological agents and transfection experiments with SHR expression vectors indicate that the membrane SHRs must share common determinants with their nuclear counterparts [34, 35] and thus originate from the same genes. The ER has been shown to physically interact with c-SRC, the insulin-like growth factor, the p85 subunit of PI3 kinase and calveolin-1, whereas the PR possesses a proline-rich domain within its amino-terminal region that mediates direct hormone-dependent interaction with the SH3 domain of a variety of cytoplasmic signaling proteins, including c-SRC [35].

Estrogen Related Receptors

The ERRs were the first orphan nuclear receptors identified through a search for genes related to ERα [36]. Initial studies of the ERRs showed that they did not bind estradiol or other physiologically relevant steroid hormones and indicated that their biological roles could be quite distinct from those of the classic ERs [37–40]. However, the recent observation that the ERs and ERRs share target genes and coactivators [41, 42], coupled with the striking discovery that diethylstilbestrol, a potent synthetic estrogen, and 4-hydroxytamoxifen, a mixed estrogen agonist/antagonist, are ERR ligands [43–45], suggests that the ERRs are bona fide SHRs [46].

Selective Steroid Hormone Receptor Modulators

Pharmacological and toxicological studies of synthetic SHR ligands have led to the realization that certain drugs can have distinct effects on the same SHR depending on the target tissue. This concept has mainly emerged from the study of tamoxifen, a molecule that acts as an estrogen antagonist in the breast but as an estrogen agonist in bone and uterus [47]. The term *selective estrogen receptor modulator* (SERM) is now being used to describe estrogenic drugs that display cell type- and context-dependent actions, and this concept has now been extended to all SHRs. The mechanisms by which selective SHR modulators exert tissue-specific effects are not yet understood and are likely to be distinct for each class of compounds, targeted receptors, and site of action. However, our better understanding of the modes of action of SHR at the molecular level indicate that these mechanisms may include selective activation of receptor isoforms (e.g., ERα versus ERβ), distinct behavior of the drug-receptor complex in the presence of different corepressor/coactivator ratios, preferential recruitment of specific coregulatory proteins (including other transcription factors involved in cross-talk with SHRs such as AP-1 and NF-κB), and possibly selective activation of nongenomic pathways [48–50]. The future development of selective SHR modulators with improved therapeutic indexes and no undesirable side effects is likely to constitute the most significant outcome of the vast effort dedicated to understanding how SHRs work, and basic molecular and genetic studies of SHR action will continue to be the main driving force behind this process.

Acknowledgments

The literature on steroid hormone receptor signaling is extensive and only selected studies and reviews were cited in this article due to strict space limitation. I apologize to all investigators whose work was not included. Many thanks to members of my group for revising the manuscript. My laboratory is funded by the Canadian Institute of Health, the Canadian Breast Cancer Research Initiative and the National Cancer Institute of Canada.

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CHAPTER 276

PPARγ Signaling in Adipose Tissue Development

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Introduction

Adipose tissue plays a central role in maintaining lipid homeostasis and energy balance in vertebrates by storing triglycerides or releasing free fatty acids in response to changing energy demands. Adipocytes also perform an important endocrine function by secreting numerous signaling molecules, which are involved in the regulation of food intake, energy balance, and whole-body lipid homeostasis. Excessive accumulation of adipose tissue leads to obesity and often to insulin resistance, whereas its absence is associated with lipodystrophic disorders. In contrast to rodents, which have no white adipose tissue until after birth [1], human preadipocytes begin to differentiate during late embryonic development, although the majority of the differentiation occurs after birth [2]. All vertebrates have the ability to induce adipose tissue differentiation throughout their lives in response to the body's energy storage demands.

During the past 10 years, work from a number of groups has outlined a pivotal role for PPARγ in adipocyte development. Evidence from both cell culture and animal models indicates that this protein is absolutely required for adipogenesis. A member of the nuclear hormone receptor superfamily, PPARγ is a transcription factor whose activity is regulated by the binding of specific small molecule ligands. Like many other nuclear receptors, PPARγ binds to DNA in a heterodimeric complex with the retinoid X receptor (RXR). The discovery that the natural ligands for PPARγ are likely to be native and modified polyunsaturated fatty acids provided a mechanism whereby changes in cellular or systemic lipid homeostasis might be translated into specific changes in adipocyte gene expression and differentiation. Obesity, the development of excess adipose tissue, is invariably associated with insulin resistance. PPARγ has also been identified as a key regulator of insulin sensitivity, an activity that is likely to be directly related to its function in adipose tissue biology. Synthetic ligands for this receptor are widely used for the treatment of type II diabetes.

PPARγ: A Dominant Regulator of Adipose Tissue Development

Early insights into the function of PPARγ in adipocyte differentiation came from studies of the aP2 gene. Through a combination of polymerase chain reaction (PCR)-mediated cloning and biochemical purification, PPARγ was identified as the factor responsible for directing adipocyte-specific expression of the aP2 enhancer [3–5]. It was shown to be highly expressed in both white and brown adipose tissue and to be induced early during the time course of adipocyte differentiation [5,6]. Subsequent studies demonstrated that expression and activation of PPARγ in fibroblastic cells is sufficient to trigger the adipocyte gene expression cascade and lead to the development of the adipose phenotype [7]. In addition to the fatty acid binding protein aP2, PPARγ target genes in adipose tissue include phosphoenol pyruvate carboxykinase [8], acyl-CoA synthetase [9,10], fatty acid transport protein 1 (FATP-1) [11,12], CD36 [13], lipoprotein lipase (LPL) [14] and liver X receptor α (LXRα) [15]. Although activation of PPARγ leads ultimately to the induction of all of the genes necessary to define an adipocyte, not all of these

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genes are direct targets for PPARγ. Execution of the differentiation program now appears likely to involve the sequential and coordinated action of PPARγ and members of the CAAT/enhancer binding protein (C/EBP) and sterol regulatory element binding protein (SREBP) transcription factor families (see later discussion).

Although PPARγ was once considered an orphan receptor, considerable evidence has emerged to suggest that the endogenous ligands of the PPARs are likely to be native or modified polyunsaturated fatty acids [16–18]. Thus, it appears that the substrates for energy storage in adipose tissue, fatty acids, are also signaling molecules that direct the differentiation of new adipocytes. The naturally occurring prostanoid 15-deoxyprostaglandin J2 binds to PPARγ with high affinity; however, its role as a signaling molecule *in vivo* is not yet clear [19,20]. Of particular pharmacological importance was the discovery that PPARγ is the biological receptor for a class of insulin-sensitizing agents known as the thiazolidinediones [19,21–23]. Two of these drugs, rosiglitazone (Avandia) and pioglitazone (Actos), are in widespread clinical use for the treatment of type II diabetes. The availability of potent and highly specific ligands has greatly facilitated analysis of PPARγ function. For example, both naturally occurring and synthetic PPARγ ligands are potent stimulators of adipogenesis in cultured systems [19,23]. The thiazolidinediones have also been shown to increase adipose tissue mass *in vivo* [24].

Analysis of PPARγ Function in Animal Models

The human and mouse PPARγ genes extend over more than 100 kb of genomic DNA (Fig. 1). Each is composed of nine exons that give rise to three distinct mRNA molecules, PPARγ1, PPARγ2, and PPARγ3, by alternate promoter usage and differential splicing [25–28]. Fat and large intestine are the only tissues, where considerable amounts of PPARγ2 and PPARγ3 transcripts have been reported [26,27]. In rodents, PPARγ appears late during embryonic development and is restricted to the brown adipose tissue (day 18.5), and only transiently appears in the central nervous system [29–31]. Rodents do not develop the white adipose tissue until after birth.

Homozygous inactivation of the PPARγ gene in mice results in embryonic lethality [32]. PPARγ-deficient fetuses can only survive until midgestation and die by day 10 of development. At day 9.5, PPARγ–/– fetuses are alive and are similar to their wild-type counterparts; however, at day 10, embryos show serious vascular abnormalities in the placenta. Barak and colleagues [32] utilized a tetraploid-rescue approach to generate a single PPARγ null pup that survived to term. This PPARγ-deficient animal was completely devoid of both white and brown adipose tissues. Studies using embryonic stem cells have also pointed to an absolute requirement for PPARγ in adipose tissue development. Injection of PPARγ-deficient cells into wild-type blastocysts produces chimeric mice in which the adipose tissue is composed exclusively of PPARγ+/+ cells [33]. Similarly, embryonic fibroblasts

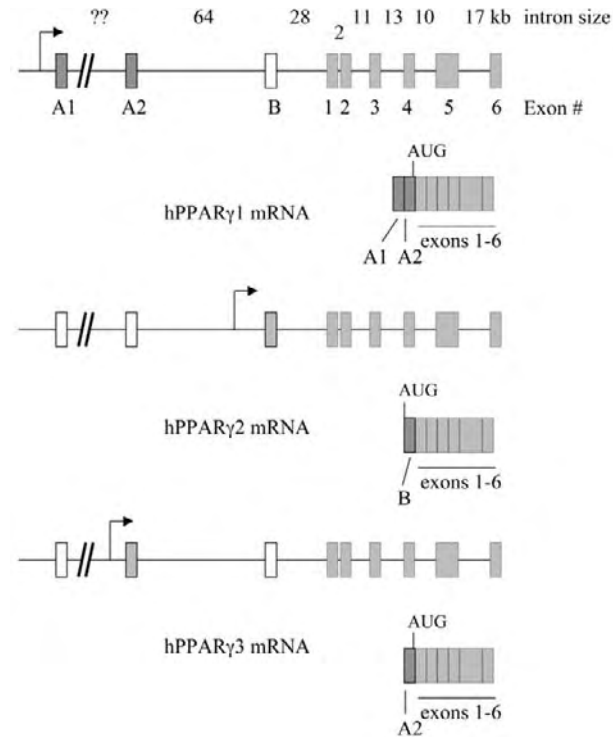


Figure 1 Structure of the murine and human PPARγ genes. Three PPARγ isoforms are produced by the differential use of three promoters and alternative splicing of the three 5'-exons (A1, A2, B). Exons 1–6 are common to all three transcripts. In the human PPARγ1 and PPARγ3 genes, the AUG translation initiation codon is located six nucleotides upstream of the one used in other species. Therefore, the human PPARγ1 protein has two additional amino acids at its N terminus compared with the rodent PPARγ1 protein. Note that transcription from the promoters 1 and 3 results in the same protein of 477 amino acids. The PPARγ2 protein of 505 amino acids is produced by transcription from the promoter 2. Distances between exons are based on the alignment of PPARγ mRNA sequences with the Human Genome build #28.

derived from PPARγ-deficient fetuses fail to differentiate into adipocytes *in vitro* [32,34]. Very recently, studies using PPARγ-deficient fibroblasts have shown that it is the PPARγ2 isoform that is specifically required for the differentiation program [35].

A limited amount of data on genetic polymorphisms in human populations corroborate studies in animal models. Mutations that result in permanent activation of PPARγ protein are associated with obesity [36]. In contrast, mutations that render PPARγ less active are generally associated with a lower body mass index (BMI) and ameliorated insulin sensitivity [37–41].

Transcriptional Networks in Adipose Tissue Development

Adipocytic differentiation has been primarily studied *in vitro*. The 3T3-L1 and 3T3-F422A cell lines [42], are the most widely used adipocyte culture models. These cells are morphologically similar to the fibroblastic preadipose cells

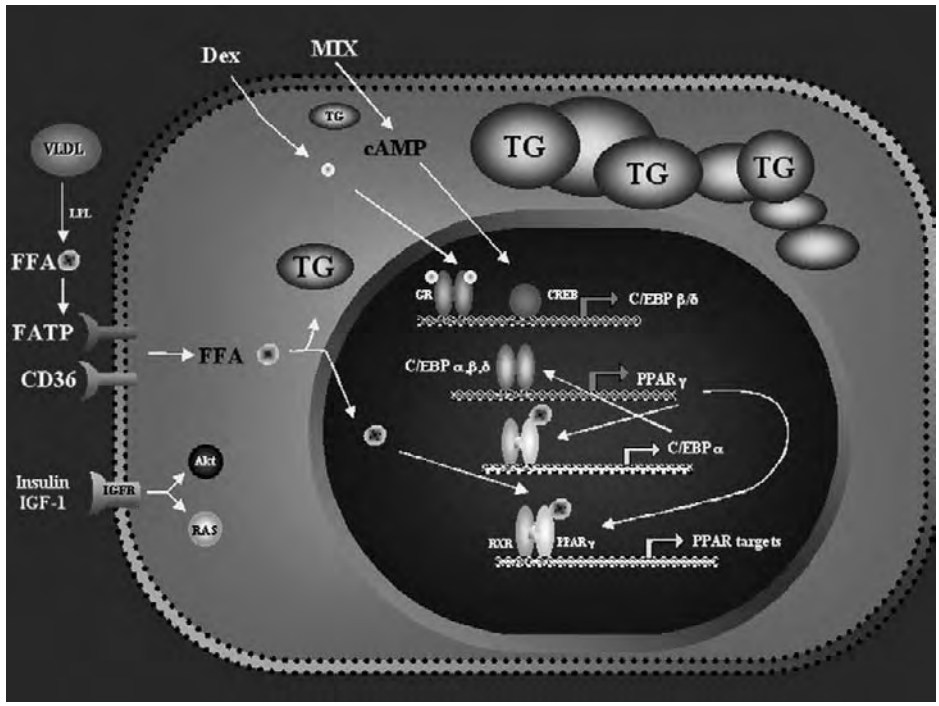


Figure 2 Transcriptional networks in adipocyte differentiation. Insulin and IGF act on the IGF receptor to activate signaling pathways involving ras and Akt. C/EBPβ and C/EBPδ are induced following the hormonal stimulation of 3T3-L1 cells with MDI. C/EBPβ expression is regulated by glucocorticoids through the glucocorticoid receptor (GR), whereas C/EBPδ expression is induced by cAMP via CREB. Both C/EBPβ and C/EBPδ activate the expression of the PPARγ gene. PPARγ in turn induces the expression of C/EBPα. PPARγ and C/EBPα cross-regulate each other to maintain their high level of expression as C/EBPβ and C/EBPδ levels decline. Finally, PPARγ and C/EBPα cooperate to induce expression of genes specific for the mature adipocyte phenotype.

found in the stroma of adipose tissue and, once differentiated, they exhibit virtually all of the characteristics associated with adipocytes present within the adipose tissue. When injected into mice, 3T3-L1 cells differentiate into adipocytes and form fat pads that are indistinguishable from normal adipose tissue [43]. Using these model systems a number of the molecular pathways that control adipogenesis have been elucidated (Fig. 2).

Confluent 3T3-L1 cells can be converted in adipocytes by a treatment with a mixture of insulin, dexamethasone, methyl-isobutyl-xanthine (MIX), and fetal bovine serum, commonly referred to in literature as MDI [44]. Insulin activates the insulin-like growth factor 1 (IGF-1) receptor in 3T3-L1 cells; therefore, IGF-1 can be substituted for insulin in the adipogenic cocktail [45]. Dexamethasone activates the glucocorticoid receptor pathway, which results in a rise in the intracellular cAMP levels. MIX, a cAMP-phosphodiesterase inhibitor, prevents cAMP hydrolysis and is used to stimulate the cAMP-dependent protein kinase pathway.

The developmental program of the 3T3-L1 preadipocytes can be divided into four distinct stages: (1) preconfluent proliferation, (2) confluence/growth arrest, (3) hormonal induction/clonal expansion, and (4) permanent growth arrest/terminal differentiation (Table I). Studies during the past 20 years have defined specific patterns of gene expression associated with each of these stages. Approximately 48 hours after induction by MDI, 3T3-L1 cells complete the postconfluent mitosis and enter into an unusual growth arrest phase, called GD [46,47]. Following the growth arrest, cells are committed to become adipocytes and begin to express late markers of differentiation by day 3. These late markers consist of lipogenic and lipolytic enzymes, as well as other proteins responsible for modulating the mature adipocyte phenotype. Finally, the cells round up, accumulate triglyceride droplets, and become terminally differentiated adipocytes by days 5–7.

At confluence, within 1 hour after the addition of MDI, the transient expression of c-fos, c-jun, junB, c-myc is observed [48]. These factors have not been implicated directly in any differentiation-specific events but they are believed to have mitogenic properties. C/EBPβ and C/EBPδ are the first transcription factors induced following the hormonal stimulation [49–52]. C/EBPβ expression is regulated by glucocorticoids, whereas the promoter of C/EBPδ is responsive primarily to MIX. The activity of C/EBPβ and C/EBPδ is believed to directly induce the expression of PPARγ2 via interaction with a C/EBP site in the PPARγ2 promoter [49,53,54]. PPARγ2 can be first detected on day 2 after addition of MDI and its expression acquires the maximum by days 3–4. In turn, PPARγ2 induces the expression of C/EBPα, which acquires its highest expression level on day 5 [55]. Finally, PPARγ and C/EBPα cross-regulate each other to maintain their high level of expression when C/EBPβ and C/EBPδ levels decline [53].

Table I Temporal Pattern of Gene Expression during *In Vitro* Adipocyte Differentiation[a]

		JunB							aP2
		c-jun	Wnt-10b						SCD
		c-fos	CUP	ADD1	C/EBPβ				Adipsin
	DAY	c-myc	Pref-1	SREBP1c	C–/EBPδ	C/EBPα	PPARγ2	LPL	PEPCK
Proliferation	–5	–	+++	–	–	–	–	–	–
Confluence	–4	–	+++	–	–	–	–	++	–
MDI simulation clonal expansion	0	+++	+++	–	–	–	–	+++	–
	0.5	–	+	–	++	–	–	+++	–
	1	–	–	++	+++	–	+	+++	–
Permanent growth arrest terminal differentiation	2	–	–	+++	+++	+	++	+++	+
	2.5	–	–	+++	++	++	++	+++	++
	3	–	–	+++	–	++	+++	+++	+++
	4	–	–	+++	–	++	+++	+++	+++
	5	–	–	+++	–	+++	+++	+++	+++
	7	–	–	+++	–	+++	+++	+++	+++

[a]Stages of differentiation are depicted in the first column: (1) preconfluent proliferation, (2) confluence/growth arrest, (3) clonal expansion, and (4) permanent growth arrest/terminal differentiation. The expression pattern of genes associated with each step of differentiation is shown.

It is now clear that PPARγ and C/EBPα cooperate to activate gene expression and the full program of adipogenesis. Although, retroviral expression of either PPARγ [7] or C/EBPα [53,56] alone is sufficient to induce *in vitro* adipogenesis in 3T3-L1 cells, this procedure is much more efficient if both factors are coexpressed [7,57,58].

Another protein implicated in the adipocyte differentiation pathway is a member of the basic helix–loop–helix protein family of transcription factors, called ADD-1/SREBP-1c (adipocyte differentiation and determination factor1 or sterol regulatory element binding protein 1c) [59]. Ectopic expression of ADD1 alone renders 3T3-L1 cells differentiation competent [60], whereas the expression of the ADD1 dominant-negative form strongly inhibits 3T3-L1 differentiation [60]. Coexpression of both ADD1 and PPARγ2 results in a synergistic effect on adipocyte differentiation [60]. The mechanism by which ADD1/SREBP1c promotes differentiation is still unclear, however, it has been postulated that genes activated by this transcription factor lead to synthesis of endogenous ligands for PPARγ [61]. Paradoxically, however, transgenic overexpression of ADD1/SREBP-1c in adipose tissue of mice was found to cause severe lipodystrophy [62]. The mechanism underlying this effect is not yet clear.

Negative Regulation of Adipocyte Differentiation

Exposure of preadipocytes to differentiating agents, such as the MDI cocktail, releases the cells from a number of inhibitory pathways that are antagonistic to terminal differentiation. Examples of such inhibitory factors include the CUP/AP-2α transcriptional repressor, Pref-1, and members of the Wnt family. CUP/AP-2α is a C/EBP family member abundantly expressed in preadipocytes, where it has been proposed to mediate repression of the C/EBPα gene through direct binding to specific sequences in the C/EBPα promoter. Expression of the CUP/AP-2α factor in preadipocytes is blocked upon hormonal stimulation [63,64]. Another postulated inhibitor of adipogenesis inhibition, Pref-1, is also highly expressed in 3T3-L1 preadipocytes. Pref-1 is a small, membrane associated, EGF-repeat-containing protein [65,66]. Pref-1 is elevated in the mouse model of congenital generalized lipodystrophy, a condition characterized by poorly developed white and brown adipose tissue [62]. Cleavage of the membrane associated Pref-1 in its extracellular domain results in the generation of a soluble signaling mediator that may interact with an as yet unidentified receptor. Pref-1 expression in preadipocytes is blocked by treatment with glucocorticoids [67]. Finally, the Wnt family of signaling factors has recently been implicated in the control of adipose differentiation. Forced expression of Wnt-1 in 3T3-442A cells inhibited the formation of adipose tissue when these cells were grafted into nude mice [68]. Furthermore, 3T3-L1 cells, which are engineered to express a dominant-negative form of the TCF4, a transcriptional mediator of the Wnt pathway, undergo adipogenesis without any hormonal induction. Activation of the Wnt pathway in these studies repressed expression of both the PPARγ and C/EBPα genes, and this has been postulated to be the mechanism by which Wnts inhibit adipogenesis.

PPARγ, TNF-α Signaling Antagonism and Insulin Resistance

Insulin resistance is a common feature of obesity, lipodystrophy, and non-insulin-dependent diabetes mellitus (NIDDM). A possible connection between PPARγ and insulin sensitivity was first suggested by the critical role of PPARγ in adipocyte development. However, the discovery that PPARγ is the biologic target for the thiazolidinedione class

of antidiabetic drugs provided the definitive link [19,21–23]. In rodents, PPARγ gene expression is down-regulated by fasting and by insulin-deficient diabetes, whereas exposure to a high-fat diet increases PPAR expression in adipose tissue and may result in obesity and insulin resistance (reviewed in [24]).

The cytokine tumor necrosis factor α (TNF-α) has long been recognized for its capacity to impair normal adipocyte physiology by inducing lipolysis, blocking insulin signaling, and antagonizing adipogenesis. Elevated TNF-α levels are observed in several rodent models of obesity and NIDDM [69]. Recent studies utilizing gene disruption in mice confirmed that absence of TNF-α or its receptor results in significantly improved insulin sensitivity [70,71]. It has been suggested that TNF-α blocks insulin signaling in adipocytes, at least in part, by down-regulating expression of the C/EBPα and PPARγ genes. Recent studies have shed light on potential mechanisms for this repression. One mechanism by which TNF-α could inhibit adipogenesis is through activation of the MEK/MAPK signaling pathway [72,73]. It has been previously shown that MAP kinase mediated phosphorylation of the PPARγ protein at Ser82 (Ser112 for PPARγ2) results in repression of its transcriptional activity [74]. Other groups have demonstrated that administration of TNF-α early during 3T3-L1 adipocyte differentiation could block clonal expansion due to the erroneous regulation of two retinoblastoma protein family members, p130 and p107. These proteins regulate cell cycle events through interactions with the E2F transcription factors. TNF-α disrupts the normal pattern of expression of both p130 and p107, leading to a complete block in mitotic clonal expansion and the activation of apoptosis [75].

TZDs, potent and specific PPARγ ligands, appear to antagonize TNF-α-mediated inhibition of insulin signaling at several different levels. First, TZD treatment was shown to decrease circulating TNF-α levels in rodents [76,77]. Second, TNF-α affects insulin action through inhibition of the insulin receptor (IR) tyrosine kinase activity [78]. TZDs can antagonize this effect of TNF-α on IR itself as well as on IR downstream targets, such as IRS proteins, and restore insulin sensitivity in fat [79]. This effect has not been observed in tissues that do not express PPARγ. Third, TZDs rapidly increase expression of genes that are crucial for insulin signaling in adipocytes, such as c-Cbl associated protein (CAP), IRS-2, and the p85 subunit of the PI3 kinase [80–83]. The question of whether these genes are direct PPARγ targets has not yet been addressed. The concept that TNF-α is an important modulator of insulin sensitivity and adipocyte development is based primarily on data in animal models. The role of this factor in human adipose tissue biology is not yet clear.

PPARγ and Cell Cycle Regulation

Given the strong correlation between growth arrest and terminal differentiation, it is not surprising that PPARγ has been linked to cell cycle regulation. For many specialized cell types, differentiation and division are mutually exclusive choices. In adipocytes, induction of the differentiation program by PPARγ is accompanied by cell cycle withdrawal. Studies using tumor cell lines have shown that binding of the E2F/DP-1 complex to its target promoters, an event usually associated with cell cycle reentry, is significantly decreased on stimulation with the PPARγ-specific ligand. PPARγ is believed to participate in the silencing of the E2F/DP-1 transcriptional activity by down-regulating the PP2A protein phosphatase [84]. Another study revealed that PPARγ, in concert with C/EBPα, may mediate cell growth arrest during adipogenesis by up-regulating the cyclin-dependent kinase inhibitors p18 and p21 [85]. It was also demonstrated that treatment with PPARγ ligand could effectively bypass the block in adipocyte differentiation imposed by the retinoblastoma protein deficiency [86]. These authors proposed that the adipocyte differentiation observed in RB null embryonic fibroblasts is related to a severely reduced level of C/EBPβ dependent transactivation and that pRB may participate in a pathway leading to the production of an endogenous PPARγ ligand.

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CHAPTER 277

Orphan Nuclear Receptors

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Classical Receptors versus Orphan Receptors

Endocrine Signaling Paradigm

Nuclear hormone receptors comprise a superfamily of ligand-modulated transcription factors that regulate gene transcription in response to their cognate ligands. The transcriptional effects of these receptors are mediated via two critical functional domains: the DNA-binding domain (DBD) and the ligand-binding domain (LBD). The DBD recognizes specific target genes, whereas the LBD modulates the gene transcription by interacting with transcriptional regulatory complexes in a ligand-dependent fashion.

Nuclear acting hormones such as steroid and thyroid hormones were identified nearly a century ago. These signaling molecules are secreted into the bloodstream at nanomolar concentrations and ultimately regulate transcription in distant target tissues. Tissues that respond to these hormones express receptors whose ligand-binding affinity closely matches the circulating concentration of the hormone. The combination of secreted hormones and high-affinity targets is a characteristic that defines the classical endocrine signaling system. This signaling paradigm provides an efficient means to simultaneously regulate gene expression at multiple sites throughout the body.

To date, 49 human nuclear receptors have been identified [1]. Although virtually all of these proteins possess the characteristic LBD, approximately 34 had not originally been associated with a cognate ligand. These proteins are known as *orphan receptors* and their existence implies that additional signaling molecules remain to be identified. Because more than 30 years had passed since a classical steroid hormone had been identified, it is reasonable to imagine that ligands for orphan receptors may have escaped detection because they are not generated via classical endocrine paradigms.

Nature of Orphan Receptor Ligands

Specialized endocrine signaling molecules are common in higher organisms, but in lower organisms, intermediary metabolites commonly possess both metabolic and signaling functions. For example, in bacteria and yeast, numerous gene networks are regulated by sugars, amino acids, fatty acids, and adenosine triphosphate (ATP) derivatives [2–4]. These regulatory networks sense specific nutrients and modulate their levels by regulating, in a coordinated manner, the expression of genes required for their synthesis, degradation, or transport. In addition to metabolic signals, single-cell organisms have also developed networks to detect and minimize exposure to exogenous toxins such as antibiotics (e.g., tetracycline) [5] and heavy metal derivatives (e.g., mercury, arsenic) [6,7]. Because higher organisms must also regulate their exposure to nutrients and foreign toxins, it is possible that they possess receptors that respond to similar signals. Indeed, studies during the past 5–6 years have established a definitive role for orphan receptors in these processes.

An important component of all signaling networks is the ability to respond to signaling molecules at physiologically relevant concentrations. In contrast to endocrine hormones that are secreted at nanomolar concentrations, intermediary metabolites are by necessity present at much higher (micromolar) concentrations. Thus, an important distinction between endocrine and metabolite/toxin-controlled receptors is that the former are high-affinity sensors, whereas the latter bind their ligands with an appropriately lower affinity.

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Orphan Receptors and Metabolite-Derived Signals

RXR, A Common Heterodimeric Partner

Many receptors function as an obligate heterodimer with the nuclear receptor RXR [8]. Although the identity of the endogenous RXR ligand remains controversial, it is clear that this receptor can be activated by synthetic rexinoids [9], 9-*cis* retinoic acid [10], and very high concentrations of phytanic [11,12] and docosahexaenoic acids [13]. Receptors that heterodimerize with RXR fall into two classes: those that are "permissive" and respond to RXR ligands and those that are "silent" and fail to respond [14–16]. The permissive receptors include the PPARs, LXR, bile acid receptor (BAR), and constitutive androstane receptor (CAR). Although these receptors respond to their specific ligands, the potential also exists for cross-activation via RXR agonists.

PPARs: Regulators of Lipid and Glucose Homeostasis

The PPARs (peroxisome proliferator-activated receptors) include three different receptor genes (PPARα, β/δ, and γ) whose ligands are lipid-derived compounds. PPARα binds to endogenous polyunsaturated fatty acids and regulates hepatic genes involved in fatty acid oxidation. PPARβ/δ also binds polyunsaturated fatty acids and increases HDL cholesterol and reverse cholesterol transport from peripheral tissues. PPARγ is expressed at high levels in adipose tissue where it regulates lipid storage and glucose homeostasis. These receptors (discussed elsewhere in this series) provide excellent examples of the nexus between metabolism and transcription in higher animals.

LXR, A Sterol and Fatty Acid Sensor

Two related LXR genes have been identified: LXRα and LXRβ. The β-subtype is expressed ubiquitously and its physiological function is unclear [17]. In contrast, LXRα is expressed in a variety of tissues that contribute to lipid homeostasis including hepatocytes, intestinal epithelia, macrophages, and adipose tissue (Table I) [18]. LXRα exhibits partial constitutive activity that reflects a combination of ligand-independent activity [19] and activation by an endogenous mevalonic acid-derived metabolite [20]. LXRα can be further activated by a variety of oxysterols. The endogenous LXR ligand is unclear and different sterol ligands may be acting in different tissues, for example, 24(*S*),25-epoxycholesterol in the liver [18] and 27-hydroxycholesterol in macrophages [21]. Whereas oxysterols activate LXRα, the transcriptional activity of this receptor can be repressed by polyunsaturated fatty acids [22] and geranylgeranyl pyrophosphate [20]. LXRα is therefore a dynamic "integrator" of multiple lipid metabolites.

Studies using knock-out mice and synthetic agonists have demonstrated that LXR is not only a lipid sensor but also an effector of cholesterol and fatty acid homeostasis. LXRα activation results in increased hepatic cholesterol degradation and a decrease in intestinal cholesterol absorption [23,24]. The increase in cholesterol turnover is due to enhanced transcription of cyp7a1, the rate-limiting step in the conversion of cholesterol to bile acids. Cyp7a1 is regulated in rodents but not humans because the LXR response element is defective in the human promoter. In contrast, the decrease in intestinal cholesterol absorption is due to activation of the ABCA1 (ATP binding cassette A1) transporter in both humans and rodents. LXRα also activates expression of ABCG1 [25] and of ABCG5/G8 [26], which have been implicated in the efflux of sitosterol and other sterols.

In macrophages, LXRα coordinately activates expression of ABCA1 [27] and apolipoprotein E (apoE) [28], a secreted cholesterol-acceptor protein. The net effect is enhanced removal of cholesterol from atherosclerotic lesions (macrophage foam cells) and its transfer to the liver via HDL cholesterol. Interestingly, this process appears to be under feed-forward control in humans as a macrophage-specific LXRα promoter is activated by PPARγ and autoregulated by LXRα [29–31].

The ability to reduce cholesterol absorption and to stimulate cholesterol efflux makes LXRα an attractive target for the treatment of atherosclerosis. Unfortunately, LXRα activation is also associated with an increase in circulating triglycerides [32]. This is likely a result of enhanced expression of SREBP-1c (sterol response element binding protein), a transcription factor that activates a battery of genes required for saturated fatty acid synthesis [33]. One of these genes, fatty acid synthase (FAS), is also a direct target of LXRα [34]. Because elevated triglycerides may be counterproductive in atherosclerosis, an ideal LXRα-based agent may be one that selectively activates certain genes (e.g., ABCA1, apoE) but not others (SREBP-1c, FAS). The identification of selective estrogen receptor modulators [35] raises the possibility that similar reagents may eventually be identified for other nuclear receptors.

FXR, A Bile Acid Receptor

As early as 1957 it had been appreciated that a biliary component could inhibit bile acid production [36]. A key feature of this negative feedback loop is the ability of bile acids to inhibit Cyp7a transcription [36]. The nature of the bile acid sensor remained elusive until 1999 when it was identified as the nuclear receptor FXR or BAR [37–39]. It is now clear that BAR plays a broader role in regulating bile acid homeostasis. In addition to inhibiting hepatic expression of CYP7A1, the rate-limiting enzyme in bile acid biosynthesis, BAR also inhibits expression of CYP8B [40], which acts downstream in the bile acid biosynthetic pathway. BAR also stimulates expression of the ABC cassette protein BSEP (bile-salt export protein, ABCB11) [41], which promotes biliary secretion of bile acids. Thus, the net effect of BAR activation is to limit bile acid accumulation in the liver. Indeed, BAR-deficient mice are highly sensitive to the toxic effects of excessive bile acids [42].

Table I Expression Patterns, Key Target Genes, Ligands and Physiological Functions of Selected Orphan Nuclear Receptors

H_2N— DNA BINDING DOMAIN ———— LIGAND BINDING DOMAIN AF2 —COOH

Receptor	Gene symbol[a]	Key tissues of expression	Critical target genes[b]	Ligands[c]	RXR ligand response?	Function
LXRα	NR1H3	Hepatocytes, Intestinal Epithelium, Macrophage, Fat	cypa7a1 (rodent), ABCA1, apoE, ABCG1/G4/G5/G8, SREBP-1c, FAS, LXRa	Oxysterols, polyunsaturated fatty acids (–)	Yes	Cholesterol, triglyceride & bile acid homoestasis
BAR FXR	NR1H2	Hepatocytes, Intestinal Epithelium	BSEP, SHP, IBABP, PLTP, apoCII ↓CYP7A1, ↓CYP8B	*Bile Acids* Chenodeoxycholic,cholic & deoxycholic acids	Yes	Cholesterol, triglyceride & bile acid homoestasis
SXR PXR	NR1I2	Hepatocytes, Intestinal Epithelium	CYP3A4, CYP2C8, CYP2C9, CYP2B10, MDR1, MRP2, OATP2	*Xenobiotics* *Human*: rifampicin,ritonavir, taxol, clotrimazole, hyperforin *Mouse*: PCN[d]	No	Clearance of xenobiotics & endogenous toxins
CAR	NR1I3 NR1I4	Hepatocytes, Intestinal Epithelium	cyp2b10, cyp3a11, MRP2	*Xenobiotics* *Human*: ? *Mouse*: androstanol(–) TCPOBOP[e]	Yes	Clearance of xenobiotics & endogenous toxins

A schematic representation of an orphan nuclear receptor is shown above the table. Target gene specificity is determined by the DNA binding domain; ligand binding and transcriptional activation are mediated by the ligand binding domain.

[a]Gene symbols are according to the unified nomenclature system for the nuclear receptor superfamily (*www.ens-lyon.fr/LBMC/laudet/nomenc.html*).

[b]↓ indicates genes that are down-regulated by agonist ligands; other genes up-regulated by agonist ligands.

[c](–) indicates an antagonist, inverse agonist or inhibitory ligand; other ligands are agonists or activating ligands.

[d]PCN, pregnenolone-16α-carbonitrile.

[e]TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene.

Bile acids are produced in the liver, secreted into the bile, and then efficiently absorbed via an ileal bile acid transporter. Thus, the ileal epithelium is exposed to extremely high levels of bile acids. The cytosolic ileal bile acid binding protein (IBABP) is thought to act as a shuttle protein that aids in transport and protects the cell from high levels of free bile acids. Interestingly, BAR is an extremely effective inducer of IBABP [43], which is consistent with the role of this receptor in protecting the body from excess bile acids.

The ability to inhibit CYP7A1 transcription implies that an antagonist of BAR could be a useful cholesterol-lowering agent. However, like LXRα, BAR induces a reciprocal relationship between cholesterol and triglyceride levels [42,44]. This is potentially related to BAR-mediated regulation of apolipoprotein CII (apoCII) [45] or phospholipid transfer protein (PLTP) [46]. Regardless of the mechanism, LXRα and BAR share similar caveats with regard to their potential use as targets for cholesterol lowering.

Orphan Receptors and Xenobiotic Signals

SXR, A Master Regulator of Drug Clearance

The ligand-binding properties of the steroid and xenobiotic receptor SXR (also known as PXR, PAR, PRR, NR1I2) are different from most nuclear receptors. Whereas most receptors possess a high degree of ligand-binding specificity, SXR is activated by a very large number of structurally unrelated pharmaceutical agents including rifampicin, clotrimazole, SR12813 [47–51], HIV protease inhibitors [52], taxol [53], and hyperforin [54], the active ingredient in the herbal remedy called St. John's wort. Many of these compounds bind directly to the receptor LBD with affinities in the micromolar range. In an effort to understand the basis for SXR's extreme promiscuity in ligand binding, Watkins and colleagues [55] determined the three-dimensional structure of human SXR. They found that SXR has a larger ligand binding cavity than most nuclear receptors as well as a flexible loop, which accommodates both small and large ligands. These studies highlight the broad ligand-binding specificity of SXR and confirm its designation as a xenobiotic receptor.

Many SXR activators were previously described as inducers of the hepatic cytochrome *P*-450 monooxygenase CYP3A (CYP3A4 in human, cyp3a11 in mouse, CYP3A23 in rat, CYP3A6 in rabbit). A variety of biochemical and genetic studies have demonstrated that SXR directly regulates CYP3A induction via SXR response elements in the CYP3A promoter. CYP3A4 is the most abundant cytochrome *P*-450 in human liver and is responsible for the metabolism of approximately 50% of all drugs [56]. Subsequent studies have expanded the number of SXR target genes to include other *P*-450 enzymes including CYP2C8, CYP2C9, and CYP2B [52,53,57]. These enzymes metabolize a variety of drugs including nonsteroidal anti-inflammatory drugs, hypoglycemic agents, anti-epileptic agents, and taxol, an antineoplastic agent [56]. Thus, SXR is not only a xenobiotic sensor, but a direct effector of xenobiotic degradation.

In addition to being expressed in the liver, SXR is also highly expressed in the intestine [47–50], which raises the question as to the function of SXR in this organ. We have found that SXR regulates expression of MDR-1/P-glycoprotein, a broad-specificity efflux pump located on the lumenal surface of intestinal enterocytes [58]. P-glycoprotein can severely limit the oral absorption of its substrates [58,59]. Because most drugs are administered orally, the SXR/P-glycoprotein linkage may limit the bioavailability of many pharmaceutical agents.

Other transporters including OATP2 (Na+-independent organic anion transporter 2) [60] and the ABC protein MRP2 (multidrug resistance protein 2, ABCC2) [52] also act as SXR target genes. OATP2 is present on the sinusoidal membrane of hepatocytes and promotes uptake of organic anions (e.g., bile acids and xenobiotics) into the liver. MRP2 resides on the canalicular membrane of hepatocytes and mediates the biliary excretion of its substrates. Taken together, these studies indicate that SXR is a master regulator of a diverse array of xenobiotic clearance pathways.

The ability to activate drug clearance has important implications. In particular, drugs that activate SXR may decrease their own bioavailability as well as the availability of coadministered drugs [61–65]. In principle, this type of drug–drug interaction could be overcome by screening for analogs that retain therapeutic activity but fail to activate SXR. An example of this is illustrated by the use of taxotere, a taxol analog that retains antineoplastic activity but fails to activate SXR [53]. The identification of such "SXR-transparent" drugs would be predicted to decrease the likelihood of drug–drug interactions. Finally, it is important to note that the LBDs of human and rodent SXR possess distinct ligand-response profiles (see Table I) [51]. This divergence is consistent with the observation that rodent models are poor predictors of drug metabolism in humans.

CAR, A Close Relative of SXR

The nuclear receptor CAR is a close relative of SXR. Both are highly expressed in the liver and intestine and these receptors share sequence identity in their DNA (66%) and ligand-binding domains (41%) [66,67]. Mouse CAR was originally identified as a constitutively active receptor whose activity could be repressed by androstanol [68]. Subsequent studies demonstrated that CAR was a receptor for TCPOBOP (1,4-bis[2-(3,5-dichloropyridyloxy)]benzene) [69–72], a highly potent inducer of cyp2b10 expression in mouse hepatocytes. TCPOBOP had previously been demonstrated to be a member of the phenobarbital class of hepatic *P*-450 inducing agents suggesting that CAR played a role in mediating this xenobiotic response. This link became definitive with the demonstration that CAR knock-out mice fail to induce cyp2b10 in response to either TCPOBOP or phenobarbital [67].

Although CAR is a strong inducer of cyp2b10, it also serves as a weaker regulator of SXR target genes [73]. Moreover, these receptors recognize a similar array of ligands, albeit with distinct dose-response profiles [74]. These findings

suggest that SXR and CAR may be partially redundant in their functions. This notion is confirmed by the observation that mice lacking either SXR or CAR are highly sensitive to the paralytic effects of zoxazolamine [67,75]. This presumably reflects a shared defect in the clearance of this drug. It is important to note that effective ligands have been identified for mouse CAR but not for its human counterpart. In the future, it will be important to test drugs for their ability to modulate human CAR and to elucidate the contribution of this receptor to drug metabolism in humans.

Future Directions

Orphan nuclear receptors provide convincing evidence that higher organisms regulate gene transcription in response to metabolic and xenobiotic cues. As metabolic disease (atherosclerosis, hypercholesterolemia, diabetes) represents the leading cause of death in industrialized societies, these receptors provide important targets for drug discovery. At the same time, discovery efforts will be enhanced by dual-screening strategies that select for therapeutic activity while minimizing interactions with xenobiotic receptors. Finally, orphan receptors represent a valuable source for future exploration because 26 orphans have yet to be associated with specific ligands.

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CHAPTER 278

Identification of Ligands for Orphan Nuclear Receptors

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Introduction

The nuclear receptors comprise a superfamily of transcription factors that are activated by small, lipophilic chemicals including the classic steroid, retinoid, and thyroid hormones. There are 48 members of this family encoded by the human genome [1], far more receptors than established lipophilic hormones. The work of a number of laboratories has shown that many of the remaining "orphan" members of the family function as metabolic sensors, linking physiological levels of intracellular metabolites to the transcriptional regulation of key biochemical pathways. In this brief review, we highlight recent progress that has been made in the identification of ligands for orphan nuclear receptors. With the exception of the hepatocyte nuclear receptor 4 (HNF4, NR2A) and estrogen related receptor (ERR; NR3B) subtypes, all of the receptors discussed in this review bind to their cognate DNA response elements as heterodimers with the 9-*cis* retinoic acid receptors (RXR, NR2B).

PPARs: Fatty Acid Sensors

A wealth of evidence now exists to support roles for the peroxisome proliferator-activated receptors (PPARs; NR1C) in lipid and carbohydrate metabolism [2]. Three PPAR subtypes are encoded by distinct genes: PPARα, PPARγ, and PPARδ. Each PPAR subtype has a unique biology. PPARα is abundantly expressed in the liver, kidney, heart, and muscle and plays a central role in fatty acid oxidation. PPARγ is expressed at high levels in adipose and has a prominent role in fat cell formation and fatty acid storage. PPARδ is expressed in most tissues and was recently shown to regulate systemic cholesterol and lipid homeostasis.

Synthetic ligands have provided tremendous insight into the functions of the PPARs [2]. PPARα is the molecular target for the fibrates (Fig. 1), a class of drugs used to lower triglyceride levels in dyslipidemic patients. PPARγ is the target for the thiazolidinedione drugs (Fig. 1), which enhance insulin sensitivity and lower glucose levels in patients with type II diabetes. Although there are no marketed drugs targeted against PPARδ, a potent, synthetic PPARδ agonist was recently shown to increase serum levels of high-density lipoprotein and to decrease fasting triglyceride and insulin levels in insulin-resistant nonhuman primates [3]. Thus, PPARδ agonists may have utility in the treatment of cardiovascular disease associated with metabolic syndrome X.

All three PPAR subtypes are activated in cell-based reporter assays by a variety of fatty acids and fatty acid metabolites [2]. Many of these fatty acids bind to the receptors at low micromolar concentrations, which are consistent with their levels in serum. These data suggest that rather than having a single, high-affinity ligand, the PPARs may function as more generalized sensors of free fatty acid levels. Recently, a higher affinity (K_d~40 nM) PPARγ ligand, hexadecyl azelaoyl phosphatidylcholine, was extracted from oxidized low-density lipoprotein [4]. Thus, higher affinity natural ligands may also exist for the PPARs.

LXRs: Cholesterol Sensors

Cholesterol is an essential component of cell membranes and serves as the precursor to the steroid hormones and

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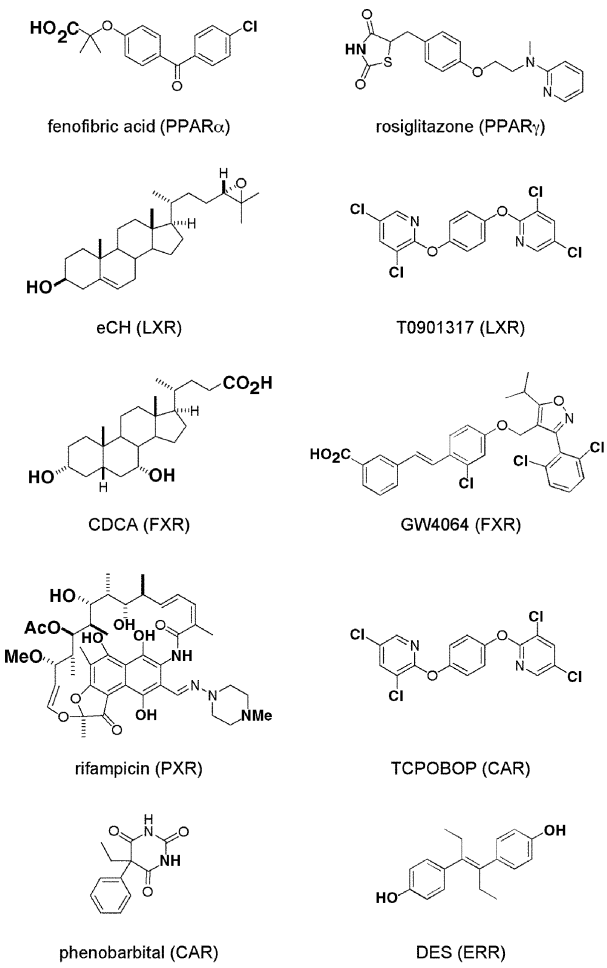


Figure 1 Orphan nuclear receptor ligands. The chemical structures of representative ligands for orphan nuclear receptors are shown.

bile acids. However, excess cholesterol is associated with diseases such as atherosclerosis and the formation of gallstones. Thus, a delicate balance must be maintained between the ingestion and synthesis of cholesterol and its elimination from the body. It is now established that the two liver X receptor (LXR; NR1H) subtypes, LXRα and LXRβ, play important roles in the elimination of excess cholesterol from the body [5]. The LXRs regulate a variety of genes involved in cholesterol metabolism including CYP7A1, which catalyzes the rate-limiting step in the conversion of cholesterol to bile acids in the liver, and the ATP-binding cassette (ABC) proteins A1 and G1, which mediate the efflux of cholesterol from enterocytes and macrophages for elimination from the body. Thus, the LXRs are components of a physiological regulatory system that senses excess cholesterol and modifies gene expression accordingly.

The natural ligands for the LXRs are oxidized cholesterol derivatives including 24(*S*),25-epoxycholesterol (eCH) (Fig. 1), 22(*R*)-hydroxycholesterol, and 24(*S*)-hydroxycholesterol [5,6]. Each of these compounds has a distinct tissue distribution profile. eCH is produced in the liver from a shunt of the mevalonate pathway in which squalene epoxide is metabolized to the diepoxide prior to undergoing cyclization. eCH is present in extracts from rat livers at 1–5 μM concentrations and rises upon cholesterol feeding. 22(*R*)-hydroxycholesterol is present at micromolar concentrations in extracts of the adrenals. Interestingly, 24(*S*)-hydroxycholesterol is generated exclusively in the brain. Its abundance in this tissue has led to it being termed *cerebrosterol.* Recently, the first potent, selective synthetic LXR agonist (T0901317) (Fig. 1) was described [7]. T0901317 binds and activates both LXR subtypes at nanomolar concentrations.

FXR: Bile Acid Sensor

Bile acids are essential for the elimination of cholesterol from the body and the solubilization and transport of lipids in the intestine. However, bile acids are also detergents that are extremely toxic at high concentrations. Thus, their levels must be tightly regulated. In 1998, several groups reported that the farnesoid X receptor (FXR, NR1H4) is a bile acid receptor [5,8]. Several different naturally occurring bile acids bind and activate FXR at physiological concentrations including chenodeoxycholic acid (Fig. 1), cholic acid, deoxycholic acid, and lithocholic acid and/or their glycine or taurine conjugates. The first potent, selective nonsteroidal FXR agonist, GW4064, was recently described (Fig. 1) [9]. FXR is highly expressed in tissues that are critical in bile acid biology including the liver, intestine, and kidney, and it regulates the expression of genes involved in bile acid homeostasis including the intestinal bile acid binding protein, the bile salt export pump, and CYP7A1 [10].

PXR and CAR: Xenobiotic Sensors

The body must protect itself against myriad xenobiotics ingested in the diet or otherwise absorbed. Two nuclear receptors, the pregnane X receptor (PXR, NR1I2) and the constitutive androstane receptor (CAR, NR1I3), have evolved to detect xenobiotics as part of the body's detoxification machinery [11]. Both receptors are highly expressed in the liver and intestine and regulate the expression of a number of genes involved in the hydroxylation, conjugation, and transport of xenobiotics. PXR is activated by a remarkable diverse collection of natural and synthetic chemicals including macrocyclic antibiotics (e.g., rifampicin; Fig. 1), antimycotics (e.g., clotrimazole), steroids (e.g., dexamethasone), and the herbal antidepressant St. John's wort. Because it is activated by many widely used prescription medicines, PXR activation represents the basis for a common class of drug–drug interaction, in which one drug stimulates the metabolism of another. Although CAR can also function as a xenobiotic sensor, it differs from PXR in two important respects. First, CAR is sequestered in the cytoplasm in its inactive state. Second, once it has entered the nucleus, CAR has a high basal level of transcriptional activity even in the absence of an exogenous ligand. CAR can be activated by compounds such as phenobarbital (Fig. 1), which promote

its translocation from the cytoplasm into the nucleus without binding directly to CAR. However, once in the nucleus, CAR can also be modulated by compounds that bind directly to the receptor such as the androstanols, which suppress CAR's high basal activity, or TCPOBOP (Fig. 1), which further increases CAR's transcriptional activity [12].

Ligands for Other Orphan Nuclear Receptors

Recently, ligands have been identified for the three ERR subtypes (α, β, γ) [13,14] and two mammalian HNF4 subtypes (α, γ) [15]. All of these receptors have high basal activity in cell-based reporter assays in the absence of exogenous ligands. The three ERR subtypes are expressed in a number of different tissues and bind to DNA efficiently as monomers. The basal transcriptional activity of all three ERR subtypes can be directly suppressed by the estrogen receptor agonist diethylstilbestrol (Fig. 1), and the estrogen receptor antagonist 4-hydroxytamoxifen can efficiently deactivate ERRγ [13,14]. These data suggest that the ERRs may function as receptors for estrogens or related steroids. The HNF4s are known to bind to DNA as homodimers and regulate genes involved in lipid and carbohydrate homeostasis [16]. X-ray crystallography and mass spectroscopy revealed that both HNF4 subtypes bind to saturated and monounsaturated fatty acids with chain lengths of 14–16 carbon atoms [15]. The transcriptional activity of HNF4α has also been reported to be higher in the presence of fatty acids in cell-based reporter assays [17]. However, unlike other nuclear receptors, the HNF4 subtypes do not appear to readily exchange their ligands [15]. Thus, HNF4 may define a new class of constitutively active nuclear receptors in which the lipophilic ligand serves as an integral component of the receptor complex rather than a reversible switch.

Conclusion

During the past decade, ligands have been identified for more than a dozen of the orphan nuclear receptors. Many of these are now known to be receptors for key intracellular metabolites including fatty acids and cholesterol derivatives. Others serve as sentinels in the body's defense mechanism against xenobiotics. Studies of the ERRs suggest that additional steroid hormones may yet exist to be discovered. Finally, recent studies with HNF4 raise the possibility that some orphan nuclear receptors have high constitutive levels of transcriptional activity because they bind irreversibly to their lipophilic ligands. The availability of natural and synthetic ligands will aid tremendously in unraveling the biological functions of these orphan nuclear receptors.

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CHAPTER 279

Orphan Receptor COUP-TFII and Vascular Development

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Introduction

Nuclear receptors belong to a large family of transcription factors, which can modulate gene expression [1,2]. Since the discovery of the steroid/thyroid hormone receptor superfamily, an increasing number of transcription factors with similar structural motif have been added. A majority of these newly added members have unknown ligands, thus they are classified as orphan nuclear receptors. Like the other members of the steroid/thyroid hormone receptor superfamily, the orphan receptors are involved in development, differentiation, and homeostasis processes. The absence of ligand makes it more difficult to dissect the functional role of orphan receptors. However, the analysis of orphan receptor null mice will provide new insights into the physiological function of these receptors during development, organogenesis, and homeostasis. In this review, we attempt to describe the functional role of orphan receptors during vascular development.

Vascular Development

During embryogenesis, the vascular system plays an important role. It is in charge of the transport of oxygen and nutrients, which are indispensable to the growth and development of the different tissues in vertebrate embryos. For that reason, the development of the vascular system is one of the first events to occur. The vascular network formation requires two distinct phenomena, vasculogenesis and angiogenesis [3,4].

Vasculogenesis

Vasculogenesis is the formation of the early vascular plexus from mesodermal cells (hemangioblasts), which proliferate and differentiate to form the precursors of the endothelial cells of the vessel wall. During early development, vasculogenesis gives rise to a primary vascular plexus including the major vessels of the embryo, such as the aorta and major veins [3]. Factors such as fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF), and VEGFR-2 are important for this process.

Angiogenesis

The next step in formation of the vascular system is *angiogenesis,* which corresponds to the formation of new blood vessels from preexisting vessels. Two different processes characterize angiogenesis: angiogenic remodeling and angiogenic sprouting. The remodeling process consists of the modification of the initial vessel plexus in order to form interconnecting branching patterns present in the mature vascular network. The sprouting process corresponds to the formation of new capillaries originating from small venules or from other capillaries. It occurs both in the yolk sac and in the embryo (neural tube and retina). It also involves many factors responsible for the proteolytic degradation of the extracellular matrix, for the chemotactic migration and proliferation of endothelial cells, for the formation of a lumen, and for functional maturation of the endothelium. The involvement of factors such as VEGF, VEGFR-2, Tie1, Tie2, angiopoietin-1 (Ang1), Ang2, EphrinB2, platelet-derived

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growth factor (PDGF), and PDGFR is primordial for the formation of a mature vascular network [3,4].

Transcription Factors Involved in Vasculogenesis and Angiogenesis

Recently, Roman and Weinstein [4] provided a concise overview of the molecules involved in the development and maintenance of the vertebrate embryonic vascular network. It appears that COUP-TFII is the only orphan nuclear receptor with a well-described role in angiogenesis during embryonic development in mice [5]. Some recent studies suggest that the orphan receptor, PPARγ, may also play a role in the vascular development.

PPARγ: Inhibitor of Angiogenesis

The peroxisome proliferator-activated receptor (PPAR, NR1C) group contains three subtypes: α, β (also known as δ or NUCI), and γ. Since the cloning of PPAR members, many pharmacological activators have been identified [6,7]. PPARs are known to regulate genes involved in lipid and glucose metabolism. Despite the ubiquitous expression of PPARδ, its physiological function is not well known. In PPARα knock-out mice, the liver accumulates droplets of lipid. PPARα ligands can inhibit inflammatory responses in vascular smooth muscle cells *in vivo*, suggesting that PPARα agonists may have a positive effect on vasculature in atherosclerosis [6,7]. The specific ligand for PPARγ, 15d-PGJ_2, can inhibit endothelial tube formation and proliferation, as well as the induction of VEGF receptors *in vitro*. Moreover, 15d-PGJ_2 was shown to be an inhibitor of angiogenesis in the cornea *in vivo* [8]. It is not clear whether PPARγ is involved because we do not know whether the ligand used acts through PPARγ or another nuclear receptor [6,9]. The study of PPARγ knock-out mice has revealed that the establishment and maintenance of the fetal and maternal vascular networks are abnormal in the placenta. However, all other vascular processes appear normal in the null embryos. Whether other PPAR members can compensate for PPARγ has not yet been defined. Therefore, it appears that the vascularization of the placenta is dictated by PPARγ-dependent trophoblast functions [10].

COUP-TFII: Positive Effector in Angiogenesis

COUP-TF (NR2F) is one of the best characterized orphan receptor among the nuclear receptor superfamily. Two members have been identified in mammals, COUP-TFI (also called v-ErbA related protein 3, EAR-3) and COUP-TFII (also called apolipoprotein regulating protein 1, ARP-1). COUP-TF members share a high degree of homology within and between species, so it is assumed they must play important physiological functions [11].

In the developing mouse embryo, COUP-TFI and COUP-TFII exhibit overlapping, but distinct, expression patterns. They are also differentially expressed in the nervous system and during organogenesis [12]. The expression pattern of COUP-TFII in the nervous system is more restricted than that of COUP-TFI. During organogenesis, COUP-TFII is expressed in the mesenchymal compartment. Expression of COUP-TFII is lower than that of COUP-TFI in the nasal septum, tongue, follicles of vibrissae, and cochlea. Expression of COUP-TFII is higher than that of COUP-TFI in the developing salivary gland, atrium of the heart, lung, stomach, pancreas primordium, mesonephros, kidney, and prostate [12]. Because COUP-TFI and COUP-TFII have different expression patterns, they may have different physiological functions.

To study the physiological function of COUP-TFI and -II *in vivo*, we have generated null mice for these genes. COUP-TFI seems to be important for the neuronal development and differentiation. Accordingly, COUP-TFI null mutant mice die perinatally from starvation due to defects in formation of the glossopharyngeal nerve [12]. In contrast, the knock-out mice for COUP-TFII die early during embryonic development at 9.5–10.5 days from heart and vasculature defects [5]. COUP-TFII mutants probably die as a result of malformation of the vasculature, leading to extensive hemorrhage and edema in the brain and heart. Analyses of the mutant embryos revealed defects in angiogenesis, in the development of the atrium chamber and sinus venosus, and malformations of the cardinal veins.

Detailed observation of COUP-TFII null mice revealed that the major vessels, which arise through vasculogenesis, were formed and expressed the specific markers VEGF-R1, VEGF-R2, and PECAM, suggesting that COUP-TFII does not play an essential role in vasculogenesis. However, the remodeling of the vascular plexus was impaired. Therefore, the sprouting and branching from preexisting vessels was defective in COUP-TFII mutants, suggesting an angiogenesis defect. The defect in vasculature development observed in COUP-TFII mutants was similar to the one observed in mice deficient for Ang1 or its receptor, Tie2 [3]. Moreover, the Ang1 expression pattern is very similar to that of COUP-TFII. In fact, Ang1 mRNA expression is down-regulated 10-fold in the mesenchymal compartments of the brain, eyes, somites, and heart in COUP-TFII mutants. Because COUP-TFII may function through the Ang1-Tie2 signaling pathway, it is likely that it acts on the vasculature formation via mesenchymal–endothelial interactions. Further experiments, using subtraction library screening or P19 cells overexpressing COUP-TFII, also showed that Ang1 is a downstream target gene of COUP-TFII (Petit, F., Tsai, M.-J., and Tsai, S.Y., unpublished data). In the COUP-TFII mutants, there is no obvious alteration in the expression of Tie2, which is regulated by its own ligand, Ang1. This observation suggests that another signaling pathway must be affected by COUP-TFII. Taken together these data suggest that, in mesenchymal cells, COUP-TFII is the upstream regulator of Ang1 expression, which in turn activates its receptor, Tie2, to exert the differentiation of endothelial cells (Fig. 1).

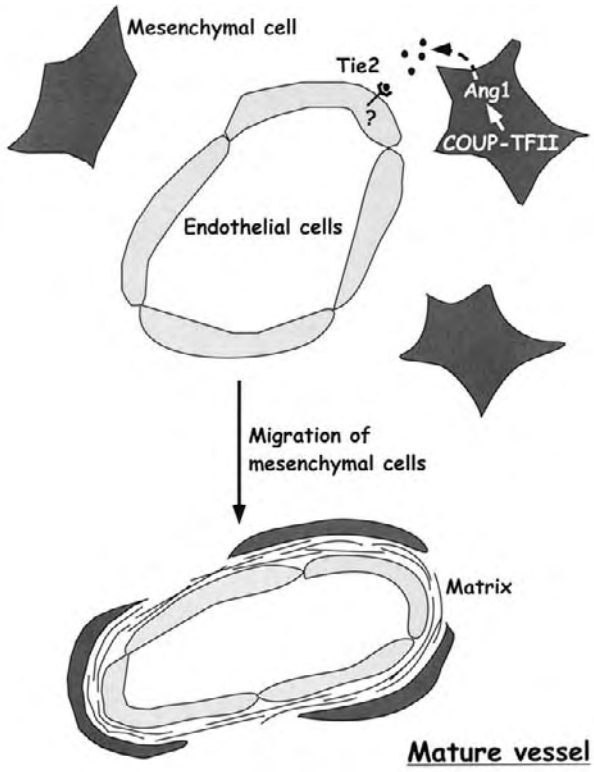


Figure 1 Role of COUP-TFII in the development of vessels. COUP-TFII induces the expression of Ang1, which is released by the mesenchymal cells, Ang1 activates its own receptor, Tie2, located on the endothelial cells, which in turn allow the migration of mesenchymal cells and subsequently the differentiation of mesenchymal and endothelial cells.

Conclusion

So far, among the orphan nuclear receptor group, COUP-TFII is the only member shown to play a role in vascular development. The involvement of COUP-TFII in angiogenesis suggests that it might play a role in tumor growth and progression. Indeed, COUP-TFII is highly expressed in several tumor cell lines. The discovery of artificial or natural ligands for COUP-TFII will provide important tools for the therapeutic treatment of disease involving angiogenesis or vascular modeling. Moreover, these putative ligands will allow us to further study the function of COUP-TFII in other signaling pathways.

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CHAPTER 280

Cross-Talk between Nuclear Receptors and Other Transcription Factors

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Introduction

All cellular processes depend on extracellular cues ranging from nutrient and oxygen supply, through cell density and contact with adverse agents, to highly specific stimuli originating within the multicellular organism. The extracellular cues are sensed by receptors and converted into responses through the intracellular signaling network. To maintain homeostasis of the cell and of the multicellular organism, the reactions to extracellular cues must be well controlled. Any change of condition requires finding a new balance of life processes achieved by constant readjustment of the cellular signaling network. Each stimulatory pathway is, therefore, counterbalanced by inhibitory components. For instance, receptor tyrosine kinases are associated with negatively regulating protein tyrosine phosphatases, which keep receptor autophosphorylation at a low level in the absence of ligand and limit the duration of activity on activation of the receptors. Similarly, dual-specificity kinases within pathways are balanced by dual-specificity phosphatases whose abundance or activities are up-regulated together with the pathway, thus limiting the extent and duration of stimulation. Upon mitogenic stimulation, cells enter and proceed through the cell cycle, itself an ordered circuit of consecutive positive and negative elements.

One can easily imagine what would happen if the control circuits did not operate: A wound-healing reaction would not stop once the damage was repaired; it would instead form excessive keloid material (scar tissue). Excessive expression and activation of metalloproteases in response to inflammatory cytokines would cause various types of tissue damage, as seen in rheumatoid arthritis. The expansion of T and B lymphocytes upon antigen-specific stimulation would lead to leukemia-like cell numbers unless negative control mechanisms and ultimately induced apoptosis limited the expansion. A bacterial invasion would lead to uncontrolled and long-lasting release of tumor necrosis factor alpha (TNF-α), for example, by macrophages, which results in septic shock and death of the organism.

This chapter deals with a particular organismic control mechanism established by hormones. Glucocorticoid hormones or retinoic acid as well as vitamin D counteract proliferative, inflammatory, and immune responses. This is why glucocorticoids are widely used in treating unwanted inflammatory reactions, asthma, autoimmune diseases, and leukemia. Vitamin D is applied to psoriatic skin to block proliferation. Retinoids are being used for certain types of cancer. The presumable mechanistic principles were discovered in 1990 and in the years thereafter. The nuclear receptors, which are activated by the hormone ligands, interfere with several of the signal transduction and transcription steps that are relevant for the panel of reactions mentioned. The mechanisms of interference with components of signal transduction are briefly reviewed here before describing in more detail the most interesting mechanism: *cross-talk* between transcription factors.

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Proliferative and Proinflammatory Pathways

If a process is to interrupt the immune response or unwanted proliferation, or even inhibit cancer cells, which signaling pathways and which gene expressions need to be addressed? Obviously, we need to address pathways and genes that turn on the cell cycle, which cause the release of cytokines and which control cellular migration, adhesion, and invasiveness. It is these properties that lymphocytes, macrophages, dendritic cells, and other inflammatory cells acquire in order to "squeeze" through endothelial barriers, to invade tissues, and to home into lymphoid organs. Cancer cells share many of these phenotypic features. Knock-out technology has revealed some of the pathways and transcription factors relevant for these processes in immune cells (see, e.g., [1]). Members of the Rel family of transcription factors, of the AP-1 family as well as NF-AT, Oct-1, and C/EBP, fulfill central functions in immune cells upon activation. These factors or the signaling pathways leading to their activation need to be targeted in order to inhibit the responses. AP-1 and NF-κB are driven also by oncogenic pathways. Inhibiting their action or activation would be antiproliferative [2–5].

For instance, components of the bacterial cell wall such as lipopolysaccharides (LPS) activate toll-like receptors that are linked to the protein kinase complex (IKK) phosphorylating I-kBα [6,7]. I-κBα keeps NF-κB in an inactive cytoplasmic state [5,8,9]. Upon phosphorylation, I-κBα is subjected to degradation and NF-κB is released and transported into the nucleus, where it acts on the promoters of numerous cytokine genes. In the case of macrophages the toll-like receptors trigger the synthesis and release of TNF-α. Important for the mechanism discussed here, nonlethal (to wild-type mice) doses of LPS become lethal in adrenalectomized mice (unable to produce glucocorticoid hormone [10]). Inflammatory cytokines can pass through the blood–brain barrier and exert central nervous system effects [11,12], including the stimulation of the hypothalamic–pituitary–adrenal axis, which leads to release of glucocorticoids from the adrenal gland if present [13].

Another central pathway involved in growth factor transcription as well as in transcription of cyclin D1, the entry step into the cell cycle, and of adhesion and migration components, originates from the small G protein Ras. The Ras– MEK–Erk pathway regulates a wide variety of features essential for the immune response as well as for a proliferative and invasiveness program. For instance, the pathway leads to expression of AP-1 subunits as well as to their activation. AP-1 and NF-κB control the synthesis of several cytokines in T cells and macrophages. Interestingly, the Ras–MEK–Erk pathway controls also alternative splicing [14], yielding different CD44 transcripts and subsequently CD44 proteins that, in turn, are required for specific activation of certain receptor tyrosine kinases [15]. Thus, interference with inflammation, proliferation, and immune responses should address predominantly the pathways leading to AP-1 and NF-κB activity.

Nuclear Receptors

The mediators of hormone-dependent interference with proliferative and inflammatory responses belong to the family of nuclear receptors that share several structural features [16,17]. Nuclear receptors are bona fide transcription factors whose action is turned on by the specific hormone ligand. The C-terminal half carries the ligand-binding domain and a transactivation domain (AF-2) whose accessibility depends on the presence of the proper ligand. AF-2 assembles coactivator complexes necessary for initiation of transcription. The monomeric nuclear receptor does not bind sufficiently to DNA; rather homodimerization (e.g., in the case of steroid hormone receptors) or heterodimerization with a common subunit, RXR (of the receptors for vitamin D, for thyroid hormone, and for retinoids) is required. A conserved structure in the middle of the nuclear receptor molecule is folded by coordinating zinc atoms into two finger-like structures, the N terminal of which confers, together with its dimer partner, binding to the specific promoter element. The C-terminal zinc finger mediates protein interactions; for example, through the extended so-called D-loop, it mediates homodimerization of steroid hormone receptors. The N-terminal half carries another transactivation domain (AF-1) that is ligand independent but requires modifications by other pathways. Recent evidence suggests that modifications by Erk or Rsk-1 enhance the transcriptional function of some nuclear receptors (e.g., ERα carries an Erk site at amino acid 118 [18] and a pp90rsk1 site at amino acid 167 [19,20]). Steroid hormone receptors are associated with chaperones that, in addition to other functions, are needed to keep the ligand-binding domain in a steroid hormone accepting conformation. Often the steroid hormone receptors are cytoplasmic in the absence of ligand [21].

Induced Expression of Inhibitory Molecules

The most straightforward explanation for an inhibitory role of nuclear receptors would be the hormone-induced synthesis of an inhibitory molecule. To what extent the transcription of such effector molecules plays a physiological role is still a matter of debate. Removal of the adrenal gland leads to thymic hypertrophy suggestive of reduced cell death because the thymus is the organ of high cellular apoptosis. Glucocorticoids induce apoptosis in isolated thymocytes for which proper transcriptional function of the GR is required [22,23]. Also several other nuclear receptors appear to exert functions in the thymus [24]. It is therefore plausible that nuclear receptors are involved in the control of synthesis of proapoptotic transcripts. Nevertheless, it is still unclear to what extent this is physiologically relevant [24].

Induced inhibitors of transcription and of signal transduction have been identified. In several types of cells, the abundance of the inhibitor of NF-κB, I-κBα, is enhanced upon treatment of cells with glucocorticoid hormone [10,23,25]. As we will see, the induction requires the presence of a

dimerization-competent glucocorticoid receptor. One could imagine that enhanced expression of I-κBα terminates the action of NF-κB and thus the release of cytokines. (For a recent discussion of NF-κB:I-κB shuttling between cytoplasm and nucleus see [5].) Another glucocorticoid receptor target is MKP-1, the phosphatase acting on Erk. The hormone-enhanced abundance (by both increased transcription and reduced turnover [26]) of MKP-1 counteracts the activation state of Erk. As one would expect for induced new synthesis, the inhibition occurs with a delay of more than 16 hr. Thus, the induced expression of inhibitory proteins represents one type of mechanism explaining how a hormone can terminate inflammatory processes. The induced synthesis will not lead to an instantaneous block.

Immediate Hormone Responses

Most of the anti-inflammatory and antiproliferative action of nuclear receptors does not depend on prior protein synthesis. Such immediate action implies that the liganded receptor exerts a molecular function by direct interaction with another component, for example, DNA or protein. The function could be exerted in the cytoplasm or in the nucleus. The steroid hormone receptors are associated with chaperones that keep the proteins in ligand-susceptible conformation, and they share prolonged periods of cytoplasmic localization. Thus, a cytoplasmic action would be feasible although difficult to reconcile with hormone-dependent activation of cytoplasmic components: The bulk of ligand-activated receptor is located in the nucleus, whereas signaling components including JNK remain by and large cytoplasmic [27,28]. Controversy still surrounds questions as to what extent and how nuclear receptors affect signal transduction pathways directly. Both inhibitory and stimulatory actions have been reported, the kinetics of which are often too slow to be taken as direct actions (e.g., 50% inhibition of JNK activity [29–32], Erk [33,34], p38 [35]). One of the signaling molecules addressed is JNK, the enzyme activating the AP-1 subunit Jun. Interference with JNK is possibly a nuclear event [28]. The more convincing data, which are based on the use of gene disruptions and coprecipitations, classify interactions with components of the signaling network as costimulatory. The associations, for instance, of the androgen or estrogen receptors with Src, PI3Kp85, Fyn, and other signaling molecules appear to enhance nuclear receptor function in transcription [36,37].

The inhibitory action of nuclear receptors is by and large nuclear. Two types of mechanisms for immediate inhibition appear to emerge: blocking of transcription by binding to a promoter element, and modulation of other transcription factors without obligatory binding of the nuclear receptor to DNA. The repressor function at the promoter element recognized by the nuclear receptor has been best documented for the thyroid receptor. In the nonliganded state, the receptor recruits corepressor molecules such as N-CoR and histone deacetylases [38]. Hormone binding converts the receptor into the transactivating molecule. Less well understood is the action of the glucocorticoid receptor on so-called nGREs, negative glucocorticoid response elements [39]. nGREs seem to play roles in the autoregulatory circuit of the hypothalamus–pituitary–adrenal axis, for example, in the transcription of the genes for promelanocorticotropin and prolactin. The binding to nGREs appears to require dimerization or multimerization of the glucocorticoid receptor [39]. nGREs possibly induce a conformational change such that the GR cannot recruit the complex of components required for transcriptional initiation and instead binds inhibitory cofactors.

The modulation of transcription factors without direct binding to DNA is the more predominant mechanism relevant for the control of proliferation and of the immune reactions. It is also the most intriguing mechanism and presumably the evolutionarily more ancient one [40]. All nuclear receptors (perhaps with the exception of the mineralocorticoid receptor) appear to share this ability [41–48]. The evidence for the glucocorticoid receptor for which most data have been collected is described next.

Direct Modulation of Transcription Factors

Mutual Modulation, A Method of Maintaining Homeostasis

Rapid repression of phorbol-ester-induced transcription of collagenase (MMP-13) absence of phorbol synthesis led to the discovery of transcription factor modulation by nuclear receptors [41]. The collagenase promoter fragments sufficient for induced transcription and hormone-dependent repression comprised neither GRE or nGRE sequence elements. Similarly, the repressible promoter for interleukin-2 carried no GRE or nGRE [10]. These data led to the recognition that nuclear receptors interfere with other transcription factors. The glucocorticoid receptor modulates negatively the transcription by CREB [4,49], by the Jun:Fos heterodimer (AP-1) [41–43], and by, for example, Oct-1 [50], Spi-1/PU-1 [51], GATA-1 [52], and NF-κB [53]. This list is probably not complete. As introduced earlier, interference with AP-1 and NF-κB function is certainly most relevant in the inhibition of inflammation and of proliferation [3,54]. Interestingly, the modulation is mutual, liganded glucocorticoid receptor down-modulating AP-1- and NF-κB-dependent transcriptions, activated AP-1 or p65 (NF-κB) inhibiting GRE-dependent transcription [41]. Interestingly, less hormone was required for repression of AP-1 than for induction of a GRE-containing promoter [41]. Accordingly, the program of gene expression can be modeled by a fine-tuned transition from totally AP-1 (NF-κB, or other factor) dependent transcription and complete inhibition of GRE promoters, through increasing repression of AP-1 and decreasing repression of GRE promoters to a totally hormone-dependent program [55]. This adjustment would be created by the relative concentrations of hormone activating the nuclear receptor versus growth factors activating AP-1 or NF-κB [55].

Tethering

The mutual modulation of transcription factors AP-1 or NF-κB by GR and the reverse is based on protein–protein interactions. By means of coimmunoprecipitations, low-affinity interactions between nuclear receptors and AP-1 were detected [41–43,56]. Although initially thought that mutual binding would interfere with DNA binding, this assumption turned out to be wrong. According to genomic footprints [4,57] and chromatin immunoprecipitations [58], the inhibited AP-1 (at the MMP-13 promoter) or NF-κB (at the promoters for interleukins 2 and 8, as well as for ICAM-1) remain bound to their promoter elements [4,57,58]. Chromatin immunoprecipitation proved that the glucocorticoid receptor was associated with the complex. The molecules "tethered" together apparently exert molecular properties different from those as individual factors; that is, they "forget" to be positive transcription factors [59,60].

This hypothesis is not far-fetched. Theoretical and experimental arguments support that the binding to a specific DNA element determines the conformation of the partners: of DNA as well as of the protein factor [60]. It is thus not too surprising that protein–protein tethering also determines protein function. Synergy has been found for the tethering of nuclear receptors with AP-1 or NF-κB in certain tissues [4,47]. Interestingly Jun:Jun homodimer function can be enhanced by glucocorticoids [4,56]. It is not clear, however, when Jun homodimers are formed under physiological conditions. In the organism, however, the negative interference of GR with AP-1 and NF-κB appears to predominate.

Dissociation of GRE Promoter Activity from Tethering Action

I turn now to a discussion of the dissociation of GRE promoter activity from the tethering action, mainly by the dimerization-defective GR, and to cross-talk as the major mechanism of action for the anti-inflammatory and immunesuppressive functions of the glucocorticoid receptor.

What was already suggested by the different hormone concentration requirements became obvious by mutations created in the glucocorticoid receptor GR [61]. One mutation in particular was most informative: a point mutation in the D-loop responsible for subunit dimerization at the GRE element. This mutation was introduced into the mouse by knock-in strategy. The resulting $GR^{dim/dim}$ mice carried a receptor that was unable to bind to GREs and to activate GRE promoters, but could perfectly repress AP-1 and NF-κB promoters [22,23]. The $GR^{dim/dim}$ mice were viable, suggesting that the induction of hormone-dependent genes was not essential for life under normal animal house conditions. The repressive action was, however, essential, a conclusion that could be derived from the nonviability of total GR knock-outs [62]. The dimerization-defective GR suffices to inhibit most proinflammatory responses [23] with the only exception being thymocyte apoptosis [63], which is counteracted by Bcl-2 in transgenic mice [64] and may involve the activation of caspase-9 [65]. Thus cross-talk between GR and AP-1 and NFκB is possibly the decisive mechanism in the control of the immune system and most relevant for the medical use of glucocorticoid hormones as anti-inflammatories. For instance, inhibitions by hormone of phorbol ester induced inflammation, of the acute phase response or of LPS-induced TNF-α release, were perfectly normal in the mutant mouse. The synthesis and release of macrophage and T-cell cytokines as well as transcription of the cyclooxygenase-2 gene were blocked to the same degree as in wild-type mice. The role of GR in thymocyte development, however, has not been resolved and remains controversial [23,24].

Transcriptional Initiation Steps That Could Be GR Targets

Ever since the discovery of transcription factors and their binding to elements at a fair distance to the TATA box or to the site of RNA polymerase and initiation complex assembly, researchers have been puzzled about how they might activate transcription. According to an early hypothesis, the DNA between the enhancing factor and the initiation complex loops out to bring all partners into proximity, where they provide an unknown start stimulus. Consequently, the modulation of AP-1 by GR was seen as interrupting the contact between AP-1 and the basic transcription complex. When additional factors were found—coactivators—competition for shared coactivators was considered as a mechanism for how a nuclear receptor could affect another transcription factor [47]. One of the coactivators, CBP/p300, is of low abundance, making competition for CBP a plausible proposal [66]. CBP indeed collaborates with both AP-1 and nuclear receptors. However, other CBP-binding transcription factors such as STATs or NF-κB should also compete for CBP, but do not repress AP-1. Even more convincingly, however, the activation domains of the receptors that recruit coactivators could be destroyed without any effect on cross-talk [67].

The discovery of corepressors and their association with histone deacetylase activity primed the hypothesis that recruitment of these negative counterplayers of coactivators may be responsible for hormone-dependent repression. However, inhibitors of histone deacetylase could not release repression [4,58,68].

What then is arrested and prevents transcription after the preinitiation complex has been formed? In chromatin immunoprecipitations, the GR-arrested preinitiation complex lacked one of the phosphorylations of the RNA polymerase II C-terminal tail [58]. This phosphorylation is thought to be important for the start of transcription and for elongation. Because the data are based on the specificity of an antibody, further confirmation is needed.

A current area of intensive research concerning gene expression addresses the state of the chromatin and its regulation. Transcribed genes are less densely packed, a state suggestive of better accessibility for protein factors. Regulated changes of chromatin accessibility occur prior to assembly

of the preinitiation complex, during or after assembly. This order of remodeling may differ among genes [69,70]. It is therefore possible that the nuclear receptor prevents the remodeling step at genes that require such a process after formation of the preinitiation complex. Studies with chromatin immunoprecipitations have yet not explored this possibility.

Whatever the mechanism, yet unknown additional factors are likely to be involved. A cofactor isolated by two-hybrid screens promises to reveal such a factor (tentatively named protein #198; unpublished data). The protein carries three LIM domains, zinc-finger-like structures that mediate protein–protein interactions. One of the LIM domains binds specifically to either Fos (one of the AP-1 subunits) or p65 (the NF-κB subunit), while nuclear receptors interact with another LIM domain, offering the possibility that the protein forms a trimeric complex. Protein #198 carrying only one LIM domain acts as dominant-negative factor abolishing cross-talk between AP-1 and either GR, thyroid hormone receptor, or retinoic acid receptor. Loss of protein #198 by morpholino antisense oligonucleotide technique also prevented the cross-talk. What exactly this new cofactor does in conferring cross-talk modulation of transcription is currently being explored.

Conclusion

The cross-talk function of nuclear receptors is essential for adult life. It is exerted by interference with the activity of another transcription factor at a step after formation of the preinitiation complex. The cross-talk is mutual and offers a way to adjust pro- and antiproliferative transcription in an elegant and fine-tuned way. A new class of cofactors appears to be involved in the cross-talk. Because long-term systemic therapy with glucocorticoids can result in severe side effects such as osteoporosis and joint necroses, current efforts are directed toward distinguishing whether the side effects are caused by GRE-dependent genetic programs or whether they are intimately connected to the cross-talk function of GR, which is unavoidably used for these therapies. It may be possible to select ligands that activate only the cross-talk function.

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CHAPTER 281

Drosophila Nuclear Receptors

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Introduction

The completion of the *Drosophila* genome sequence in early 2000 revealed 21 nuclear receptor (NR) genes [1], about half of the 49 NR genes identified to date in the human genome [2]. In striking contrast, ~270 NR genes have been described in *Caenorhabditis elegans* [3]. It seems likely that the ~255 nematode-specific NRs reflect an adaptation unique to the nematode group. The 21 *Drosophila* NRs represent all major subfamilies described previously [4], and 16 receptors appear to have orthologs in either vertebrates or nematodes, or both (Table I).

Only five receptors have not been identified outside the arthropod branch. Knirps, Knirps-related, and Eagle form a unique class of highly related proteins (often referred to as the Knirps group) that lack a ligand-binding domain (LBD).

The other two receptors, DHR39 and DHR83, are closely related to FTZ-F1 and dFAX-1, respectively, and probably represent—in evolutionary terms—relatively recent splits within the arthropod lineage. In general, it appears that most *Drosophila* NRs are more similar to their human counterparts than to the corresponding *C. elegans* homolog (Table I). The fruit fly, *Drosophila melanogaster*, provides an ideal system to analyze NR function in the context of a developing organism, with a wide range of genetic tools that allow the dissection of regulatory pathways and in-depth characterization of gene function.

Nuclear Receptors and Embryonic Pattern Formation

Drosophila embryogenesis is characterized by a succession of events that transform the early embryo into a segmented first instar larva. Maternally deposited transcripts create opposing protein gradients along the anterior-posterior axis of the early embryo that trigger spatially restricted activation of the so-called gap genes, establishing more defined subdivisions. The gap genes, in turn, activate the next set of genes, the pair-rule genes, that divide the embryo into regions representing the precursors of the body segments.

The NR genes *tailless* (*tll*) and *knirps* (*kni*) are gap genes. Embryos mutant for *tll* exhibit deletions in the terminal domains of the embryo [5]. Once Tll expression is activated through the Ras signaling pathway, it sets up a posterior boundary, restricting the expression of other gap genes like *kni*, *krüppel,* and *giant* [6]. The *tll* gene also plays a role in establishing the optic lobe primordium, which, at a later stage, develops into the optic lobe neuropiles and the larval photoreceptor [7]. The *kni* gene is required for the proper development of A1–A7 abdominal segments [8]. Closely related to *kni*, *knirps-related* (*knrl*) has partially overlapping functions with *kni* in development of the nervous system [9]. Kni and Knrl also play a critical role in cell migration and branch morphogenesis during embryonic tracheal development [10].

The NR gene *FTZ-F1* encodes two isoforms with distinct functions. During embryogenesis, maternally supplied αFTZ-F1 interacts with the Fushi tarazu (FTZ) homeodomain protein. These two proteins constitute mutually dependent cofactors required for correct segmentation [11,12]. FTZ contacts the AF-2 domain of αFTZ-F1 via an LXXLL motif, a signature typically found in NR cofactors and previously unknown to exist in homeodomain proteins [13,14]. The other isoform, βFTZ-F1, which plays a crucial role in molting and metamorphosis at later stages, is first expressed in the late embryo and appears to be required for the completion of embryogenesis [15].

Ecdysone Regulatory Hierarchies

Following embryogenesis, *Drosophila* progresses through three larval stages and metamorphosis, a dramatic process in

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Table I ***Drosophila*** **Nuclear Receptors**

D. melanogaster	Cytology	IF	*C. elegans*	*H. sapiens*	Ecd	Lethal phase	Function
dERR	66B12-13	1	N/A	ERRβ (88/39)	?	?	?
dFAX-1	51F7	1	FAX-1 (93/22)	PNR (83/51)	?	?	?
dHNF4	29E3-4	2	NHR-64 (69/37)	HNF4α (92/65)	?	?	gut formation?
DHR3	46F4-5	2	CHR3 (92/32)	ROR (79/39)	+	embryo	pupariation, activates βFTZ-F1, PNS development
DHR4	2C1-2	1	NHR-91 (68/27)	GCNF (63/20)	+	?	?
DHR38	38E2-3	1 (2?)	NHR-6 (73/22)	NURR1 (92/58)	-	pharate adult[1]	adult cuticle formation, dimerizes with Usp
DHR39	39B1-3	1	NHR-25 (63/25)	LRH-1 (65/32)	+	viable	?
DHR78	78D6-7	1	NHR-41 (73/20)[2]	TR2 (76/25)	(+)	3rd instar	required in mid 3rd instar, trachea molting
DHR83	83E4	1	FAX-1 (67/20)	PNR (62/25)	?	?	?
DHR96	96B14-15	1	DAF-12 (68/29)	VDR (59/25)	(+)	?	?
Dissatisfaction	26A6-7	1	NHR-67 (71/19)	TLX (78/43)	?	sterile	sexual behaviour, serotonin neuron development
E75	75A9-B6	3	NHR-85 (82/12)	REVERBα (82/37)	+	1st instar	oogenesis, embryonic midgut constrictions
E78	78C4-7	2	SEX-1 (76/26)	REVERBα (73/34)	+	viable	follicle cell migration/patterning
Eagle	79A3-4	1	N/A	N/A	?	embryo, 1st instar	neuroblast determination
EcR	42A10	3	N/A	FXR (76/31), LXR (69/38)	+	embryo	20E receptor, molting, pupariation
FTZ-F1	75D4-6	2	NHR-25 (83/24)	LRH-1 (88/38)	+	embryo	FTZ cofactor, mid-prepupal competence factor
Knirps	77E1-2	1	N/A	N/A	?	embryo	embryonic pattern formation, gap gene
Knirps-related	77D1-4	1	N/A	N/A	?	?	embryonic tracheal development
Seven-up	87B8-9	2	UNC-55 (69/24)	COUP-TF1 (95/92)	?	embryo	photoneuron determination
Tailless	100B1	1	NHR-67 (80/16)	TLX (83/36)	?	embryo	embryo termini (gap gene), optic lobe cell fate
Ultraspiracle	2C1-2	1	N/A	RXR (88/44)	+	2nd instar	EcR-partner in 20E receptor, molting, pupariation

Cytology: Chromosomal location was based on published polytene chromosome *in situ* hybridizations and on estimated positions by the genome project. IF: isoforms, only mRNA isoforms encoding different proteins are considered. Number is based on published cDNA and EST data. *C. elegans* and *H. sapiens*: homologs of corresponding *D. melanogaster* NR and percent identity in the DBD/LBD. N/A = no homolog was found, closest match was not significantly more similar than matches to other members of the NR family. Ecd: regulated by ecdysone, tested by organ culture experiments, (+) indicates a weak induction. lethal phase: The zygotic lethal phase of known null mutations. function: summary of key functions

[1]T. Kozlova, personal communication.

[2]The NHR-41 sequence was kindly provided by A. Sluder and C. Gissendanner.

which the crawling larva is transformed into a reproductively active adult fly. These postembryonic developmental transitions are triggered by pulses of the steroid hormone ecdysone. An ecdysone pulse at the end of larval development signals the onset of metamorphosis and initiation of prepupal development, followed by another ecdysone pulse ~12 hr later that triggers the prepupal–pupal transition. The ecdysone signal is transduced through a heterodimer of two NRs: the EcR ecdysone receptor and an ortholog of vertebrate RXR, USP, which appears to function as an obligatory partner [16–18]. USP can also dimerize with DHR38, a NR involved in adult cuticle formation [19,20]. The EcR/USP and DHR38/USP dimers can recognize similar DNA sequences and may directly compete for binding to common regulatory targets [21]. Upon binding ecdysone, the EcR/USP receptor directly induces a set of primary-response genes, some of which encode transcription factors that, in turn, induce a larger set of secondary-response target genes. These gene cascades not only transduce and amplify the ecdysone signal, but also diversify it to achieve temporal and spatial specificity [22].

A surprising number of the key players induced in the ecdysone-regulatory hierarchies at the onset of metamorphosis are members of the NR family [23]. At least 10 NR genes are regulated by ecdysone (Ecd, Table I); most are induced by the hormone, whereas βFTZ-F1 is repressed by ecdysone [24]. The *E75* primary-response gene encodes three NR isoforms, designated E75A, E75B, and E75C [25]. *E75* plays a critical role in oogenesis [26] and is required for proper midgut constrictions during embryonic development [27]. *DHR3* is induced by ecdysone at puparium formation and is required for induction of βFTZ-F1 in the mid-prepupa [28]. E75B, a NR without a functional DNA-binding domain (DBD), can bind directly to DHR3, blocking its ability to induce βFTZ-F1 [29]. The timing of βFTZ-F1 expression thus appears to be controlled by induction of the DHR3 activator and decay of the E75B repressor. βFTZ-F1 provides competence for prepupal responses to ecdysone and is required for larval salivary gland cell death through stage-specific induction of the *E93* death gene [24,30]. *E78* and *DHR39*, two ecdysone-inducible NR genes, are expressed during the early stages of metamorphosis but do not carry out essential functions in the fly [31,32]. Among *Drosophila* NR genes, *E78* is most similar to *E75*. Intriguingly, one of the *E78* isoforms, E78B, is expressed in synchrony with E75B and is also missing its DBD, suggesting possible functional redundancy. DHR39 is closely related to FTZ-F1 and is almost as similar to DHR4, indicating that this NR group might share overlapping functions. *DHR4* mutations have not yet been described, although *DHR4* expression in early prepupae and its induction by ecdysone in organ culture suggest a role at the onset of metamorphosis (K. King-Jones, unpublished results).

Null mutations in the ecdysone-inducible *DHR78* NR gene lead to breaks in the tracheal respiratory system of the larva and lethality during the third instar [33]. Another *Drosophila* NR gene known to be regulated by ecdysone, *DHR96* [34], is an ortholog of *C. elegans daf-12*, which functions at the intersection of pathways that regulate dauer larva formation and adult longevity [35,36]. No specific mutations have yet been reported in *DHR96*, or in two other *Drosophila* NR genes, *dERR* and *dHNF4*. Recent studies, however, have shown that vertebrate HNF4α transcriptional activity can be modulated by long-chain fatty acids, and that diethylstilbestrol inactivates ERRβ [37,38]. It will be interesting to determine whether dERR and dHNF4 are capable of binding similar ligands and if *in vivo* ligands for these NRs can be identified.

The Neuronal Connection

Several NRs are involved in neuronal development, and their expression is often limited to specific neuronal cells. In embryos, *eagle* (*eg*) is almost exclusively expressed in four neuronal lineages that arise from a specific neuroblast (NB 7-3), which ultimately develops into a set of serotonin neurons. Mutant alleles of *eg* exhibit the correct number of *eg*-expressing NB 7-3 progeny, but fail to establish the correct projections, suggesting that the specification of cell identity in this lineage is altered [39,40]. *seven-up* (*svp*) encodes a COUP-TFI-like NR and represents the most conserved member of the NR family between humans and flies. SVP is expressed specifically in four of the eight photoreceptor neurons in the developing eye (R1, R3, R4, R6). *svp* mutations cause a transformation of these cells toward an R7 cell fate [41]. Conversely, ectopic expression of SVP in R7 photoneurons drives them toward an R1–R6 cell fate [42]. Another NR gene known to be expressed in the nervous system is *dissatisfaction* (*dsf*), a *tll*-like gene. Curiously, the LBD of the human Tll ortholog, TLX, is more similar to Dsf than to Tll itself, suggesting that both *dsf* and *tll* are orthologs of the *TLX* gene. *Dsf* is required for appropriate courtship behaviors and sex-specific neural development, and is expressed in a few cells in the larval and adult brain. Sexually dimorphic transcripts of *dsf* have not yet been identified, suggesting that its distinct roles in males and females may be mediated by a sex-specific dimerization partner and/or ligand(s) [43,44].

The genome project revealed two previously unknown NR genes that we refer to here as *DHR83* and *dFAX-1*. Both are closely related, but *DHR83* appears to have no orthologs in either humans or worms. dFAX-1 was named after its *C. elegans* counterpart, FAX-1, which is required for axonal pathfinding [45]. Whether dFAX-1 plays a similar role in *Drosophila* is unknown, although the absence of any ESTs, as well as the fact that this gene was not identified before the completion of the genome project, argues that its expression is either highly cell-type specific or of low abundance. *DHR83* appears to encode a rare transcript as well, but it remains to be seen whether this gene has functions similar to *dfax-1*. Finally, *DHR3* mutants display defects in embryonic peripheral nervous system development, but it is not clear whether this contributes to the early lethality seen in these mutants [46].

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SECTION B

Transcription Factors

Marc Montminy, Editor

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CHAPTER 282

JAK-STAT Signaling

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Introduction

Characterization of the ability of interferon α (IFN-α) to rapidly induce genes led to the discovery of the JAK-STAT pathway. Subsequent studies determined that JAKs (Janus kinases) and STATs (signal transducers and activators of transcription) represent a families of proteins that play a critical role in mediating the biological response to the ~50 hematopoietins, all members of the mammalian cytokine family of ligands (see Table I). Although components of this pathway can be found in the most primitive metazoans, the pathway has grown in complexity as multicellular organisms evolved. In mammals, this pathway regulates pivotal aspects of cellular homeostasis and inflammatory response. This review will provide a brief overview of the significant progress that has been made in understanding this pathway since it was first discovered a decade ago. For a more detailed discussion, readers are directed to more comprehensive reviews [1–4].

The JAK-STAT Paradigm

Hematopoietins represent a family of secreted ligands, which exert their potent biological effects on target cells that express the corresponding receptors. These receptors can be divided into five subgroups based on their structures and the STATs that they activate (see Table I). Of note, a number of receptor tyrosine kinases and G-protein-coupled receptors have also been shown to activate STATs, but these signals do not appear to be as critical to their biological response [2]. Upon binding ligand, the dimeric receptors undergo a conformational change, bringing together and thereby activating associated JAKs (see Fig. 1; [5]). Activated JAKs in turn phosphorylate receptor tyrosine motifs, leading to the recruitment of specific STATs (Table I). At the receptor, these STATs become activated by a single tyrosine phosphorylation event.

After release from the receptor, they dimerize through reciprocal phosphotyrosine–SH2 domain interactions ("classical dimerization"; [6,7]). Intriguingly, recent evidence indicates that STATs may actually form stable dimers prior to activation, but through a different mechanism (J. Braunstein and C. Schindler, unpublished observation). "Classically dimerized" STATs are competent for both DNA binding and robust nuclear translocation. Although structural studies have provided detailed insight into STAT DNA binding activity [6,7], little is known about the structural motifs that regulate nuclear import [2]. Once in the nucleus, STAT dimers bind to members of the gamma-activated site (GAS) family of enhancers, which culminates in the activation of target genes [2,8]. One well-established exception is the Stat1-Stat2 heterodimer. This unique heterodimer, which forms in response to stimulation with type I IFNs, associates with a DNA binding protein, IFN regulatory factor 9 (IRF-9), to form the transcription factor IFN stimulating gene factor 3 (ISGF-3). This factor binds to the IFN stimulated response element (ISRE) subset of the IRF family of enhancers [2].

STAT signals decay within a period of hours and the STATs are re-exported back to the cytoplasm, effectively resetting the cell for the next round of signaling. This appears to entail nuclear dephosphorylation and a Crm1/Ran-GTPase-dependent nuclear export process [2,9]. Some activated STATs may also undergo targeted degradation [10]. Intriguingly, recent studies suggest that the predominantly cytoplasmic distribution of STATs found in resting cells reflects a steady state, which represents a balance between basal (i.e., in the absence of tyrosine phosphorylation) nuclear import and export (S. Bhattacharya and C. Schindler, unpublished observation). This raises the possibility that STATs may regulate the expression of some genes in unstimulated cells [11].

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Table I Hematopoietins Signal through JAKs and STATs

Ligands	JAKs	STATs
IFN family		
IFN-α's[†]/β/ω/Limitin	**Jak1**, Tyk2	**Stat1**, **Stat2**, (Stats3–6)
IFN-γ	**Jak1**, **Jak2**	**Stat1**, (Stat5)
IL-10[*]	**Jak1**, Tyk2	**Stat3**
IL-19	?	?
IL-20	?	Stat3
IL-22	?	Stat3, (Stat5)
IL-24	?	Stat3
gp130 family		
IL-6	**Jak1**, (Jak2)	**Stat3**, Stat1
IL-11	Jak1	**Stat3**, Stat1
OSM	Jak1, (Jak2)	**Stat3**, Stat1
LIF	**Jak1**, (Jak2)	**Stat3**, Stat1
CNTF	Jak1, (Jak2)	**Stat3**, Stat1
NNT-1/BSF-3	Jak1, (Jak2)	**Stat3**, Stat1
G-CSF	Jak1, (Jak2)	**Stat3**
CT-1	Jak1, (Jak2)	**Stat3**
Leptin	Jak2	**Stat3**
IL-12	**Tyk2**, Jak2	**Stat4**
IL-23	?	**Stat4**
γC family		
IL-2	**Jak1**, **Jak3**	**Stat5**, (Stat3)
IL-7	**Jak1**, **Jak3**	**Stat5**, (Stat3)
TSLP[¶]	?	Stat5
IL-9	Jak1, Jak3	**Stat5**, Stat3
IL-15	**Jak1**, Jak3	**Stat5**, (Stat3)
IL-21	Jak3, (Jak1),	**Stat3**, Stat5, (Stat1)
IL-4	**Jak1**, **Jak3**	**Stat6**
IL-13[¶]	Jak1, Jak2	Stat6, (Stat3)
IL-3 family		
IL-3	**Jak2**	**Stat5**
IL-5	**Jak2**	**Stat5**
GM-CSF	**Jak2**	**Stat5**
Single Chain family		
Epo	**Jak2**	**Stat5**
GH	Jak2	**Stat5**, (Stat3)
Prl	Jak2	**Stat5**
Tpo	**Jak2**	**Stat5**

The JAKs and STATs activated by members of the hematopoietin family are indicated. Confidence in these assignments vary: very strong (based on knockout studies and shown in bold), reasonably strong (plain lettering) and least strong (shown in brackets).

[†]There are 12 IFNα's.

[*]IL-10 homologue AK155 has not yet been functionally characterized.

[¶]Bind to a related, but γc independent receptor.

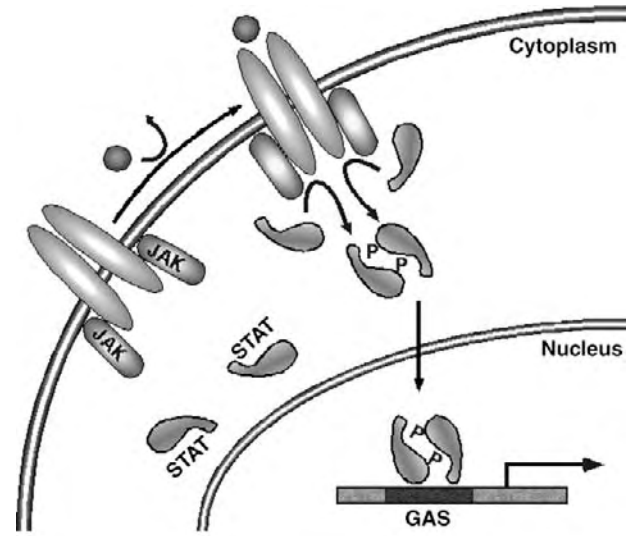


Figure 1 The JAK-STAT signaling paradigm. See text for details.

The JAK Family

Four members of the mammalian JAK family, Jak1, Jak2, Jak3, and Tyk2, are associated with a proline-rich, membrane proximal receptor domain [1]. JAKs range in molecular weight from 120 to 130 kDa. Jak1, Jak2 and Tyk2 are expressed ubiquitously, but the expression of Jak3 is more restricted [3]. Seven conserved JAK homology (JH) domains have been identified. The JH1 and JH2 domains constitute the carboxy-terminal kinase and pseudokinase domains, which distinguish JAKs from most other protein tyrosine kinases [1]. The amino-terminal JAK homology domains, JH3–JH7, constitute a FERM (four-point-one, Ezrin, Radixin, Moesin) domain, which mediates receptor association [2].

Although biochemical studies have implicated differing sets of JAKs for each hematopoietin receptor subgroup, it appears they can be interchanged without affecting signaling specificity [1,2]. Gene targeting studies have demonstrated that Jak1 plays an important role in the biological response to members of the interleukin (IL)-2, IL-6, and IFN/IL-10 receptor families [12]. Because the Jak1–/– mice exhibit a perinatal lethal phenotype, more complete analysis awaits adoptive transfer and tissue-specific knockout studies. The Jak2 knock-out mice exhibited a midgestational lethal phenotype (i.e., day 12.5), which has been attributed to a block in definitive erythropoiesis [13,14]. *Ex vivo* studies with Jak2–/– fetal liver cells indicate defects in their responses to Tpo, IL-3, members of the IL-2 family, IFN-γ, but not IL-6 or IFN-α. Consistent with biochemical studies demonstrating a robust association of Jak3 with γC [3], both the Jak3–/– and γC[–/–] mice demonstrate severe SCID-like defects in lymphopoiesis [3,15–17]. The phenotype of the Tyk2 knock-out mice is the most surprising of all. In contrast to earlier biochemical and genetic studies, these mice exhibit only relatively modest defects in their response to type I IFNs and IL-10. Defects in their response to IL-12 and LPS are considerably more remarkable [18,19].

The STAT Family

Mammals express seven STAT proteins, which range in size from 750 to 900 amino acids. Both the chromosomal distribution of these STATs and the identification of homologs in more primitive eukaryotes suggest that this family arose from a single primordial gene [2,20]. Duplications of this locus appear to reflect an increasing need for cell-to-cell communication. Homologs, most closely related to Stat3 and Stat5, have been identified in model eukaryotes, including *Dictyostelium*, *Caenorhabditis elegans,* and *Drosophila* [2]. Whereas in *Drosophila* the single STAT transduces signals through a "classical" JAK-STAT pathway, the STAT homologs in *Dictyostelium* and *C. elegans* appear to signal through different pathway(s).

STATs can be divided into five structurally and functionally conserved domains (see Fig. 2; [6,7]). The amino-terminal domain (NH_2; ~125 amino acids) is well conserved and promotes cooperativity in DNA binding [2,21]. This domain has also been implicated in nuclear translocation [2]. The coiled-coil domain (amino acids ~135 to ~315) consists of a four-helix bundle that protrudes laterally (~80 Å) from the core. This domain has been shown to associate with a number of potentially important regulatory modifiers including IRF-9 and StIP [2,4]. It has also been implicated in nuclear export [2]. The DNA-binding domain (DBD; amino acids ~320 to ~480) recognizes the palindromic GAS element, upon dimerization, and may also participate in nuclear export [6–9]. However, the Stat2 DBD, despite its sequence conservation, is unable to bind DNA directly. The linker domain (amino acids ~480 to ~575) structurally translates the dimerization signal to the DNA binding motif. Recent studies suggest it regulates a basal (i.e., in resting cells) nuclear export process [22]. The SH2 domain (amino acids ~575 to ~680) is the most highly conserved motif. It mediates both specific recruitment to the appropriate receptor, as well as STAT dimerization [2]. The tyrosine activation motif represents a conserved tyrosine near residue 700 that is activated by phosphorylation. This phosphotyrosyl residue is specifically recognized by a SH2 domain of the partner STAT [2]. Each STAT, except Stat2, has been shown to homodimerize *in vivo.* The carboxy termini of all STATs vary considerably in both length and sequence. They encode the transcriptional activation domain (TAD), which is conserved between mouse and man for every STAT, except Stat2 [2,23].

Gene targeting studies have provided important insight into the functional specificity of the STATs. Consistent with its identification during the purification of ISGF-3 and an IFN-γ induced factor, Stat1 knock-out mice exhibit defects in their biological response to both type I and type II IFNs, but not other ligands [2,24,25]. Likewise, Stat2 knock-out mice are only defective in their response to type I IFNs [23]. Analysis of these mice has highlighted the role type I IFNs play in regulating both innate and acquired immunity ([23]; E. Cha and C. Schindler, unpublished observation). The Stat3 knock-out mice exhibit an embryonic lethal phenotype, reflecting the pleiotropism of Stat3 activating ligands (see Table I). Tissue-specific Stat3 knock-outs have identified important defects in responses to IL-6, IL-10, KGF, CNTF, and LIF [2]. The diverse role Stat3 plays is reminiscent of the more pervasive role STATs play in primitive metazoans. The Stat4 and Stat6 knock-out mice exhibit specific defects in their response to the cytokines that regulate the polarization of naïve T-helper cells into effector Th1 and Th2 subsets, where IL-12 (as well as closely related IL-23) promotes Th1 cells and IL-4 (as well as closely related IL-13) promotes Th2 cells [26–30]. Finally, the Stat5 knock-out mice have yielded the most unexpected findings [1,31–33]. Stat5a and Stat5b, which are 96% identical, are not completely redundant, as earlier biochemical studies had suggested. Stat5a null mice exhibit a defect in PRL-dependent mammary gland development. In contrast, Stat5b single knock-out mice exhibit defects in their response to GH. The Stat5a/b double knock-out mice have the most severe defects, yet these mice still develop a full complement of hematopoietic lineages. Defects in the response to GH and PRL are striking, while those to IL-2, IL-3, IL-5, IL-7, GM-CSF, G-CSF, and Tpo are more subtle [1,2]. The double knock-out mice have also been found to be defective in TEL/Jak2-dependent leukemogenesis [1].

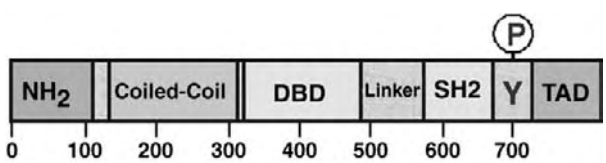


Figure 2 STAT structure. See text for details.

A Promising Future

STATs, which were first identified as IFN-dependent transcription factors, are now known to transduce vital signals for all ~50 hematopoietins (see Table I). Although the basic outline for these JAK-STAT signaling cascades has been established, much work remains to be done in understanding how this pathway is regulated and how it mediates its potent biological effects. Future developments in STAT regulation are likely to include further insight into STAT modifications and signal decay. Likewise, studies on the wide variety of biological responses mediated by STATs are likely to include more precise and comprehensive identification of STAT target genes, as well as a better understanding as to how STATs interact with other transcription factors to achieve desired gene regulation.

The most compelling evidence that STATs are modified at residues in addition to the activation tyrosine comes from work on serine phosphorylation, which appears to enhance transcriptional activity of several STATs [34]. Gene targeting studies, which are under way, are likely to provide valuable insight into this potentially important regulatory loop. Data on other modifications, including acetylation, arginine-methylation, ubiquitination, and potentially SUMO-lation,

are more limited, but promising avenues for future study [2,4,10,35–37].

Studies on STAT signal decay have made considerable progress with the recent characterization of the SOCS family of counter-regulatory proteins. Several SOCS family members are themselves STAT target genes, thereby establishing a classic negative feedback loop [2,38]. Another important focus has been on phosphatases that dephosphorylate receptors, JAKs and STATs, but many remain to be identified [1,2]. Other studies have suggested that some signal decay is achieved by targeting activated STATs for ubiquitin-dependent degradation [10]. In addition, the role that structural changes play in both the activation and decay of STAT signaling has not been explored, but is likely to be important.

Lastly, there is increasing evidence that STATs interact with other transcription factors to regulate the expression of target genes. Although, a detailed characterization of STAT target gene promoters has not been completed, ongoing studies will provide important insight into the nature of these interactions and how they may regulate acetylation, nucleosome phasing, and stimulation of the basal transcriptional machinery [39,40]. Thus, it seems likely that the coming decade will continue to bring important developments in our understanding of how STATs mediate their potent biological responses.

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CHAPTER 283

FOXO Transcription Factors: Key Targets of the PI3K-Akt Pathway That Regulate Cell Proliferation, Survival, and Organismal Aging

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Introduction

The development and integrity of multicellular organisms depend on the ability of each cell to integrate a wide range of external and internal cues and to trigger appropriate cellular responses. In recent years, Forkhead transcription factors of the FOXO subfamily have emerged as key players in controlling cell cycle progression, cell survival, detoxification, and DNA damage repair by integrating extracellular signals and triggering changes in gene expression. At the organismal level, FOXO transcription factors may play a critical role in the control of life span, a function that may be conserved throughout evolution.

Identification of the FOXO Subfamily of Transcription Factors

Forkhead transcription factors all share a conserved DNA-binding domain (DBD) of 100 amino acids that folds into a winged helix structure termed the *forkhead box.* The Forkhead family is evolutionarily conserved from yeast to mammals, and in humans comprises around 30 members that have been divided into 17 subgroups (FOX for "Forkhead Box" A to Q) [1]. FOX transcriptional regulators play a wide range of roles during development, from organogenesis (FOXC) [2] to language and speech acquisition (FOXP) [3].

Among the large Forkhead family, the FOXO subgroup first attracted interest because all three members of this family, namely, FOXO1/FKHR, FOXO3a/FKHRL1, and FOXO4/AFX, were identified at the site of chromosomal translocations in human tumors. The chromosomal translocations involving FOXO family members result in the generation of a chimeric protein in which the transactivation domain of FOXOs is fused to the DBD of another transcription factor, creating a dysregulated and highly active transcriptional fusion protein (Table I) [4–7]. These initial findings suggested that, when mutated, FOXO transcription factors might play a role in tumor development.

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Table I FOXO Family Members

New name	Old name	Chromosomal translocation	Fusion protein	Type of cancer	Expression
FOXO1	FKHR	2–13 1–13	PAX3-FOXO1 PAX7-FOXO1	Alveolar rhabdomyosarcoma	Ubiquitous but high in ovary
FOXO2 FOXO3a	AF6q21 FKHRL1	6–21	MLL-FOXO3a	Acute myeloblastic leukemia	Ubiquitous but high in brain and kidney
FOXO4	AFX	X-11	MLL-FOXO4	Acute myeloblastic leukemia	Placenta and muscle

Regulation of FOXO Transcription Factors by the PI3K-Akt Pathway

In recent years, a series of genetic and biochemical studies revealed that FOXO family members are regulated by the PI3K-Akt pathway in response to growth factor stimulation. Binding of growth factors or insulin to their receptors triggers the activation of the phosphoinositide kinase (PI3K), which in turn is responsible for the activation of several serine/threonine kinases, including Akt or SGK (serum glucocorticoid inducible kinase) [8]. The PI3K-Akt pathway is conserved in the nematode *Caenorhabditis elegans*, where this pathway is activated by an insulin-like signal [9,10]. Inactivation of the PI3K-Akt pathway during development of the nematode results in arrest in dauer, a long-lived stress-resistant larval stage, whereas in the adult, inactivation of this pathway increases life span by two- to threefold. Strikingly, two independently conducted genetic screens found that all suppressor alleles of the PI3K-Akt pathway mutants map to the gene encoding a FOXO transcription factor termed DAF-16 [11,12]. These results indicate that DAF-16 is a key target of the PI3K-Akt pathway.

Several studies conducted in mammalian cells provide a molecular mechanism that appears to explain the genetic link between the PI3K-Akt pathway and FOXOs. These studies show that the protein kinase Akt phosphorylates all FOXO family members at three key regulatory sites (Thr32, Ser253, and Ser315 for FOXO3a) that are conserved from *C. elegans* to mammals and are part of a perfect consensus sequence for Akt phosphorylation [RXRXX(S/T)] (Fig. 1) [13–17]. Akt is not the only protein kinase that phosphorylates FOXOs at these regulatory sites; SGK also phosphorylates FOXO3a. However, the efficacy with which SGK and Akt phosphorylate the three phosphorylation sites of FOXO transcription factors. Whereas Thr32 is phosphorylated by both kinases, Akt preferentially phosphorylates Ser253, and SGK favors the phosphorylation of Ser315 [18].

The three FOXO regulatory sites are phosphorylated in response to a wide range of stimuli that activate Akt and SGK, including insulin-like growth factor 1 (IGF-1) [13], insulin [19], interleukin 3 [20], erythropoietin [21], epidermal growth factor [22], and transforming growth factor β [23]. The phosphorylation of FOXO transcription factors by Akt and SGK in response to growth factors triggers a change in the subcellular localization of FOXOs [13,14] (Fig. 2). In the absence of growth factors, when Akt and SGK are inactive, FOXOs are localized within the nucleus. When cells are exposed to growth factors, the PI3K-Akt/SGK cascade is activated and leads to the relocalization of FOXOs from the nucleus to the cytoplasm, away from FOXO target genes (Fig. 2). Mutation analyses of the three regulatory sites have shown that the phosphorylation of each site contributes to the relocalization of FOXOs from the nucleus to the cytoplasm [18]. This apparent redundancy of phosphorylation may represent a way of modulating the extent of the relocalization of FOXOs to the cytoplasm in different cell types or in response to different combinations of signals.

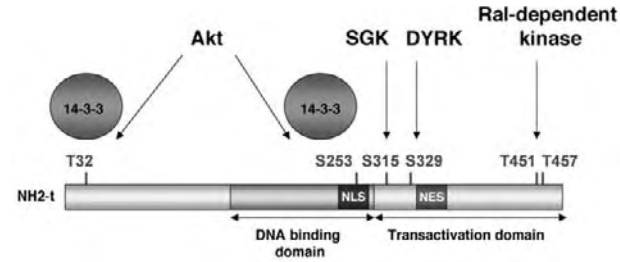


Figure 1 Domain structure and phosphorylation sites within FOXO family members. Numbering for FOXO3a: T32, S253, S315; numbering for FOXO1, S329; numbering for FOXO4: T451, T457.

Other Regulatory Phosphorylation Sites in FOXOs

In addition to the sites on FOXOs that are phosphorylated in response to growth factors, several other sites of phosphorylation have recently been identified (Fig. 1). FOXO4 (AFX) is phosphorylated at two residues, Thr 447 and Thr 451, in response to the activation of the small G protein termed Ral, although the kinase directly responsible for the phosphorylation of these two threonines is not yet known [24]. Phosphorylation of Thr 447 and Thr 451 does not affect the subcellular localization of FOXO4, but instead appears to abolish FOXO4 transcriptional activity. Another study has reported that the MAP kinase family member DYRK catalyzes the phosphorylation of Ser 329 in FOXO1, a residue that is conserved in all FOXO family members [25]. However, the phosphorylation of Ser 329 is not modulated by growth factors and its role in the regulation of FOXO function is still unclear.

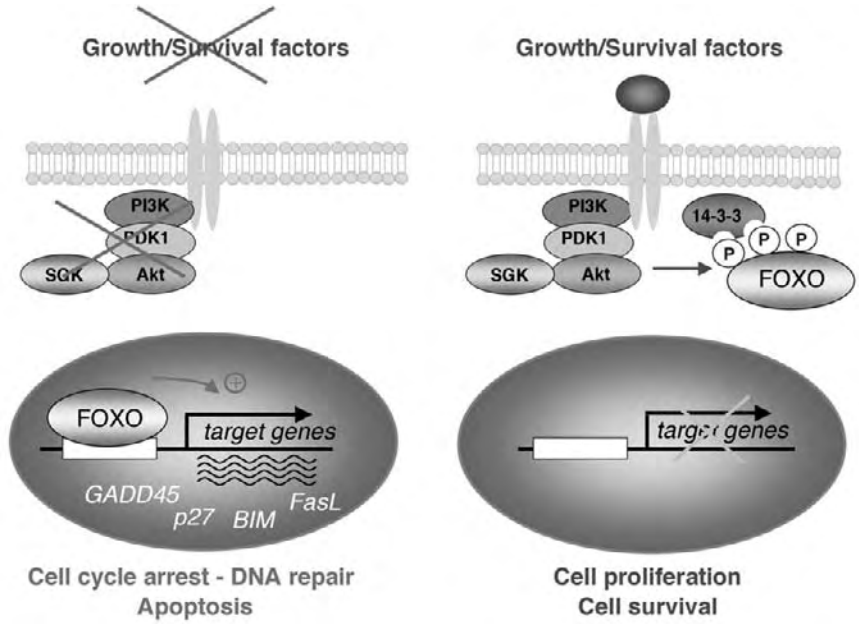


Figure 2 Regulation of FOXO family members by growth and survival factors. In the absence of growth/survival factors, FOXO family members induce the expression of death genes, cell cycle arrest genes or stress response genes, including Fas ligand, the proapoptotic Bcl-2 family member BIM, the cell cycle inhibitor p27KIP1, and the growth arrest and DNA damage response gene GADD45. In the presence of growth/survival factors, the PI3K-Akt/SGK pathway is activated. Akt and SGK promote survival and cell cycle progression by phosphorylating and inhibiting FOXOs.

Mechanism of the Exclusion of FOXOs from the Nucleus in Response to Growth Factor Stimulation

The translocation of FOXOs from the nucleus to the cytoplasm appears to be the primary mechanism of regulation of these transcription factors in response to growth factors. Experiments using leptomycin B, a specific inhibitor of nuclear export, show that the translocation of FOXO transcription factors from the nucleus to the cytoplasm is mediated by a nuclear export signal (NES)-dependent mechanism [14,26,27]. Mutation analyses have revealed that one (FOXO1) or two (FOXO3a) leucine-rich domains in the conserved C-terminal region of FOXOs function as NES [14,26,27] (Fig. 1). Furthermore, the phosphorylated forms of FOXOs have been shown to specifically interact with 14-3-3 proteins [13], which appear to serve as chaperone molecules. The phosphorylation of the first FOXO regulatory site (Thr 32 in FOXO3a) creates a perfect 14-3-3 binding site, and the phosphorylation of the second FOXO regulatory site (Ser 253 in FOXO3a)—although it does not create a canonical 14-3-3 binding site—also participates in 14-3-3 binding [13]. Several mechanisms have been proposed to explain how 14-3-3 binding to FOXOs may promote the relocalization of FOXOs from the nucleus to the cytoplasm. One study suggests that 14-3-3 binding decreases the ability of FOXOs to bind DNA, releasing FOXOs from a nuclear DNA anchor and allowing the relocalization of FOXOs to the cytoplasm [28]. A second study suggests that 14-3-3 binding to FOXOs occurs within the nucleus and actively promotes the nuclear export of FOXOs, perhaps by inducing a conformational change in FOXOs that would expose the NES and allow interaction with Exportin/Crm1 [27].

Finally 14-3-3 binding to FOXOs has been shown to prevent the nuclear reimport of FOXOs by masking FOXOs' nuclear localization signal (NLS) [26,29], consistent with a known function of 14-3-3 in regulating the subcellular localization of several of its other binding partners. These various mechanisms for regulating the translocation of FOXOs from the nucleus to the cytoplasm may serve as a fail-safe mechanism to ensure a complete sequestration of FOXOs in the cytoplasm, away from FOXO target genes in the nucleus. The sequestration of a transcription factor in the cytoplasm upon phosphorylation is a mechanism by which the activity of a variety of transcriptional regulators, including NFAT [30,31] and the yeast transcription factor Pho4 [32], are controlled, and this mechanism may have been selected by evolution because it represents an efficient way to inhibit the function of a transcription factor.

Transcriptional Activator Properties of FOXOs

Like other FOX family members, FOXOs bind to DNA as a monomer via the Forkhead box, a 100-amino-acid region located in the central part of the molecule (Fig. 1). The NMR structure of the DBD of FOXO4 has been solved and shows that this domain adopts a winged helix structure similar to those of the other FOX family members that have been solved, FOXA (HNF3 γ) and FOXD (Genesis) [33]. The consensus

recognition site for FOXOs on DNA has been determined by three independent groups [34–36] and the core motif, GTAAA(C/T)A, is very similar to that of other Forkhead transcription factors [37]. Both more extensive DNA sequence motifs in promoters of FOXO target genes and regions of FOXO proteins outside the Forkhead box are likely involved in conferring binding specificity to particular FOXO family members under different conditions of stimulation.

When present in the nucleus and bound to DNA, FOXOs act as potent activators of transcription. The phosphorylation of FOXOs by Akt or SGK appears to regulate the transcriptional activity of FOXOs primarily by excluding this transcription factor from the nucleus [13,15,17,18,38]. When the phosphorylated form of FOXOs is artificially maintained within the nucleus either by leptomycin B treatment or by mutation of the FOXOs NES, FOXOs are transcriptionally active even in the presence of growth factors, indicating that the main function of phosphorylation at the growth factor-regulated sites is to relocalize FOXOs to the cytoplasm rather than to directly affect the DNA binding or transcriptional activity of FOXOs [26]. Deletion analyses have revealed that the transactivation domain of FOXOs spans the C-terminal region of the molecule (Fig. 1). In the chimeric protein PAX3-FOXO1 present in some human rhabdomyosarcomas, it is the transactivation domain of FOXO1 that is fused to PAX3 and that confers potent transactivation properties to PAX3 [39]. FOXOs' ability to bind to the transcriptional coactivator CBP (CREB binding protein) may provide a connection with the basal transcriptional machinery resulting in FOXO/CBP-dependent changes in target gene expression [40].

FOXOs and the Regulation of Apoptosis

The expression of constitutively active mutants of FOXOs triggers cell death in several different cell types, ranging from primary neurons to lymphocytes [13,17,41–43]. FOXO-induced apoptosis is dependent on the ability of FOXOs to bind to DNA and to induce transcription, indicating that a subset of target genes that FOXOs transactivate consists of death genes (Table II). Thus, one way in which Akt and SGK promote cell survival is by sequestering FOXOs away from death genes. Several death genes contain FOXO binding sites in their promoters, including the genes encoding for death cytokines (Fas ligand, TNF α, CD30 ligand) or death cytokine receptors (Fas, TNFR). In particular, FOXO3a has been shown to bind to and activate the promoter for the Fas ligand gene [13,44]. Consistent with the hypothesis that FOXOs regulate Fas ligand transcription, the inactivation of Akt has recently been shown to induce Fas ligand expression [45]. Because FOXO3a-induced apoptosis is reduced in cells in which Fas ligand signaling is blocked [13], Fas ligand may relay in a paracrine manner the effects of FOXOs

Table II FOXO Target Genes

Genes	Function	Gene induction	Binding to promoter	Promoter activation	FOXO binding sites
Cell Death					
Fas ligand	Death cytokine	ICC	EMSA; ChIP	+	+
Bim	BH3-only Bcl-2 family member	NB; WB			
bNIP3	BH3-only Bcl-2 family member	Microarray			
Legumain	Cysteine protease	Microarray			+
Bcl-6	Transcriptional repressor	Microarray; NB	EMSA	+	+
Cell Cycle					
p27KIP1	Cdk inhibitor	WB; NB		+	+
WIP1	Phosphatase	Microarray			+
EXT1	Tumor suppressor	Microarray			+
Cyclin G_2	G_2 delay	Microarray			+
Polo-like kinase (PLK)	Mitosis control	WB	ChIP	+	+
Cyclin B	Cdc2 activator	WB	ChIP	+	+
Antioxidant					
Selenoprotein P	Detoxification of ROS	Microarray; NB			+
SOD3		NB			+
DNA Repair					
GADD45	Growth arrest and	Microarray; NB; WB		+	+
PA26	DNA damage response	Microarray; NB			+
Metabolism					
Glucose-6-phosphatase	Glucose metabolism	NB	EMSA	+	+
Phosphoenolpyruvate carboxykinase	Glucose metabolism	NB		+	
IGF-BP1	Regulation of IGF-I levels	NB	EMSA	+	+

Key: ICC, immunocytochemistry; NB, Northern blot; WB, Western blot; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation.

by triggering cellular pathways that culminate in apoptosis. FOXOs also appear to control the expression of several members of the Bcl-2 family, which are known to play a critical role in the balance between cell survival and cell death. FOXO3a induces the expression of Bim, a potent prodeath Bcl-2 family member, and apoptosis induced by the inactivation of the PI3K-Akt/SGK pathway is reduced in lymphocytes from Bim-deficient mice, indicating that Bim may be an important target gene of FOXO3a that mediates cell death [43,46]. In addition, FOXO4 indirectly downregulates the expression of the prosurvival Bcl-2 family member Bcl-xL via the induction of a transcriptional repressor Bcl-6 [47]. Thus, one way in which FOXOs appear to trigger apoptosis is by modulating the ratio of prodeath and prosurvival members of the Bcl-2 family.

FOXOs Are Key Regulators of Several Phases of the Cell Cycle

In cycling cells such as fibroblasts, the main effect of the expression of FOXO family members is not apoptosis but cell cycle arrest at the G_1/S boundary [48]. One target gene that mediates FOXO-induced cell cycle arrest is the Cdk inhibitor p27KIP1, which inhibits Cdk2 activity, thereby blocking cell cycle progression at the G_1/S transition [20,48,49] (Table II). FOXOs induce p27 promoter activity, and FOXOs' ability to induce G_1 arrest is diminished in p27-deficient fibroblasts, suggesting that p27 is a critical FOXO target that mediates G_1 arrest [48]. The FOXO-mediated arrest at the G_1/S transition may be a critical first step in the differentiation process because transgenic mice expressing a dominant-negative form of FOXO1 have impaired T-cell differentiation [36].

Endogenous FOXO3a is localized to the nucleus in cells passing through the G_2 phase of the cell cycle, suggesting a role for FOXO3a at the G_2/M checkpoint [50]. Cells in which FOXO3a is activated in the S phase display a delay in their progression through the G_2 phase of the cell cycle. Microarray analyses led to the identification of several FOXO3a target genes that may mediate FOXOs' effect at the G_2/M boundary (Table II) [50]. As with the G_1/S checkpoint, the G_2/M checkpoint is critical in the cellular response to stress, in part to allow time for detoxification and the repair of damaged DNA. Indeed, when active, FOXO3a induces DNA repair [50]. One of the genes that may mediate both FOXO3a-induced arrest at the G_2/M checkpoint and DNA repair is the growth arrest and DNA damage response gene 45 (GADD45) since the GADD45 promoter is activated by FOXO3a, and FOXO3a-induced DNA repair is diminished in GADD45-deficient fibroblasts [50].

FOXO3a also promotes the exit from the M phase and allows the transition to the following G_1 phase [44]. FOXO3a binds to and induces the promoter of two genes that play a critical role in the exit from M phase, cyclin B and polo-like kinase (PLK) (Table II) [44]. These findings are consistent with the observation that the activation of PI3K and Akt prevents the completion of the M phase [44]. As the two yeast Forkhead transcription factors Fkh1 and Fkh2 have been found to play a role in the completion of M phase [51], this function of FOXOs may have been conserved throughout evolution.

How can FOXOs induce both an arrest in G_1 and G_2 and promote the exit from the M phase? One possible explanation is that during normal cell cycles, FOXOs would be recruited to promoters of genes that promote the exit from the M phase. In contrast, under conditions of stress or absence of growth factors, FOXOs may be recruited to promoters of genes controlling cell cycle arrest at the G_1/S and G_2/M checkpoints, allowing repair of damaged DNA. Thus, FOXOs may integrate different extracellular cues, possibly by undergoing various posttranslational modifications, and elicit the appropriate cell cycle response by triggering a specific array of target genes.

FOXOs in Cancer Development: Potential Tumor Suppressors

In mammals, FOXOs' ability to induce a G_1 arrest, a G_2 delay, DNA repair, and apoptosis makes it an attractive candidate as a tumor suppressor. Loss of FOXO function may lead to a decreased ability to induce cell cycle arrest, leading to tumor development. A decreased ability to repair damaged DNA due to the absence of FOXOs may result in genomic instability. Finally, in the absence of FOXOs, abnormal cells that would normally die may instead survive, resulting in tumor formation. FOXOs' ability to induce cell cycle arrest, DNA repair, and apoptosis are reminiscent of the functions of the tumor suppressor protein p53. In that respect, it is interesting to note that genes such as GADD45, WIP1, and PA26 (Table II) that are induced in response to FOXOs have also been found to be regulated by p53 [52–54]. These observations raise the possibility that FOXOs and p53 may under some circumstances function in a cooperative manner.

One further link between the FOXO family and cancer is that all FOXO members had been initially characterized because of their presence at chromosomal breakpoints in cells from human tumors (Table I). However the expression of these human tumor fusion proteins in transgenic mice is not sufficient to promote cancer [55]. This finding suggests that the human tumors may have arisen not only as a result of the chimeric molecules but perhaps also as a consequence of the loss of one FOXO allele. If FOXOs function as tumor suppressors, then the haploinsufficiency of one FOXO family member could be a contributing factor to the development of the tumor.

Role of FOXOs in the Response to Stress and Organismal Aging

All mutants of the nematode that lead to the activation of the FOXO transcription factor DAF-16 are not only long lived but also display resistance to oxidative stress, heat

shock, and UV [9,10,56,57]. This observation suggests that one way in which DAF-16 activity may lead to an increase in organismal life span is by augmenting the resistance of cells to various stresses. In the nematode, expression of the manganese-dependent superoxide dismutase SOD3 is induced when DAF-16 is active [58]. The promoter of SOD3 contains a Forkhead binding site that is in a region conserved across species, suggesting that SOD may be a conserved target of FOXOs that detoxify cells of reactive oxygen species (ROS), thereby conferring resistance to oxidative stress [34]. Another gene that is induced when DAF-16 is active and contains FOXO binding sites in its promoter is the tyrosine kinase receptor OLD-1, which is distantly related to the mammalian PDGF receptor. Expression of OLD-1 increases nematode longevity in part by increasing the resistance to oxidative stress [59].

In mammals, FOXOs induce cell cycle arrest at two critical checkpoints that allow repair of damaged DNA [48,50]. An organism's ability to respond to stress and in particular to induce detoxification and repair damaged DNA, has been shown to correlate with an increased longevity in many organisms [60]. The GADD45 gene, which in part mediates FOXO-induced arrest and DNA repair [50], is up-regulated in old mice [61]. These correlative evidence suggest that this pathway may be up-regulated to protect against oxidative stress produced by metabolism as an organism ages. Thus, by up-regulating a program of gene expression that protects cells against both internal and external cellular stresses, FOXO transcriptional regulators might promote longevity in mammals as well as in *C. elegans.*

Mosaic analyses in *C. elegans* indicate that the longevity phenotype of the nematode mutants that leads to DAF-16 activation is noncell autonomous [62]. In addition, neurons are the cell types that appear to be critical regulators of the aging phenotype in the nematode [63]. These findings, combined with the indications that DAF-16 may protect cells against stress, raise the possibility that a subset of neurons in the central nervous system may represent a "longevity control center" that might be more sensitive than other organs to oxidative stress [63]. It is tempting to speculate that factor(s) released from these neurons would convey the life span signal to the whole organism. It will be interesting to determine if a central neuronal regulator of life span also operates in mammals.

FOXOs and the Regulation of Metabolism in Relation to Organismal Aging

Reduction in nutrient intake consistently correlates with an increase in life span from *C. elegans* to mammals [60]. In the nematode, nutrient deprivation induces the relocalization of the DAF-16 to the nucleus [56], suggesting that one way starvation may result in increased life span is by relocalizing FOXO transcription factors to the nucleus where they may in turn induce target genes that lead to the cellular resistance to stress.

In addition, FOXOs appear to themselves regulate target genes that are involved in the control of metabolism (Table II). FOXO1 induces the expression of glucose-6-phosphatase, a gene that catalyzes the hydrolysis of glucose-6-phosphate into glucose [64,65]. FOXO1 also regulates the gene encoding phosphoenolpyruvate carboxykinase (PEPCK), which mediates the conversion of pyruvate to glucose, although the mechanism by which FOXO1 up-regulates PEPCK expression is probably not via direct binding of FOXO1 to the promoter of the PEPCK gene [66,67]. The regulation of glucose metabolism by FOXOs may be one indirect mechanism by which FOXOs increase organismal longevity. Indeed, switching the cell metabolism toward glucose production and away from the oxidative phosphorylation at the mitochondria may decrease the production of ROS by this organelle. Thus, FOXOs may extend organismal life span both by decreasing the production of ROS and by increasing detoxification and repair of DNA damage caused by ROS.

Conclusion

A series of recent studies have shown that FOXO transcription factors are regulated in a conserved manner throughout evolution by the PI3K-Akt pathway. In mammalian cells, FOXOs' functions include cell cycle regulation at various key checkpoints, apoptosis, repair of damaged DNA, and the regulation of glucose metabolism. How can the same transcription factor induce such a range of biological responses, responses that appear in some cases even to be antagonistic? Depending on their posttranslational modifications or interaction with protein partners, FOXOs may be recruited to particular subsets of promoters. It is possible that FOXOs may act as rheostats depending on the stress level: In low stress conditions, FOXOs may promote cell cycle arrest and DNA repair, whereas in high levels of stress, FOXOs may induce apoptosis. This graded response to stress stimuli would protect cells from damage, but also facilitate the elimination of terminally damaged cells. The result would be a suppression of tumor formation or an increase in the life span of the organism.

Understanding the molecular mechanisms that generate specificity among the FOXO family members will help to define the role of this family in various cellular responses. In addition, the generation of mice models in which FOXO family members are either inactivated or constitutively activated will help to uncover the respective contribution of the FOXO family members to tumor development and the control of life span in mammals.

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CHAPTER 284

Multiple Signaling Routes to Histone Phosphorylation

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Introduction

The eukaryotic genome is organized in a nucleoprotein structure that enables essential biological processes such as regulation of transcription, DNA repair, apoptosis, and cell division [1–3]. A number of remodeling events lead to dynamic changes in the architecture of chromatin. These appear to be prerequisite steps to evolve from a condensed to a decondensed state, and vice versa, each state being coupled to specific cellular functions. Various mechanisms have been implicated in inducing modifications in chromatin structure, some involving the active participation of ATP-dependent remodeling factors such as Swi/Snf and NURF [4–7]; others implicate enzymatic activities that elicit a number of histone modifications, including acetylation, methylation, and phosphorylation. In the past years evidence has accumulated indicating that these covalent modifications occur on histones H2A, H2B, H3, and H4, all assembled with DNA to form nucleosomes, and on H1, the linker histone. In this chapter, we focus on histone phosphorylation and the influence that phosphorylation may have on other modifications.

Although the nucleosome is a highly compacted structure [8], the N-terminal regions, or tails, of the histones are exposed [9]. Indeed, the N-terminal tails of core histones are not essential for maintaining the integrity of nucleosomes since removal of these tails by trypsin treatment does not diminish nucleosome stability [10,11]. Instead, histone tails are thought to confer secondary and more flexible contacts with DNA that allow for dynamic changes in the accessibility of the underlying genome. In addition, the disposition of the tails renders these domains accessible to modifications that could reversibly modulate chromatin structure [12]. The concept that chromatin modifications had to be reversible in nature was already implicit in some important early observations. For example, specific lysine residues were found to be acetylated and/or methylated on the histone H3 tail [13], and it was proposed that acetylation was a hallmark of active chromatin [14,15]. The cAMP-dependent kinase PKA was found to phosphorylate at least *in vitro* various chromatin substrates [16], and phosphrylation of H3 was thought to promote interactions with other chromatinic proteins [17]. In recent years fundamental advances have been made in the identification and characterization of histone modifications. These have pushed forward a unifying concept: the presence of a *histone code*, established by distinct combinations of histone modifications that can then be translated into different nuclear responses and dictate a particular biological outcome [18]. This model is strongly supported by experimental evidence indicating that posttranslational modifications are involved in gene-specific regulation [19]. Whether histone modifications modulate chromatin structure directly, by altering the contacts between histones and DNA, or indirectly, by presenting a special surface for interaction with other regulatory proteins, is still a subject of investigation.

Histone phosphorylation has been associated with extensive alteration of chromatin structure, and phosphorylation of the five individual histones has been shown. Although we focus here primarily on the role of histone phosphorylation, it is important to note that the central concepts are also applicable to other covalent modifications known to influence histone structure and function.

Histone Phosphorylation and Gene Activation

Phosphorylation of histones occurs by serine-threonine kinases at specific sites on the N-terminal tails. With the

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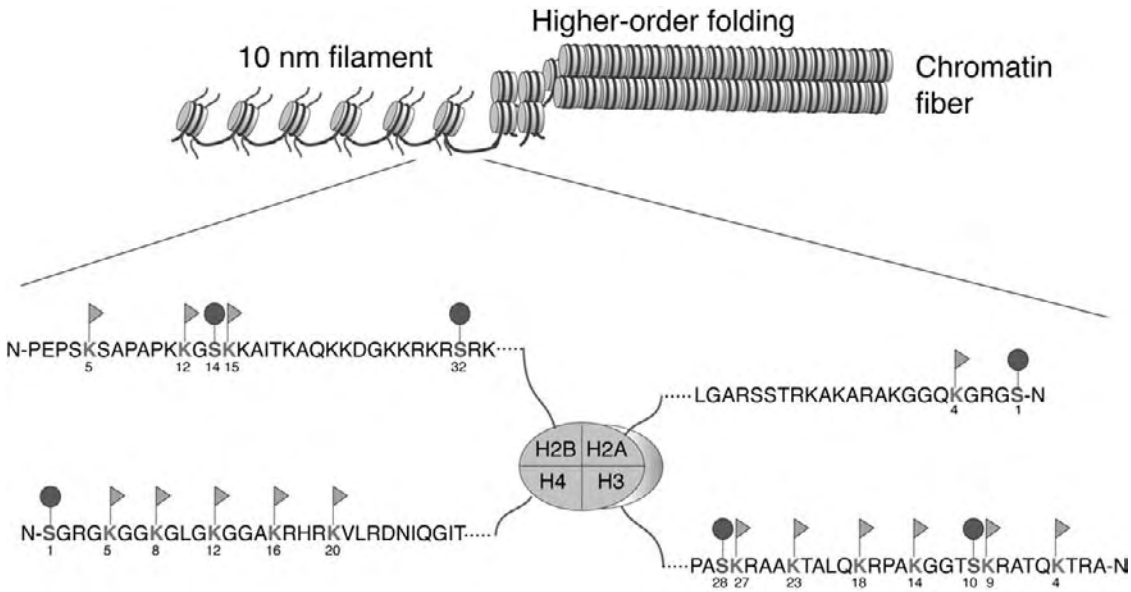


Figure 1 Chromatin organization and amino-terminal tails of core histones. Simplified representation of general chromatin organization. Histone tails (represented in fuchsia) are likely to be exposed or extended outward from chromatin fibers. The amino acid sequence of the N-terminal tails of the four human core histones is represented: red dots indicate known phosphorylation sites; whereas green flags are acetylation sites.

exception of H2A, where only one site has been demonstrated, for each histone tail there are two phosphoacceptor sites: histone H2A is phosphorylated on Ser1 (and possibly on Ser18), H2B on Ser14 and Ser32, H3 on Ser10 and Ser28, and H4 on Ser1 and Ser18. Strikingly, the distance between phosphoacceptor sites is conserved among all histones: It is always 18 residues (Fig. 1). The significance of this constant spacing is unclear, but further supports the above-mentioned hypothesis of the histone code. Of all of these modifications, the best characterized is phosphorylation of H3 on Ser10 (also because of the very successful generation of phosphospecific antibodies directed to the site [20]).

The Remarkable Case of Histone H3

Since the first description of histone H3 phosphorylation upon mitogenic stimulation of quiescent fibroblasts [21], significant evidence has contributed to establishing a direct link between this posttranslational modification and gene activation. In fact, in a variety of cellular systems, it has clearly been shown that a given stimulation causes a rapid and transient induction in Ser10 phosphorylation, with kinetics that are reminiscent of the transcriptional induction of immediate-early genes (IEGs). Importantly, only a subset of histone H3 molecules becomes phosphorylated upon mitogenic stimulation [22], generating a speckled pattern of phospho-H3 immunostaining in the nucleus. The possibility that these speckles correspond to physical sites of active transcription in fascinating. This hypothesis seems supported by chromatin-immunoprecipitation (ChIP) assays demonstrating that the induced expression of a number of IEGs is associated with Ser10 transient phosphorylation [23]. Table I summarizes the described cases of Ser10 H3 transient phosphorylation induced by various treatments and through different signaling pathways.

Although most of our knowledge of histone modifications is based on studies in cell culture systems, an important challenge is to reach a deeper understanding of the role that chromatin modifications play *in vivo* in physiological systems. We have observed that chromatin remodeling occurs in the vertebrate nervous system in response to a photic stimulation in neurons that constitute the mammalian circadian clock (SCN, suprachiasmatic nucleus) and after GABAergic stimulation in the supraoptic nucleus (SON) [24].

In most of the cellular systems analyzed, phosphorylation of Ser10 H3 involves the ERK/MAPK and p38 cascades, as shown by drug sensitivity studies (Fig. 2). The MAPK-activated RSK-2 (ribosomal S6-kinase) kinase has been identified as a candidate for EGF-induced H3 phosphorylation [25]. Interestingly, RSK-2 is also involved in EGF-induced CREB phosphorylation and c-*fos* expression in fibroblasts [26], establishing a direct link between signaling to chromatin and induction of IEGs. Also MSK-1 (mitogen- and stress-activated kinase-1) appears to function as H3 kinase at the same Ser10 site [23,27]. Both MAP and SAP kinase pathways can activate MSK-1; therefore, it is likely that different signaling pathways converge on a single kinase to phosphorylate serine 10 of histone H3. Salvador and collegues [28], on the other hand, have described Ser10 H3 phosphorylation upon stimulation with follicle-stimulating hormone (FSH) in rat ovarian granulosa cells. FSH-stimulated H3 phosphorylation seems correlated with cAMP-dependent protein kinase A (PKA) activation. More recently the first Ser10 histone H3 kinase complex from *Saccharomyces cerevisiae* was

Table I Association of Phosphorylated Histone H3 with Promoters of IEGs in Response to Various Stimuli[a]

Stimulus	Cell type	Activated gene(s)	Signaling cascaede	Ref.
Oncogenic transformation	Mouse fibroblasts	c-*myc* c-*fos*	Ras-MAPK	22
UV	10T1/2 fibroblast	c-*fos* c-*jun*	p38	23
EGF	10T1/2 fibroblast	c-*fos* c-*jun*	ERK/MAPK	24,25
Heat shock	*Drosophila* larvae	Heat shock genes		26
Arsenite	10T1/2 fibroblast	MKP-1		27
FSH	Granulosa cells	Serum glucocorticoid kinase Inhibin α c-*fos*	PKA	28
	Yeast	INO1 ACT1	Snf1/AMPK	29
Inflammatory stimuli (lipopolysaccharide)	Primary human dendridic cells	IL-6 IL-8 IL-12p40 Macrophage chemoattractant protein 1	p38	30
Retinoic acid	P19 cells	Retinoic acid recptor β	Not tested	31
TPA	10T1/2 fibroblast	c-*fos*	ERK/MAPK	32

[a]ChIP assays were performed on different cell types stimulated in various ways, using an antibody that recognizes histone H3 when phosphorylated on serine 10.

purified and identified as the catalytic subunit of Snf1. The Snf1/AMPK kinase phosphorylated Ser10 H3 to enhance the transcription of the INO1 and ACT1 genes [29].

Interestingly, all of the above-mentioned kinases belong to the AGC branch of cyclic nucleotide-regulated protein kinase, underscoring their fundamental role in transducing the signal to the nuclesomes. However, how one single class of kinases activated by separate pathways can elicit different responses remains to be clarified.

Coupling Acetylation and Phosphorylation

How phosphorylation at Ser10 of H3 affects gene expression is unclear. One possibility could be that—similar to what has been proposed for histone acetylation—the addition of negatively charged phosphate groups to the H3 tail may disrupt electrostatic interactions with the negatively charged DNA backbone, thereby increasing the accessibility of nuclear factors to underlying regulatory promoter elements. Another important observation concerns studies that have have shown that H3 phosphorylation and acetylation may be coupled in response to EGF stimulation [30–32]. Indeed, nucleosomes containing phosphorylated and acetylated H3 are preferentially associated with the EGF-activated genes, suggesting that both modifications may operate in concert to elicit induced transcription. Kinetics studies suggest that H3 phosphorylation precedes acetylation in the formation of multiply modified H3 [31].

A mechanistic link between the enzymatic reactions of phosphorylation and acetylation is suggested by the fact that various coactivators involved in signaling-dependent activation of early response genes act as histone acetyltransferases (HATs) and display strong preferences for H3 phosphorylated at serine 10 as substrate [29,31]. Therefore, it appears that a significant interplay exists between the different modifications occuring on the same histone tail.

As mentioned earlier, mitogen-induced H3 phosphorylation is involved in the regulation of only a selected set of genes, hinting at the existence of a highly regulated mechanism that targets the H3 kinase (and probably acetyltransferase) to the appropriate gene loci. These notions indicated that coupling of phosphorylation and acetylation at the level of the histone substrates could possibly be paralleled by a physical and functional interplay of the respective effectors, kinases, and acetylases. At least one case has been described that supports this view and concerns the coactivator CBP (CREB-binding protein), which has HAT activity [33]. In quiescent cells, CBP and RSK-2 proteins are found associated in a complex with low kinase and HAT activities. In serum-starved cells the basal phosphorylation levels of two RSK-2 substrates, CREB and histone H3, are also low. Upon EGF mitogenic stimulation, and consequent activation by phosphorylation of RSK-2 at Ser227, CBP and RSK-2 dissociate. Thus, in contrast to the well-characterized CREB–CBP interaction, where association requires CREB phosphorylation at Ser133, formation of the RSK-2–CBP complex is efficient only when RSK-2 is not phosphorylated. The dissociation results in increased Ser133 CREB and Ser10 H3 phosphorylation. Indeed, following the dissociation, RSK-2 becomes available to phosphorylate CREB in response to EGF. At the same time, CBP

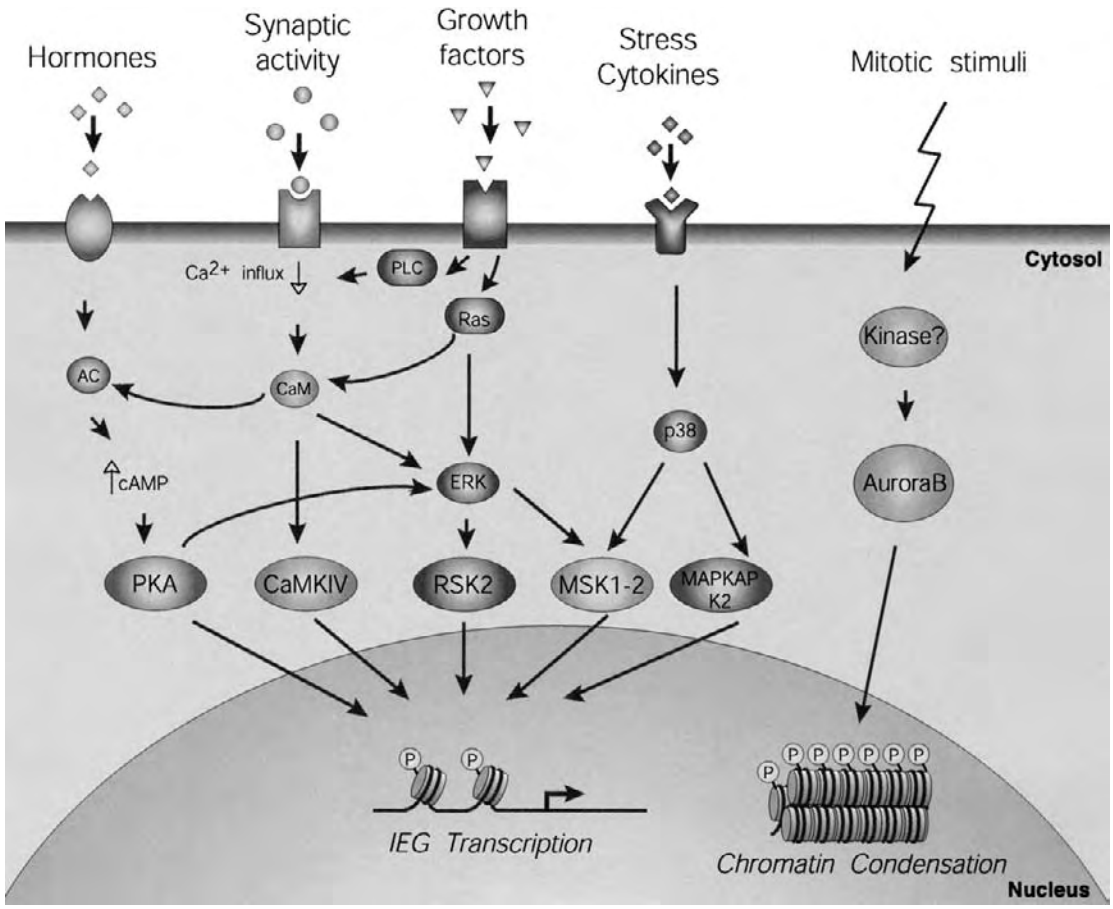


Figure 2 Duality of histone H3 phosphorylation in mammalian cells. A rapid and transient phosphorylation of hitone H3 molecules, located in the nucleosomes of IEG promoters, is achieved after activation of different signaling pathways, upon stimulation of cells with hormones, cytokines, growth factors, or UV light. During mitosis, yet ill-defined cell cycle signals induce a robust histone H3 phosphorylation, which is likely to trigger the chromosome condensation process.

becomes available to interact with the newly phosphorylated CREB. The dynamics of this tripartite regulation fits well the kinetics of early gene transcriptional activation [33].

Histone Phosphorylation and DNA Repair

In addition to transcription regulation, histone phosphorylation is also linked to other cellular processes. For example, several reports have shown that following exposure to various agents that cause double-strand DNA breaks, the histone H2A.X, one of the H2A variants, is rapidly phosphorylated at its C-terminal tail, at serine 139 in mammalian cells [34,35], and at serine 129 in yeast [36]. The number of H2A.X molecules becoming phosphorylated is directly proportional to the amount of double breaks induced and it spans the megabase of DNA around the lesion [34]. H2A.X phosphorylation is not limited to repair of DNA breaks in somatic cells, but it is involved in a number of programmed genomic rearrangements, including recombination in lymphocytes [37,38] and germ cells [39].

Recently mice lacking H2AX have been generated [40]. Mutant mice show pleiotropic phenotypes, including radiation sensitivity, growth retardation, immune deficiency, and male infertility. Those defects seem to be associated with chromosomal instability, repair defects, and impaired recruitment of many DNA repair factors to irradiation-induced foci, implying that H2AX is critical for facilitating the assembly of specific DNA repair complexes on damaged DNA.

Members of the phosphatidylinositol-3-OH kinase-related kinase (PIKK) family have been largely implicated in the response of mammalian cells to double-strand breaks [41], thus making them ideal candidates for the kinase responsible for H2A.X phosphorylation. Although PIKK family members are able to phosphorylate H2A.X *in vitro* and inhibtion of PIKK kinase activity prior to double-strand break abolished H2A.X phosphorylation, repair-deficient cells still display normal phosphorylation of H2A.X, indicating functional redundancy with other as yet ill-defined kinases [42].

Histone Phosphorylation and Apopotis

Chromatin condensation is a morphological hallmark of apoptosis and although commonly used as an indicator of

apoptosis, its biochemical basis remains unclear. Different lines of evidence indicate that histone phosphorylation occurs during programmed cell death, but at present there is little evidence of a mechanistic involvement of histone modification in triggering apoptosis in mammalian cells.

Using different mammalian cell lines and a wide range of apoptosis-inducing agents, H2A.X and H2B have been shown to be phosphorylated during apoptosis at, respectively, serine 139 and serine 32 [35,43]. These phosphorylation events initiate around the time of nucleosomal DNA fragmentation at early stages of apoptosis and seem to be dependent on activation of caspases, suggesting that caspase-induced pathways result in phosphorylation of multiple histone species.

More controversial are the data concerning the phosphorylation of histones H3 and H1 during apoptosis. Phosphorylation of histone H3 at serine 10 and histone H1 was observed upon treatment of rat thymocytes with phosphatase inhibitors [44]. Analogous results were obtained using gliotoxin, but not dexamethasone or thapsigargin [45]. In contrast to these findings, others have reported that apoptotic cells show no H1 and H3 phosphorylation [43,46]. The reasons for this discrepancy are not clear.

An analysis by mass spectrometry of posttranslational modification of histones after induction of apoptosis by okadaic acid (OA), a serine-threonine phosphatase inhibitor, has provided some important information [47]. Indeed, very soon (1 hr) after OA treatment, H2A becomes phosphorylated, whereas H3 and H4 phosphorylation became evident only 18 hr after treatment. Interestingly, H4 phosphorylation was not observed when apoptosis was induced by UV light, indicating that this event it is unlikely to occur as a secondary effect of apoptosis. Therefore, the identities of the residues phosphorylated in association with apoptosis—and the roles these modifications may play—still need to be clarified.

Histone Phosphorylation and Mitosis

Phosphorylation of histones H1 and H3 has been linked to mitotic chromatin condensation [48]. H1 hyperphosphorylation is temporally associated with entry into mitosis and depends on Cdc2 kinase activity [49]. Recent studies, however, have revealed that chromatin condensation can occur without H1 hyperphosphorylation [50] or even without H1 itself [51]. Therefore, the biological significance of mitotic H1 hyperphosphorylation remains undefined.

Histone H3 is phosphorylated during mitosis on at least two serine residues located on the N-terminal tail, serines 10 and 28 (for a review, see [52]). Thus, at least for Ser10, two distinct natures characterize its phosphorylation—one linked to gene transcription and local chromatin decondensation (see earlier section), and one coupled to condensation during mitosis. Whereas mitogenically induced Ser10 phosphorylation concerns only a subset of H3 molecules—generating the nuclear speckles mentioned previously—mitotic phosphorylation appears to involve all H3 proteins.

Phosphorylation at serine 10 begins in early G_2 in the pericentrometric heterochromatin of each chromosome and by metaphase spreads throughout all chromosomes, whereas phosphorylation on serine 28 starts to be evident only in early mitosis. The different timing of serine 10 and 28 phosphorylation suggests that diverse signaling routes are utilized for the two sites. Mitotic H3 phosphorylation at serine 10 is required for proper execution of mitosis in *Tetrahymena*, whereas *S. cerevisiae* strains bearing serine>alanine mutations at positions 10 and/or 28 in the H3 tail have no apparent mitotic defects [53]. This may indicate redundancy with modifications on other histones in some specific systems versus others. It has been proposed that H2B phosphorylation can compensate for loss of serine 10 of histone H3 in yeast [53,54]. Moreover, there is evidence for histone H2B as essential for chromosome condensation in *Xenopus* egg extracts [55]. Indeed, phosphorylation on H3 serine 10 appears to be involved in the initiation, but the maintenance, of mammalian chromosome condensation [56]. Additional evidence indicates that the N-terminal tail of histone H3 is able to stably bind its mitotic kinase, inhibiting chromatin condensation in an *in vitro* system [57]. In this study an H3 kinase activity could be sequestered *in vitro* by nucleosome complexes in peptide competition experiments.

Solid evidence has accumulated showing that in yeast, *Drosophila*, nematodes, *Xenopus* and mammals [53,58–61] members of the Aurora kinase family are responsible for mitotic phosphorylation at Ser 10 of histone H3. These findings have not been confirmed in *Aspergillus*, where NIMA was found to act as H3 kinase [62].

The role of Aurora kinases in phosphorylating H3 has also been explored by different approaches. RNAi experiments in *Drosophilla* and *Coenirhabcitis elegans* indicate that targeting Aurora-B causes a reduction of histone H3 phosphorylation during mitosis [36,49], whereas at least in *C. elegans* Aurora-A does not appear to be implicated [49]. In *Xenopus*, both Aurora-A [59] and Aurora-B [58] were identified as potential histone H3 mitotic kinases. In mammals both Aurora-A and Aurora-B can directly phosphorylate H3 Ser 10 *in vitro* and *in vivo*. However, the spatiotemporal expression of Aurora-A and Aurora-B during the mitotic cell cycle of mammalian cells shows that the two kinases have a mutually exclusive localization: While Aurora-A is present in the centromeres, Aurora-B colocalizes with the phosphorylated form of histone H3. These results strongly implicate Aurora-B as the mitotic kinase for H3 (Fig. 2).

Conclusions

This review focused on a major histone modification, phosphorylation, and on its implication in different cellular process (Fig. 3). Increasing evidence shows that several types of modifications are linked and, in particular, one modification may influence the presence of another nearby modification. This has been demonstrated for acetylation at Ser10 and phosphorylation at Lys14 on the histone H3 tail

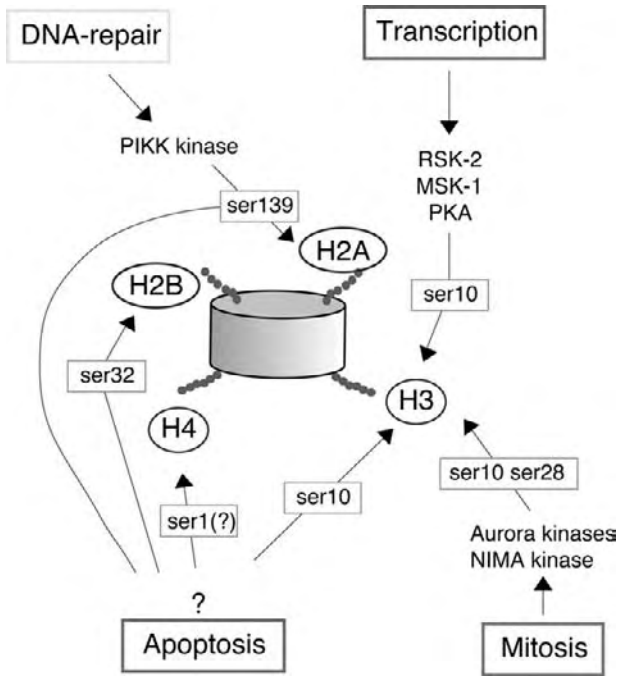


Figure 3 Multiple cellular processes are associated with histone phosphorylation. DNA damage induces phosphorylation in H2A-Ser132; transcription, upon mitogenic stimulation, on H3-Ser10; mitosis on H3-Ser10 and Ser28; apoptosis, depending on the stimulus used, on H4-Serl, H3-Ser10, H2B-Ser32, and H2A-Ser32. The putative kinases responsible for these phosphorylation events are indicated.

(see earlier section) and for phosphorylation at Ser10 and methylation at Lys9 on the same tail [63]. While the first pair modifications have been coupled to activation of gene expression, increasing evidence indicates that methylation at Lys9 results in decreased Ser10 phosphorylation and is thereby responsible for silencing. In addition to an intrahistonic *histone code* operating on the individual tail of a specific histone, interhistone codes may exist. An area of exciting research will be to study the influence that phosphorylation on a specific histone may have on the phosphorylation (or other modifications) on a tail of a different histone. The large number of possible signaling pathways involved in the many physiological scenarios discussed here (Fig. 2) provides a picture in which an impressive number of combinatorial possibilities exist. How can the same histone modification be linked with such a wide range of "chromatin states"? We favor a scenario in which histone tails function to integrate upstream signals that, in turn, impart regulatable dynamic changes to the chromatin structure of localized regions of the genome. Mitogenically induced phosphorylation of histone H3 on serine 10 is thought to cause a reduction of the overall positive charge, inducing a local decondensation. This decondensation event may be crucial for the binding of regulatory factors that could induce either chromatin condensation or transcription. Gaining further understanding of the molecules involved in signaling to chromatin and how phosphorylation can be targeted in a determined locus will be an exciting area for future investigations.

Note added in proofs

The field of histone modifications is moving very fast and some additional references need to be pointed out.

1. Two recent reports indicate that phosphorylation of histone H3 at Ser10 may occur in response to cytokine stimulation by IKK-α. This event was coupled to activation of NF-κB-responsive genes:
   Y. Yamamoto, U. N. Verma, S. Prajapati, Y. T. Kwak, and R. B. Gaynor, *Nature* **423**, 655 (2003); V. Anest, J. L. Hanson, P. C. Cogswell, K. A. Steinbrecher, B. D. Strahl, and A. S. Baldwin, *Nature* **423**, 659 (2003).
2. An histone modification event that is uniquely associated with apoptotic chromatin, in species ranging from frogs to humans, is H2B phosphorylation at position Ser14. The kinase involved in this event was recently identified as Mst1 (mammalian sterile twenty), whose function is directly regulated by caspase-3.
   W. L. Cheung, K. Ajiro, K. Samejima, M. Kloc, P. Cheung, C. A. Mizzen, A. Beeser, L. D. Etkin, J. Chernoff, W. C. Earnshaw, and C. D. Allis, *Cell* **113**, 507 (2003).
3. A case of regulation of inter-histone modifications has been reported, suggestive that several others may exist, all that will contribute to the 'histone code'.
   Sun, Z. W. and Allis, C. D. *Nature* **418**, 104 (2002).

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CHAPTER 285

Multigene Family of Transcription Factor AP-1

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Introduction

Much of our present knowledge about transcription factors comes from the discovery and study of the activating protein 1 (AP-1) family. AP-1 (and the transcription factor NFκB) has served to detect one of the decisive DNA-binding motifs required for regulation of both basal and inducible transcription of several genes containing AP-1 sites (5'-TGAG/CTCA-3′), also known as TPA-responsive elements (TRE), by a variety of extracellular signals. These include growth factors; cytokines; tumor promoters, such as the phorbol ester TPA (12-*O*-tetradecanoyl-phorbol-13-acetate) and carcinogens; UV irradiation; and other DNA-damaging agents. The core of the AP-1 family of transcription factors is formed by heterodimeric associations of members of the Jun (c-Jun, JunB, and JunD), Fos (with c-Fos, FosB, Fra-1, and Fra-2), and ATF (ATFa, ATF-2, and ATF-3) proteins. In this chapter we summarize our current knowledge of (1) the characteristic structural features of AP-1 subunits, (2) mechanisms involved in modulation of AP-1 activity, and (3) function of AP-1 proteins in cellular processes using mouse genetics.

General Structure of AP-1 Subunits

According to their function in controlling gene expression, AP-1 subunits are composed of a region responsible for binding to a specific DNA recognition sequence (DNA-binding domain, DBD) and a second region that is required for transcriptional activation (transactivation domain) once the protein is bound to DNA. The DBD of AP-1 proteins, also known as the *bZip* region, can be divided into two evolutionarily conserved, independently acting domains: the *basic domain* ("b"), which is rich in basic amino acids and responsible for contacting the DNA, and the *leucine zipper* ("Zip") region, which is characterized by heptad repeats of leucines forming a coiled-coil structure that is responsible for dimerization (Fig. 1). In addition to the leucines, other hydrophobic and charged amino acid residues within the leucine zipper region are responsible for specificity and stability of homo- or heterodimer formation between the various Jun, Fos, or ATF proteins. Jun-Jun and Jun-Fos dimers preferentially bind to the 7-base-pair (bp) motif 5′-TGAG/CTCA-3′ whereas Jun-ATF dimers or ATF homodimers prefer to bind to a related, 8-bp consensus sequence 5′-TTACCTCA-3′ [1,2]. Therefore, the characteristics of the AP-1 DNA-binding sites in promoters as well as the abundance of the individual AP-1 subunits are decisive for the selection of target genes.

In addition to the "classical" AP-1 members (Jun, Fos, ATFs), other bZip proteins have been discovered, some of which can heterodimerize with the core AP-1 subunits, for example, Maf, Maf-related proteins, Nrl, Smads, and Jun-dimerizing partners (JDPs). Binding of AP-1 to DNA may also support binding of other transcription factors to adjacent or overlapping binding sites (composite elements) to allow the formation of "quaternary" complexes. The interaction of NF-AT and Ets proteins with the interleukin 2 (IL-2) and collagenase promoters, respectively, may serve as paradigms for this type of protein–protein interaction [3,4]. AP-1 has been reported to associate with NFκB [5], the glucocorticoid receptor [6,7], and hypoxia-inducible factor 1α [8].

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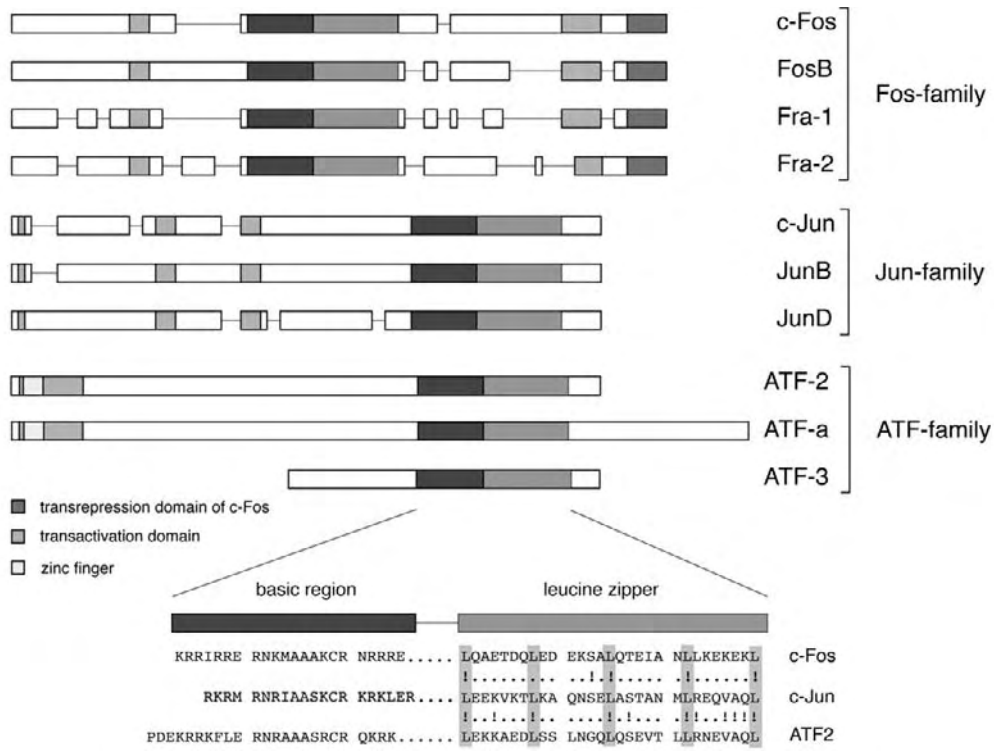


Figure 1 Structural organization of the Fos, Jun, and ATF proteins. Conserved regions are highlighted. On the bottom, the amino acid sequences of the bZip region of c-Fos, c-Jun, and ATF-2, the most extensively analyzed members of the Fos, Jun, and ATF protein families, are shown.

In contrast to the well-defined DBDs of AP-1 proteins, the structural properties of the domains mediating transcriptional activation of target genes (transactivation domain, TAD) are still ill defined. The TAD (and its function) can be transferred to heterologous DBDs that do not require heterodimerization, for example, to that of the yeast transcription factor GAL4. Such chimeric proteins are permitted to identify critical amino acids in TADs and to recognize that the TADs of individual Jun, Fos, and ATF proteins differ in their transactivation potential. Under specific circumstances, some subunits, such as Fra-1 or JunB, may even act as repressors of AP-1 activity by competitive binding to AP-1 sites, or by forming inactive heterodimers with other AP-1 members [9–11].

Transcriptional and Posttranslational Control of AP-1 Activity

Given the aspects of subunit heterogeneity, their dimerization and DNA binding rules, the control of transcription of AP-1 subunit genes, the time course of subunit synthesis, and the regulation of their function become important issues for an evaluation of the effect on target gene expression and on dependent phenotypes. The *jun* and *fos* genes represent the prototype of the class of "immediate-early" genes that are characterized by a rapid and transient activation of transcription in response to changes of environmental conditions, such as growth factors, cytokines, tumor promoters, carcinogens, and expression of certain oncogenes [12]. Because this type of regulation of promoter activity is also observed in the absence of ongoing protein synthesis, it is generally accepted that preexisting factors, whose activity gets altered by changes in posttranslational modification (in particular phosphorylation), are responsible for the regulation of promoter activity.

Most of our current knowledge on transcriptional activation of immediate early genes is derived from studies on deletion and point mutations the *c-fos* and *c-jun* promoters, combined with *in vitro* and *in vivo* footprinting analyses. The serum-response element (SRE), which is bound by a ternary complex containing the transcription factors p67-SRF and p62-TCF, is required for the majority of extracellular stimuli including growth factors and phorbol esters. Changes in the phosphorylation pattern of SRF and, predominantly, TCF regulate *c-fos* promoter activity by these stimuli. Other elements include the c-AMP response element (CRE) and the Sis-inducible enhancer (SIE), which is recognized by the STAT group of transcription factors. The very transient induction of promoter activity by extracellular stimuli can be explained by increased phosphatase activity counteracting the activity of upstream protein kinases, which address promoter-associated transcription factors. On the other hand, negative autoregulation by newly synthesized c-Fos may also play an important role [13,14].

Analysis of deletion mutants of the *c-jun* promoter identified two AP-1-like binding sites (Jun1, Jun2), which are recognized by Jun/ATF heterodimers or ATF homodimers

and which are involved in transcriptional regulation in response to the majority of extracellular stimuli affecting *c-jun* transcription. In response to G-protein-coupled receptor activation, EGF, and other growth factors, the AP-1 sites and an additional element in the *c-jun* promoter recognized by MEF2 proteins cooperate in transcriptional control of the *c-jun* gene. Similar to the factors binding to the *c-fos* promoter, the activity of factors binding to the *c-jun* promoter is regulated by their phosphorylation status (for review, see [12,15]).

The most critical members of the class of protein kinases regulating the activity of AP-1 in response to extracellular stimuli are mitogen-activated protein kinases (MAPKs). Depending on the type of stimuli, these proline-directed kinases can be dissected into three subgroups: the extracellular signal-regulated kinases (Erk-1, Erk-2), which are robustly activated by growth factors and phorbol esters, but only weakly activated by cytokines and cellular stress-inducing stimuli (UV irradiation, chemical carcinogens). In contrast Jun-N-terminal kinases (JNK-1, -2, -3), also known as stress-activated kinases (SAPKs), and a structurally related class, p38 MAP kinases (p38α, -β, -γ), are strongly activated by cytokines and environmental stress, but are poorly activated by growth factors and phorbol ester. These kinases themselves are under strict control of upstream kinases and phosphatases, which are part of individual signaling pathways initiated by specific classes of extra- and intracellular stimuli (growth factors, DNA-damaging agents, oncoproteins). This network, which exhibits a high degree of evolutionary conservation between yeast, *Drosophila*, and mammals is, however, far too complex to be discussed in greater detail in this review (for in-depth information on this subject, see [16,17]).

Erk1 and Erk2 mediate mitogen-stimulated phosphorylation of sites in the TAD of TCF proteins, resulting in enhanced transactivation activity. The JNK/SAPKs were originally identified by their ability to specifically phosphorylate c-Jun at two positive regulatory sites (Ser-63 and Ser-73) residing within the TAD [18]. Hyperphosphorylation of both sites is observed in response to stress stimuli and oncoproteins. The JNKs can also phosphorylate and stimulate the transcriptional activity of ATF-2 [19,20]. The same positive sites on ATF-2 also serve as phosphoacceptor sites for p38, whereas Ser-63 and -73 of c-Jun are not affected by p38 [21,22]. Most likely, hyperphosphorylation of Jun, ATF, and TCF proteins results in a conformational change of the TAD, allowing more efficient interaction with cofactors, which facilitate and stabilize the connection with the RNA polymerase II/initiation complex to enhance transcription of target genes. Interestingly, in addition to enhanced transactivation, hyperphosphorylation of the TAD of c-Jun also regulates the stability of c-Jun by reducing ubiquitin-dependent degradation of c-Jun [23,24]. Similarly, phosphorylation-dependent changes in the half-life of c-Fos have been observed [25].

The DBD of c-Jun is phosphorylated at multiple sites by GSK-3 and/or casein kinase II (CK-II), which results in reduced DNA binding. In response to extracellular stimuli, such as UV, phosphorylation is reduced, thus enhancing DNA binding [26,27]. The mechanism (reduced activity of the kinase or enhanced activity of a phosphatase) has not yet been definitively clarified.

In addition to phosphorylation, other posttranslational modifications regulate AP-1 activity [28]. Oxidation of a cysteine in the basic region inhibits DNA binding and there is pronounced regulation of nuclear localization. Moreover, positive and negative interference of other cellular proteins with AP-1 activity (in addition to the protein kinases and coactivators described earlier) has been identified in, for example, Maf, MyoD, YY1, STAT, SMADs and Menin [2]. The mutual interference between AP-1 and nuclear receptors, particularly the glucocorticoid receptor (GR), which may be involved in the anti-inflammatory and immunosuppressive activities of glucocorticoids, represents the most extensively analyzed example for this type of cross-talk [6,7].

Function of Mammalian AP-1 Subunits: Lessons from Loss-of-Function Approaches in Mice

As described earlier, AP-1 activity is enhanced when cells are stimulated by agents that promote cell proliferation. Moreover, oncogenic versions of c-Jun and c-Fos have been isolated from retroviruses, and various membrane-associated or cytoplasmic oncogenes (Ras, Src, Raf) permanently upregulate AP-1 abundance as part of their transforming capacity, suggesting that AP-1 members play an important role in cell proliferation and transformation [28–30]. Initial evidence for this assumption has been obtained by overexpression studies in mice and various cell lines, by blocking AP-1 activity either through expression of a transdominant-negative c-Jun mutants, by expression of antisense sequences, or by microinjection of Jun- and Fos-specific antibodies. Under these conditions cell cycle progression was disturbed and the efficiency of oncoprotein-mediated cell transformation was reduced. However, already in these systems, different lines of evidence, including variations in the expression pattern and phosphorylation status of AP-1 members during the cell cycle [31,32], suggested that the members of the Jun and Fos families play specific roles during these processes or may even antagonize each other. The generation of mice harboring genetic disruption and/or transgenic overexpression, as well as the availability of genetically defined mutant cells isolated from these animals, represents a major breakthrough in our understanding of the regulatory functions of AP-1 subunits. The distinct phenotypes of the individual knock-out mice (Table I), induced by defects in cells or tissues in which the subunit was particularly important or where its absence became first limiting, support the notion that AP-1 dimers exhibit specific and independent functions *in vivo*. As a general rule derived from all studies, the AP-1 subunits must be present in a complementary and coordinated manner to ensure proper development or physiology of the organism.

Loss of c-Jun, JunB, or Fra-1 results in embryonic lethality. c-Jun null embryos die at midgestation (E12.5 to 13.5 [33,34]) due to dysregulation in liver and heart development [35]. Mice carrying a conditional (floxed) allele of *c-jun*, survive

Table I

AP-1 member	Knockout/Knockin mice	References
c-Jun	Complete loss: embryonic lethal E13, hepatic failure, heart defect	33–35
	Liver-specific loss: viable, impaired postnatal hepatocyte proliferation and liver regeneration	36
junB in *c-jun* locus	Rescue of *c-jun*$^{-/-}$ phenotype by JunB (cardiac and liver defects); rescue of c-Jun/c-Fos-, but not c-Jun/ATF-dependent gene expression	39
JunB	Embryonic lethal E8.5-E10.0, placentation defect, Adult: myeloproliferative disease	40, 41
JunD	Viable, reduced postnatal growth, age-dependent defects in male reproductive function	50
c-Fos	Viable, defective bone remodelling, osteopetrosis, light-induced apoptosis of photoreceptors	43–46
fra-1 in *c-fos* locus	Dose-dependent rescue of the *c-fos*$^{-/-}$ phenotype (bone remodeling, photoreceptor apoptosis)	48
FosB	Viable, nurturing defect	50
Fra-1	Embryonic lethal E10, placenta defect	42
ATF-2	Hypomorth allele: decreased postnatal viability and growth, defects in endochondral ossification, ataxic gait, hyperactivity, decreased hearing, decreased number of Purkinje cells	53
	Complete loss: Neonatal lethality, Meconium aspiration syndrome, decreased cytotrophoblasts in placenta	54

the specific postnatal gene inactivation in hepatocytes but show an impaired liver regeneration in response to partial hepatectomy [36].

Knock-in mice expressing a mutated c-Jun protein in which the N-terminal phosphorylation sites at serines 63 and 73 were changed into alanines (JunAA mice) developed normally and were viable and fertile as adults [37]. However, these JunAA mice are resistant to epileptic seizures and neuronal apoptosis induced by the excitatory amino acid kainate, and JunAA fibroblasts show proliferation defects as well as apoptotic defects on stress induction [37]. By contrast, T-cell proliferation and differentiation appear to be independent of c-Jun N-terminal phosphorylation, whereas efficient T-cell-receptor-induced thymocyte apoptosis is affected [38].

Interestingly, the JunB protein, when expressed at sufficient levels, can substitute at least for some of c-Jun's functions. Knock-in mice having the *c-jun* allele replaced by *junB* undergo normal embryogenesis and develop a normal liver and heart. Analysis of indicative marker genes showed that expression of genes regulated by Jun/Fos, but not those regulated by Jun/ATF dimers, are restored, thereby rescuing c-Jun-dependent defects *in vivo* as well as in primary fibroblasts and fetal hepatoblasts *in vitro* [39].

Targeted inactivation of JunB resulted also in embryonic lethality between days 8.5 and 10.0 due to multiple defects in extraembryonic tissues [40]. Affected cell types/organs comprise the trophoblast giant cells, yolk sac mesenthelium and placental labyrinth. The observed phenotypes in JunB null embryos appear to result from severe impairment of general vasculogenic and angiogenic processes resulting in a failure to establish proper vascular interactions with the maternal circulation. The lethal phenotype can be rescued by an ubiquitously expressed *junB* transgene [40]. In such rescued mice lacking JunB in the myeloid lineage, a transplantable myeloproliferative disease resembling human chronic myeloid leukemia was observed [41].

Fra-1 is so far the only Fos member essential for embryonic development. Fra-1 null embryos died between E10.0 and 10.5 [42]. Similar to JunB null embryos, the vascularization of the placental labyrinth was impaired, suggesting that JunB and Fra-1, possibly as heterodimers, address common target genes responsible for the generation of a functional placental labyrinth. Fra-1-deficient embryos can be rescued from embryonic death by JunB overexpression [42].

Fos-null mice are viable and fertile but suffer from severe osteopetrosis caused by lack of mature osteoclasts [43–45]. Moreover, light-induced apoptosis of photoreceptors is lost in c-Fos-deficient mice [46]. The c-Fos-dependent functions in bone cells can be substituted by Fra-1, when the *fos* locus is deleted and replaced by the *fosl1* gene encoding Fra-1. *Fosl1* is a transcriptional target of c-Fos during osteoclast differentiation [47] and can fully complement for the lack of c-Fos in bone development in a gene-dosage-dependent manner. However, Fra-1 is not able to induce expression of c-Fos target genes in fibroblasts derived from the knock-in mice [48], suggesting the need for additional tissue-specific factors.

The embryonic development of FosB-deficient mice is normal [49]. Adult *fosB*$^{-/-}$ females, however, nurture insufficiently [50]. JunD-null mice develop normally but postnatal growth of homozygous *junD*$^{-/-}$ animals is reduced. JunD null males develop age-dependent defects in reproduction, hormone imbalance, and impaired spermatogenesis [51]. ATF-2 mutant mice that express small amounts of a mutant ATF-2 protein (ATF-2$^{m/m}$; [52]) are chondrodysplastic and neurologically abnormal [53], which might be explained by reduced expression of cyclin D1 in chondrocytes [52]. ATF-2-null mutant mice die shortly after birth and suffer from a disease resembling a severe type of human meconium aspiration syndrome [54].

Knock-out mice represent an excellent system for the isolation of genetically defined mutant cells. Primary and

immortalized fibroblasts can be isolated from almost all mice lacking individual AP-1 members. Analysis of these cells revealed that c-Jun acts as positive regulator of the cell cycle by suppressing p53 and, indirectly, the p53 target gene p21 [55]. Moreover, loss of c-Jun results in reduced cyclin D1 activity [56]. JunB, on the other hand, serves as a negative regulator of cell cycle progression by induction of the cyclin-CDK inhibitor p16 and down-regulation of c-Jun and cyclin D1 expression [32,57]. Data from fibroblasts lacking both c-Fos and FosB established a critical role for these AP-1 subunits in cyclin D expression [58]. Moreover, fibroblasts lacking either *c-jun* or *c-fos* cannot be transformed by oncogenes, such as Ras and Src [59]. JunD-deficient fibroblasts exhibit specific alterations in cell proliferation, depending on p53 and p19-ARF expression [60].

In addition to these cell-autonomous effects, critical and antagonistic functions of c-Jun and JunB in cell proliferation and differentiation *in trans* were observed. In an *in vitro* skin equivalent model system (which mimics many characteristics of cutaneous wound healing) composed of primary keratinocytes and mouse embryonic fibroblasts of wild-type, *c-jun*$^{-/-}$ or *junB*$^{-/-}$ genotype, c-Jun- and JunB-dependent expression of critical cytokines (KGF, GM-CSF) could be demonstrated [61,62].

As described before, AP-1 activity is greatly enhanced upon treatment of cells with genotoxic agents, implying that AP-1 target genes are involved in the cellular "stress response," such as DNA repair, induction of survival functions, or initiation of the apoptotic program. Indeed, analysis of fibroblasts from mice lacking specific AP-1 subunits provided experimental proof for this assumption. Fibroblasts lacking c-Fos are hypersensitive to UV irradiation when compared to wild-type cells, which is caused by a higher rate of apoptosis. In contrast to cells lacking c-Fos, the ability of *c-jun*-deficient fibroblasts to undergo apoptosis is greatly reduced. In contrast, overexpression of c-Jun induced apoptosis in fibroblasts. Reduced apoptosis in response to genotoxic agents was also observed in mice lacking members of the JNK/SAPK family of protein kinases, suggesting that c-Jun and ATF proteins are the major substrates of JNK/SAPKs to mediate the cellular stress response [30,63]. There is evidence that JunD participates in an anti-apoptotic pathway [60], whereas JunB appears to be part of a pro-apoptotic pathway, at least in myeloid cells, through negative regulation of anti-apoptotic genes [41]. However, it is important to note that AP-1 members depending on the cell type and extracellular stimuli may be involved in both apoptotic and anti-apoptotic responses. For example, primary liver cell cultures and erythroblasts derived from *c-jun*$^{-/-}$ embryos exhibit increased apoptotic rates. On the other hand, the lack of c-Fos results in the loss of light-induced apoptosis of photoreceptors in retinal degeneration [30]. Obviously, in addition to the abundance of individual AP-1 subunits and other cellular proteins modulating AP-1 activity on the posttranslational level some of the specificity of the function of a given AP-1 subunit is presumably based on the choice of the heterodimeric partner, dictating sequence specificity and, in turn, the subsets of AP-1 target genes to be addressed [64]. A shift in the equilibrium of gene expression of such distinct classes of c-Jun target genes, in conjunction with alterations in c-Jun-independent pathways, will contribute to the decision of the cell to either proliferate, to activate survival factors or to induce the genetic program of cell death in response to extracellular signals.

Despite the fact that AP-1 has been identified more than a decade ago, it still maintains a lot of its mystery. Further research on tissue-specific inactivation of AP-1 members and the identification of subunit-specific target genes may yield an even more complex picture of function and regulation of AP-1 than exists at present.

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CHAPTER 286

NFκB: A Key Integrator of Cell Signaling

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Eukaryotic cells possess a number of distinct signal transduction pathways that couple environmental stimuli to specific changes in gene expression. One such pathway regulates the transcription factor NFκB, which is known to orchestrate the expression of a diverse array of genes essential in host defense, inflammatory, cell survival, and immune responses. NFκB is activated by a surprisingly broad array of cellular stimuli [1]. Likewise, NFκB has been shown to induce the expression of a large functionally diverse array of genes [2]. The seemingly promiscuous nature of NFκB biology raises the question as to how NFκB proteins elicit a specific transcriptional program in response to a given environmental challenge. The discovery of several key regulatory proteins within this pathway has provided insight into the mechanism by which the NFκB achieves specific coupling of these distinct cellular processes. As would be predicted of such a central signaling pathway, aberrant regulation of NFκB activation has been associated with the pathogenesis of several diseases, including autoimmunity, arthritis, asthma, and cancer [3]. This chapter summarizes the mechanisms of NFκB regulation and discusses emerging opportunities for target-based therapeutic intervention in various disease settings.

NFκB was originally identified as a transcription factor required for B-cell-specific gene expression [4]. However, it was quickly recognized that NFκB activity could be induced in most cell types in response to myriad stimuli, including proinflammatory cytokines, bacterial lipopolysaccharides, viral infection, DNA damage, oxidative stress, and chemotherapeutic agents (Fig. 1) [1]. The classic experiment by Baeuerle and Baltimore, which demonstrated that NFκB exists in latent form in the cytosol of unstimulated cells and undergoes rapid translocation to the nucleus upon stimulation, set the stage for what is now the hallmark of NFκB regulation [5]. This paradigm provides a mechanism for NFκB to undergo rapid induction in response to cellular stress, resulting in the up-regulation of NFκB target genes. A wide variety of genes are regulated by NFκB, including those encoding cytokines, chemokines, adhesion molecules, acute phase proteins, inducible effector enzymes, antimicrobial peptides, adaptive immune response, regulators of apoptosis, and cell proliferation (Fig. 1). Remarkably, specific subsets of NFκB responsive genes can be activated in a cell- and stimulus-specific fashion. Biochemical and genetic characterization of molecular components that impinge on the NFκB signaling pathway has greatly facilitated our understanding of this process.

NFκB exists as a multigene family of proteins that can form stable homo- and heterodimeric complexes that vary in their DNA-binding specificity and transcriptional activation potential. Five proteins belonging to the NFκB family have been identified in mammalian cells: RelA (p65), c-Rel, RelB, NFκB1 (p50 and its precursor p105), and NFκB2 (p52 and its precursor p100) (Fig. 2) [1]. NFκB/Rel proteins share a highly conserved 300-amino-acid N-terminal Rel homology domain (RHD) responsible for DNA binding, dimerization, and association with the IκB inhibitory proteins. The prototype NFκB complex is comprised of p50 and p65, but a variety of NFκB/Rel-containing dimers are also known to exist. The p50/p65 complex displays strong transcriptional activation, whereas the p50/p50 and p52/p52 homodimers function to repress transcription of NFκB target genes [1]. Thus, the existence of a multigene family provides one tier of regulation by which the cell can fine-tune NFκB-mediated gene expression. Moreover, knock-out mice lacking distinct NFκB subunits display distinct phenotypes, further implicating

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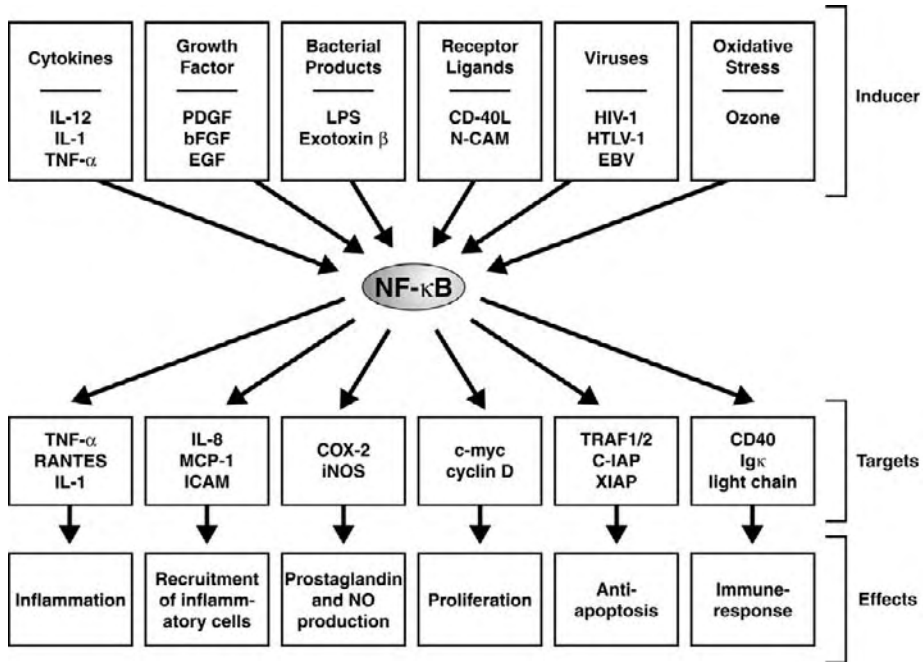


Figure 1 NFκB plays a central role in gene regulation: Stimulus-dependent activation of NFκB results in the modulation of specific subsets of NFκB target genes that are involved in distinct cellular processes. IL, interleukin; TNF, tumor necrosis factor; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; LPS, lipopolysaccharide; CD-40L, CD 40 ligand; N-CAM, neural cell adhesion molecule; HIV, human immunodeficiency virus; HTLV, human T-cell leukemia/lymphoma virus; EBV, Epstein-Barr virus; RANTES, regulated on activation, normal T cells expressed and secreted; MCP, monocyte chemoattractant protein; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthetase; TRAF1/2, TNF receptor associated factor; c-IAP, cellular inhibitors of apoptosis protein; XIAP, X-chromosome-linked inhibitor of apoptosis protein.

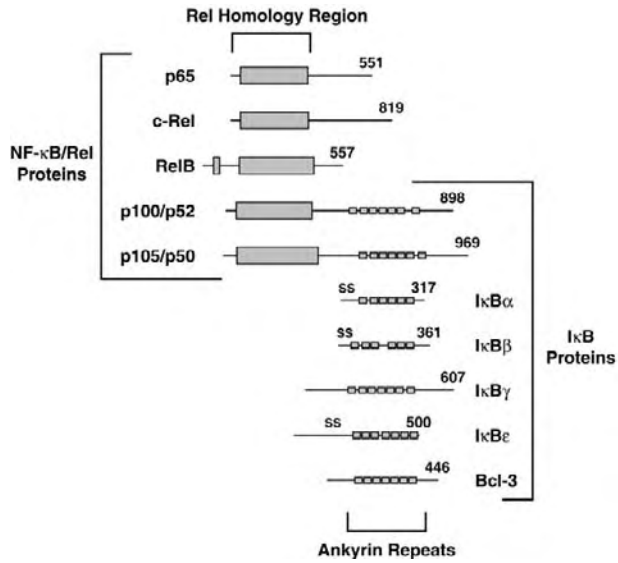


Figure 2 Schematic representation of members of the NFκB/Rel and IκB family of proteins. The position of the Rel homology region and the ankyrin repeat domain are indicated in the figure. p100/p52 and p105/p50 Precursor proteins comprise a unique subgroup of proteins that contain both a Rel homology and an ankyrin repeat domain. p100 and p105 Proteins have been shown to function in an IκB-like capacity. The number of amino acids of each protein is indicated to the right. For RelB, the shaded box N terminal to the Rel homology region represents a putative leucine zipper region.

specific NFκB dimers as activators of distinct sets of NFκB target genes [6–10].

NFκB resides in the cytoplasm in an inactive form by virtue of its association with a class of inhibitory proteins termed *IκBs*. Seven IκBs have been identified: IκBα, IκBβ, IκBε, IκBγ, Bcl3, NFκB1 precursor (p105), and the NFκB2 precursor (p100) (Fig. 2) [11]. The IκB family members, having in common ankyrin repeat domains, regulate the subcellular localization, and hence the DNA-binding and transcriptional activity, of NFκB proteins. The basis for the cytoplasmic localization of the inactive NFκB:IκB complex is thought to be due to masking of the nuclear localization signals (NLS) on the NFκB subunits by the IκB proteins. Thus, IκB degradation would lead to unmasking of the NLS, allowing NFκB to undergo translocation to the nucleus (Fig. 3). The IκBs display a preference for specific NFκB/Rel complexes, which may provide a means to regulate the activation of distinct Rel/NFκB complexes. Interestingly, NFκB induces the expression of IκBα; the newly synthesized IκB molecules enter the nucleus and remove NFκB from DNA [12–14]. The NFκB:IκB is then expelled from the nucleus as a result of potent nuclear export signals on IκB and p65 [15]. The IκBs provide yet another tier of regulatory complexity to modulate NFκB-mediated gene expression.

Activation of NFκB is achieved primarily through the signal-induced proteolytic degradation of IκB that is mediated by

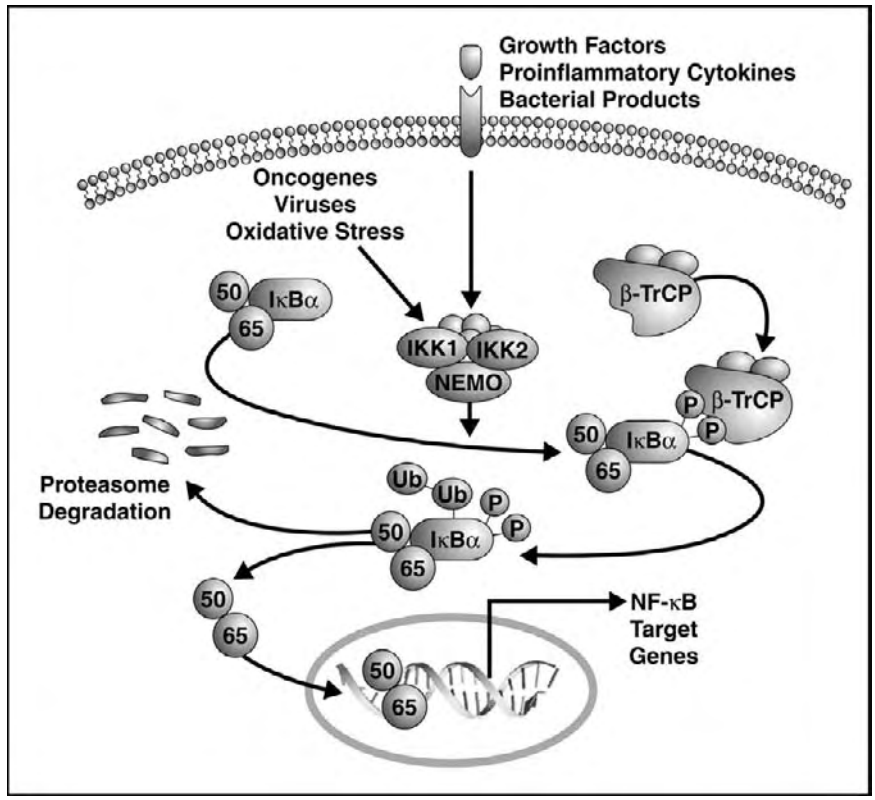


Figure 3 Schematic representation of components of the classical NFκB signal transduction pathway. In response to extracellular signals, the IKK complex becomes activated by a poorly defined series of membrane proximal events. The activated IKK complex phosphorylates (P) IκBα at serines 32 and 36, leading to site-specific ubiquitination (Ub) and degradation by the proteasome. The released NFκB complex (p50/p65) is then free to undergo nuclear translocation and activate transcription of NFκB target genes.

the ubiquitin/proteasome system [2]. The critical event that initiates the degradation of IκB is the stimulus-dependent phosphorylation of IκB at specific N-terminal residues (S32/S36 for IκBα, S19/S23 for IκBβ) [16,17]. Mutation of IκBα at either of these residues was found to block stimulus-dependent IκB phosphorylation, thereby preventing IκB degradation and subsequent activation of NFκB. Phosphorylation of IκB serves as a molecular tag leading to its rapid ubiquitination and subsequent degradation by the proteasome. β-TrCP, an F-box/WD-containing component of the Skp1-Cullin-F-box (SCF) class of E3 ubiquitin ligases, is required for recognition of phosphorylated IκB and recruitment of the degradation machinery [18,19].

Phosphorylation of IκBα on serines 32 and 36 is mediated by IκB kinases (IKKs), whose activity is induced by activators of the NFκB pathway [20–24]. IKK activity exists as large cytoplasmic multisubunit complex (700–900 kDa) containing two kinase subunits, IKK1 (IKKα) and IKK2 (IKKβ), and a regulatory subunit, NEMO (IKKγ, IKKAP1, FIP3) [25–28]. Hence the "core" IKK complex is comprised of IKK1, IKK2, and NEMO. IKK1 and IKK2 are highly homologous kinases, both containing an N-terminal kinase domain and a C-terminal region with two protein interaction motifs, a leucine zipper (LZ), and a helix–loop–helix (HLH) motif. The LZ domain is responsible for dimerization of IKK1 and IKK2 and is essential for activity of the IKK complex. The IKK1/2 complex associates with NEMO through a short interaction motif located at the very C terminus of either catalytic subunit. Short peptides derived from the interaction motif can be used to disrupt the IKK complex and prevent its activation [29]. NEMO is thought to serve an important regulatory function by connecting the IKK complex to upstream activators through its C terminus, which contains a Zn finger motif (Fig. 4). Further support for the regulatory function of NEMO is found by the observation that NEMO undergoes stimulus-dependent interaction with components of the TNF receptor complex [30]. More recently, a report described two potentially novel components of the IKK complex, namely, Cdc37 and Hsp90 [31]. Apparently, formation of the core IKK complex with Cdc37/Hsp90 is required for TNF-induced activation and recruitment of the core IKK complex from the cytoplasm to the membrane.

Sequence analysis revealed that both IKK1 and IKK2 contain a canonical MAP kinase kinase (MAPKK) activation loop motif. This region contains specific sites whose phosphorylation induces a conformational change that results in kinase activation. Phosphorylation within the activation loop typically occurs through the action of an upstream kinase or through transphosphorylation enabled by regulated proximity between two kinase subunits. IKK2 activation loop mutations,

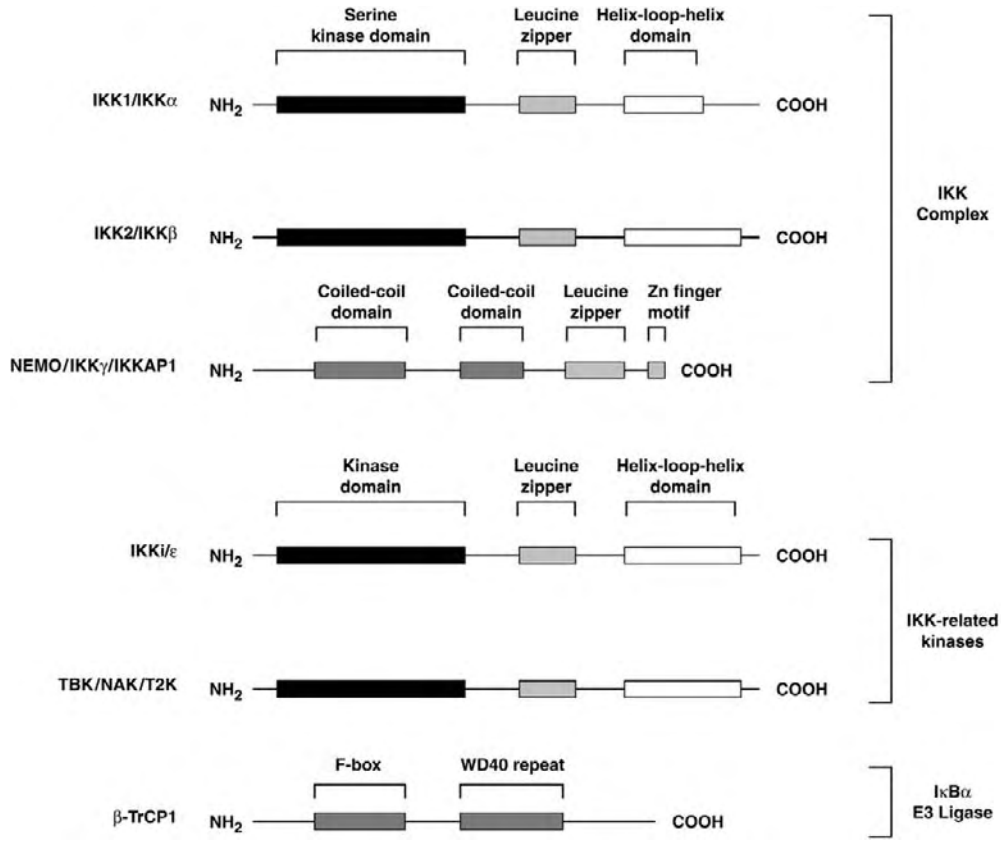


Figure 4 Schematic diagram showing the known subunits of the IKK complex, IKK-related kinases and β-TrCP, the IκBα E3 ubiquitin ligase. The name(s) of the protein is indicated to the left and the putative structural and functional motifs are indicated on top.

in which serines 177 and 181 were replaced with alanine, render the kinase refractory to stimulus-dependent activation. In contrast, replacement of serine 177 and 181 with glutamic acid, to mimic phosphoserine, yielded a constitutively active kinase, and was capable of inducing NFκB-mediated gene expression in the absence of cell stimulation [21]. The corresponding mutations in IKK1 did not interfere with NFκB activation in response to IL-1 or TNF, providing the first data suggesting that IKK2 plays a more prominent role in NFκB activation in response to proinflammatory cytokines [21,23,24]. Subsequent studies with IKK knock-out mice further validate that IKK2, and not IKK1, is required for NFκB activation in response to most proinflammatory stimuli [32–35]. The mechanism by which diverse stimuli converge to activate the IKK complex remains unresolved. For example, a number of kinases have been reported to function in the capacity of an IKK kinase, including NIK, IKKι/ε, NAK/T2K/TBK, MEKK1, MEKK3, and Cot/TPL2 [36]. However, with the exception of MEKK3, no effect was observed on either IKK activation or induction of NFκB DNA-binding activity in mice devoid of these kinases [37].

Biochemical and genetic analyses demonstrate that IKK1 and IKK2 have distinct cellular functions [2]. Disruption of the IKK2 gene results in embryonic lethality due to extensive liver apoptosis [32,34,38]. This phenotype is remarkably similar to that seen previously for RelA$^{-/-}$ mice [39]. Interestingly, IKK2$^{-/-}$- and RelA-mice could be rescued by ablation of the TNF receptor I gene, which is consistent with the role of NFκB in preventing TNF-induced hepatocyte apoptosis [34,40]. In addition, IKK2$^{-/-}$ mouse embryonic fibroblasts were demonstrated to be refractory to activation of NFκB in response to inducers of NFκB, including TNF, IL-1, LPS, and dsRNA [34,38]. In contrast, the IKK1$^{-/-}$ mice were born alive but died within 30 min, and IKK1$^{-/-}$-derived mouse embryonic fibroblasts display normal activation of IKK activity and induction of NFκB DNA binding in response to proinflammatory stimuli. These mice exhibit a plethora of developmental defects, the most striking of which is defective epidermal differentiation [32,33]. Interestingly, IKK1 was found to play a prominent role in regulating keratinocyte differentiation, which is independent from its kinase activity or modulation of NFκB [32,41].

More recently, a series of eloquent studies revealed a unique function of IKK1 in the lymphoid system [42]. Specifically, lethally irradiated mice that were reconstituted with IKK$^{-/-}$ hematopoietic stem cells displayed a diminution in B-cell maturation, germinal center formation, and antibody production, as well as defective splenic microarchitecture. Interestingly, it was discovered that IKK1$^{-/-}$ B cells exhibit a specific deficiency in NFκB2/p100 processing [42]. Moreover, independent studies demonstrated that NIK induces ubiquitin-dependent processing of NFκB2/p100 [43]. Subsequently, it

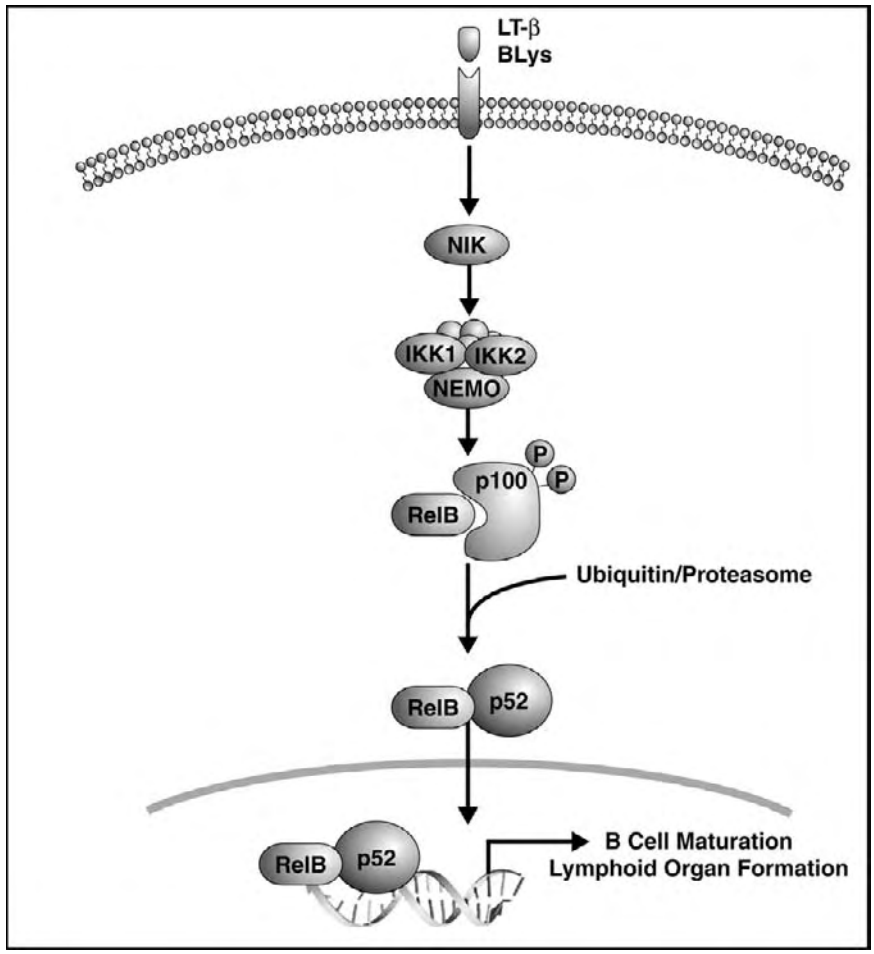


Figure 5 Activation by IKK1 is involved in an alternative NFκB signaling pathway. Select members of the TNF family, namely, lymphotoxin-beta (LT-beta) and B-lymphocyte stimulator (BLyS), activate IKK1 through NIK (NFκB-inducing kinase). Activation of this pathway leads to IKK1-dependent phosphorylation (P) and processing of NFκB2/p100. The active p52:RelB heterodimer undergoes nuclear translocation and regulates specific NFκB target genes involved in B-cell maturation and lymphoid organ formation.

was demonstrated that an inactive variant of IKK1 blocked NIK-induced NFκB2/p100 processing. Together, these studies suggest that NIK-mediated activation of IKK1 may lead to the phosphorylation and ubiquitin-dependent processing of NFκB2/p100 (Fig. 5). NFκB2/p100 is unique in that it contains both a Rel homology domain and an ankyrin domain and, consequently, possesses an intrinsic IκB-like capacity. Thus, enhanced proteolytic removal of the inhibitory C-terminal ankyrin domain will generate increased levels of transcriptionally active p52- containing NFκB complexes, perhaps leading to up-regulation of a specific subset of NFκB target genes. It is quite intriguing that, although IKK1 and IKK2 display high similarity and form stable heterodimers, they modulate distinct signaling pathways with unique biological consequences.

NFκB proteins may play a role in any disease possessing one of the cytokines, including TNF, IFNγ, IL-2, IL-6, IL-8, and IL-12, as a component of their pathophysiology (Fig. 1). The likely diseases for an NFκB-targeted therapeutic strategy may be those with a chronic, unresolved inflammatory component characterized by constitutively elevated systemic or local cytokine levels. Rheumatoid and osteoarthritis, inflammatory bowel disease, atherosclerosis, diabetes, multiple sclerosis and cachexia, among other conditions, fall into this category. Note that the chronic inhibition of NFκB likely to be necessary to ameliorate these conditions may have unknown repercussions. However, agents such as glucocorticoids inhibit NFκB and are used chronically, so we may predict that long-term NFκB-based therapy can be tolerated [44].

Despite the emphasis on NFκB's role as a regulator of cytokine gene expression and immune function, the initial application of therapeutic modulators of NFκB is likely to be in the field of oncology. If this is indeed the case, it will recapitulate the initial discovery of v-Rel proteins as transforming oncogenes nearly 20 years ago [45]. While the genetics of viral Rel proteins were being worked out, simultaneous work was proceeding on the transcription factors (including NFκB) regulating immunoglobulin and MHC genes [4,46]. It was several years before these divergent fields of research coalesced with the discovery that NFκB

proteins are the cellular homologs of v-Rel [47,48]. The functional roles of NFκB proteins, of course, extend far beyond regulation of immunoglobulins and are quite widely expressed.

More recent information has shown a wide variety of genetic aberrations of these genes in human neoplasms, from overexpression to rearrangement and amplification. The list of such observations is beyond the scope of this review, and details can be found elsewhere [49,50]. Transforming viral proteins of SV40, EBV, adenovirus E1A, and HTLV-1 activate NFκB [51–54]. Regardless of the mechanism, it is clear that the NFκB transcription factor is constitutively active in a wide variety of human cancers, including but not limited to colon, gastric, pancreatic, ovarian, hepatocellular, breast, head and neck carcinomas, melanoma, lymphoblastic leukemias, and Hodgkin's disease [55–65].

As described earlier, NFκB activity is controlled to a large extent by the activity of the IKKs, and this represents the most tractable point for therapeutic intervention. However, other mechanisms for activation and avenues for intervention exist. For example, the DNA-binding and transcriptional activity of NFκB proteins can be controlled by phosphorylation of multiple sites in both the Rel homology and transactivation domains [66–69]. Well-characterized oncogenes such as Ras, Rac, and Bcr-Abl as well as integrin-activated signals induce NFκB signaling both through the traditional IKK/IκB pathway and through direct phosphorylation of the p65 subunit of the transcription factor [66–70]. Recent results with agents selectively inhibiting the IKKs (e.g., PS-1145) or acting in part on other aspects of NFκB signaling (e.g., proteasome inhibitors, NSAIDs, and thalidomide) are beginning to show great promise in the treatment of various cancers [50,71,72].

From a functional standpoint, NFκB proteins have been suggested to play a role in cellular transformation via two general routes: direct stimulation of growth (cell cycle dysregulation) and/or inhibition of differentiation, and inhibition of programmed cell death (apoptosis). Both are likely to be involved to varying degrees based on the type of neoplasm, but the protection from apoptosis has garnered the most attention of late and may be the most important aspect from a therapeutic standpoint. With respect to direct cell cycle control, NFκB has been shown to up-regulate cyclin D1 transcription, a crucial step in the G_1 to S phase cell cycle transition [73,74]. NFκB activity peaks at multiple points in the mammalian cell cycle, including G_0/G_1, late G_1, and S phase [75]. In support of this concept, analysis of tumor cells treated with selective inhibitors of IKKs reveals a G_2/M phase blockade as well (unpublished observation), suggesting the existence of additional cell cycle targets.

NFκB activates central players in the programmed cell death pathways, including c-IAP-1 and -2, Bcl-Xl, and A1/Bfl-1 [76,77]. NFκB activation of protective proteins holds the key to the promise of NFκB inhibitors as chemotherapeutic agents. Of particular importance is the observation that diverse classes of chemotherapeutic agents are potent activators of NFκB, a response that proceeds at least in part through the activation of IKKs. Agents known to activate NFκB include etoposide, CPT-11, and TRAIL [78–80]. *In vitro* and *in vivo* experiments using the IκB super-repressor, a mutant version of IκB resistant to stimulus-induced degradation, have shown that inhibition of NFκB activity acts synergistically with chemotherapeutic agents to decrease tumor cell growth and tumor burden. Small molecule inhibitors of IKK2 show the same effect *in vitro* (unpublished observations). It is likely that agents previously used in chemotherapeutic regimens, such as glucocorticoids and proteasome inhibitors in multiple myeloma, are working at least in part through the inhibition of NFκB. It is interesting to note that specific inhibitors of IKK are only partially efficacious when used as monotherapy compared to a general proteasome inhibitor, suggesting other proteasome targets contribute to efficacy [71]. On the other hand, the side effect profile of more specific inhibitors is likely to be superior.

The development of potent and selective inhibitors of NFκB, targeting multiple aspects of NFκB signal transduction, is imminent. An important task for the near future is to determine the human diseases most likely to be resolved with these agents. Chronic inflammatory conditions are intriguing possibilities, but we must first define the consequences of long-term NFκB inhibition. The optimal tumor types to approach have not been completely defined, but are likely to include multiple myeloma [71] and one of the many neoplasms with chronically elevated NFκB (as described earlier). Tumors in which traditional chemotherapy elicits a robust (and presumably activating or protective) induction of NFκB will be likely targets for combination therapy. The molecular correlates of sensitivity to NFκB inhibitors, alone or in combination with other agents, need to be identified. For example, tumors lacking the PTEN tumor suppressor may be more sensitive to NFκB inhibitors [81]. It is clear that continued work on the basic aspects of NFκB biology is necessary. In concert with development of potent and specific modulators, these efforts have the potential to yield novel therapeutics for a wide variety of human diseases.

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CHAPTER 287

Transcriptional Regulation via the cAMP Responsive Activator CREB

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The Transcriptional Response to cAMP

Phosphorylation constitutes a predominant mechanism by which transcription factors are regulated in response to extracellular signals. The first phosphorylation-dependent activator to be described, the cAMP responsive factor CREB, belongs to the basic region/leucine zipper family of activators, which includes, among others, jun, fos, and myc [1,2]. CREB binds as a homodimer to a conserved cAMP responsive element (CRE), which often appears as a palindromic sequence (TGACGTCA), but also occurs as a lower affinity half site (TGACG) [3]. Treatment with cAMP agonist stimulates cellular gene expression by promoting phosphorylation of CREB at Ser133 [4]. A characteristic burst-attenuation pattern of transcription ensues, with maximal rates of transcription and Ser133 phosphorylation evident after 30 min [5]. This burst in transcription is rate limited by nuclear entry of a PKA catalytic subunit (PKAc), a passive process that is triggered by liberation of PKAc from the inactive holoenzyme in response to cAMP. The amplitude of target gene expression achieved in response to cAMP agonist is proportional to signal strength [5]. Following induction, target gene expression declines during the next 2–4 hr, reflecting the PP-1 and PP-2a mediated dephosphorylation of CREB [6,7].

Mechanism of Transcriptional Activation via CREB

Although phosphorylation can regulate activators by modulating their nuclear targeting or DNA-binding activities, CREB belongs to a group whose transactivation potential is specifically affected. The CREB transactivation domain is bipartite, consisting of kinase inducible (KID) and constitutive (Q2) activators that act synergistically in response to cAMP stimulus [8,9]. Following phosphorylation at Ser133, KID promotes transcription via an association with the KIX domain of the histone acetylase (HAT) paralogs CBP and P300 [10–12]. By contrast, Q2 stimulates target gene expression via an interaction with the $hTAF_{II}130$ component of TFIID [13–15].

The mechanism by which the KID and Q2 domains promote transcription has been studied extensively by *in vitro* transcription analysis on naked DNA and chromatin templates. CREB behaves as a constitutive activator when assayed with crude nuclear extracts on a naked DNA template; and Ser133 phosphorylation has only marginal effects on transcription [15,16]. Indeed, the glutamine-rich Q2 domain is highly active in this context; and reconstitution studies using purified basal transcription factors indicate that Q2 stimulates target gene expression by recruiting TFIID to the promoter [13,17].

By contrast, basal CREB activity is strongly repressed on a chromatin template [18]. And recruitment of P300/CBP in response to Ser133 phosphorylation is required for target gene activation. CBP/P300 HAT activity is essential for transcriptional induction in this setting [19]; disruption of CBP/P300 HAT activity either by mutagenesis or by addition of CBP/P300 specific inhibitor (LysCoA) impairs transcription [18]. Coincident with this observation, promoter bound histones H4 and H3 are inducibly acetylated *in vivo* on CREB target promoters in response to cAMP agonist.

Recruitment of CBP HAT activity to the promoter does not appear sufficient for transcriptional induction, however. CREB polypeptides lacking the Q2 domain are not competent for target gene activation despite wild-type CBP binding

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activity in the Ser133 phosphorylated state [18]. These experiments suggest that the activity of the Q2 domain, and more specifically its ability to associate with TFIID, is impaired in the chromatin context under basal conditions. The recruitment of CBP HAT activity to the promoter via association with Ser133 phosphorylated KID may promote target gene expression by disrupting the chromatin imposed barrier to the Q2–TFIID interaction.

A key aspect to this regulatory model is the presence of repressive complexes that inhibit CREB activity under basal conditions. In this regard, histone deacetylases (HDACs) have been shown to repress transcription from a variety of promoters, often by associating with certain activators and blocking recruitment of the transcriptional apparatus. Recent studies suggest that CREB associates with type I HDAC complexes, and these complexes appear important for maintaining low levels of histone acetylation over the promoter [20]. Indeed, treatment with HDAC inhibitors such as butyrate or trichostatin A (TSA) has been found to potentiate target gene expression in part by prolonging Ser133 phosphorylation of CREB during the attenuation phase [21]. The mechanism by which TSA and butyrate potentiate CREB phosphorylation is unclear, but current data suggest that HDACs inhibit CREB activity via an association with Ser/Thr phosphatases.

Signal Discrimination via CREB

Although first characterized as a cAMP-dependent activator, CREB also promotes cellular gene expression in response to a wide range of signals including growth factors, cytokines, UV irradiation, and neuronal depolarization [22]. At least three major intracellular pathways have been implicated in this process: cAMP, MAPK, and calcium. Despite inducing Ser133 phosphorylation with comparable kinetics and stoichiometry, non-cAMP pathways appear far less potent in stimulating target gene expression [3].

Activation of the MAPK pathway in response to growth factors promotes Ser133 phosphorylation of CREB in large part via the mitogen and stress-activated kinase MSK1 [23]. Highly related to pp90RSK, MSK1 and its paralog MSK2 appear to phosphorylate CREB with high efficiency in response to most if not all non-cAMP stimuli [23]. Importantly, CREB phosphorylation in response to non-cAMP stimuli is severely impaired in MSK1/2 null cells [24].

Despite the high stoichiometry of CREB phosphorylation via MSK1 and MSK2, CREB target gene activation via these kinases is much lower than that via PKA. Using a CREB–CBP complex specific antiserum, Wagner and colleagues [25] found that this association was strongly induced in response to cAMP but not other stimuli, suggesting that signal discrimination via CREB reflects the relative ability of CBP to be recruited to the promoter. Using fluorescence resonance energy transfer (FRET), Mayr and colleagues [26] found that relevant interaction domains in CREB and CBP, referred to as KID and KIX, respectively, are sufficient for signal discrimination in response to cAMP and non-cAMP stimuli. The process of signal discrimination appears to reside in the nucleus; cytoplasmic KID and KIX peptides showed no preference for various stimuli.

Secondary Phosphorylation of CREB: Ser142

The nature of the discriminatory signal remains elusive; but current work has drawn considerable attention to a second phosphorylation site in CREB: Ser142. In earlier studies, Sun and colleagues [27] and Sun and Maurer [28] had observed that the calcium/calmodulin-dependent kinase (CaMKII) strongly induces Ser133 phosphorylation of CREB. But target gene induction via this kinase was blocked due to concomitant phosphorylation of CREB at Ser142. Indeed, mutagenesis of Ser142 to alanine rescued target gene induction by CaMKII, whereas a Ser142Asp substitution, which mimicked Ser142 phosphorylation, reduced CREB activation in response to cAMP.

The solution structure of the KID/KIX complex has provided insight into the mechanism by which Ser142 phosphorylation blocks CREB activity [29,30]. The structure reveals that KID undergoes a random coil-to-helix transition upon binding to KIX. Ser133 appears to nucleate this interaction by forming direct contacts with residues in KIX. Hydrophobic residues lining one face of the inducible helix in KID further stabilize the complex via interactions with a hydrophobic groove in KIX. Remarkably, Ser142 projects into this groove and appears to destabilize the KID–KIX interaction by electrostatic repulsion [30].

Whether Ser142 is phosphorylated *in vivo* and whether this site explains signal discrimination via CREB are somewhat controversial questions. Substitution of Ser142 with alanine does not appear to rescue target gene in response to certain non-cAMP stimuli [26]; and Ser142 phosphorylation is not detectable using two-dimensional tryptic mapping studies on ^{32}p-labeled cells [31]. Using phosphospecific antisera, however, Kornhauser and colleagues [31] found that Ser142 and Ser143 were indeed phosphorylated in neurons following depolarization in response to KCl or glutamate. Despite their inhibitory effect on the CREB–CBP interaction, Ser142/143 phosphorylation appeared essential, in conjunction with Ser133 phosphorylation, for target gene activation in response to these stimuli. Confirming this notion, Gau and colleagues [32] found that light-induced shifts in locomotion and in CREB target gene activation in the suprachiasmatic nucleus were compromised in Ser142/Ala knock-in mice. In contrast with these findings, Impey and colleagues [33] found that CBP was actually critical for CREB target gene expression in neurons following exposure to glutamate, and that inducible phosphorylation of CBP at Ser301 was important in this process. One clear limitation in the former studies is the absence of data concerning the stoichiometry of Ser142/143 phosphorylation, particularly since phosphorylation at this site is not detectable using standard 2-D tryptic mapping experiments.

Methylation of the KIX Domain

In addition to secondary phosphorylation of KID at Ser142, formation of the KID/KIX complex is also modulated by modification of the KIX domain in CBP. In the process of evaluating the mechanism by which the methyltransferase CARM1 potentiates nuclear hormone receptor activity, Xu and coworkers [34] found unexpectedly that CARM1 blocked target gene activation via CREB in response to cAMP stimulus. Biochemical studies reveal that CARM1 disrupts CREB activity by catalyzing the methylation of CBP at Arg600 within the KIX domain. Indeed, Arg600 was first identified in a genetic screen for mutants in KIX that were unable to bind to phospho (Ser133) CREB *in vitro* [35]. NMR studies reveal that Arg600 does not participate directly in surface interactions with KID; rather, modification of this residue appears to disrupt KIX activity by destabilizing its structure. Thus Arg600 may serve as a methylation-sensitive switch that regulates the ability of CBP to associate with phospho (Ser133) CREB. The development of methylation-specific antisera should reveal under what conditions this switch is activated.

Cooperative Binding with MLL

In addition to covalent modification, CREB–CBP complex formation also appears to be regulated via allosteric mechanisms. In an elegant three-hybrid screen, Ernst and colleagues [36] found that the *Drosophila* trithorax homolog mixed lineage leukemia (MLL) protein stabilized CREB–CBP complex formation following its association with the KIX domain. NMR studies indicate the MLL stabilizes the CREB–CBP complex upon binding to a surface in KIX that is distinct from the CREB binding surface [37]. Whether MLL potentiates CREB activity *in vivo*, however, remains unclear.

The presence of several distinct mechanisms by which CREB–CBP complex formation is modulated suggests that signaling pathways may impose specific constraints on target gene activation by employing a combination of modifications and allosteric regulators. A complete characterization of these modifications will be critical in understanding how CREB has such different effects on cellular activity in response to distinct stimuli.

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CHAPTER 288

The NFAT Family: Structure, Regulation, and Biological Functions

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Introduction

Originally identified as regulators of the immune response in T cells, the NFAT family of transcription factors controls various biological processes in diverse tissues and stages of development. NFAT proteins share a high degree of structural homology with the Rel/NFκB family of transcription factors. Four calcium-regulated family members, NFAT1–4, mediate gene transcription in response to calcium/calcineurin signals, while NFAT5 transduces the hypertonic stress response and is also regulated by signals through an integrin receptor and the T-cell receptor. Once activated, NFAT proteins cooperate with other transcriptional factors and induce specific patterns of gene expression that control distinct biological programs. This chapter reviews recent data on NFAT structure and regulation and summarizes some biological functions controlled by NFAT transcription factors.

NFAT is a small but important family of transcription factors originally described in T cells [1–6]. It is now well established that NFAT proteins direct specific biological programs in a variety of cells and tissues. The NFAT family consists of five members. The primordial family member NFAT5 (TonEBP) is found in *Drosophila,* and the genes encoding the four calcium-regulated NFAT proteins, NFAT1–4 (also known as NFATc1–c4), appear to have emerged simultaneously early in the course of vertebrate evolution. The five NFAT proteins are classified into one family based on the sequence and structural similarity of their DNA-binding domains: the degree of sequence identity is ~60% to 70% when NFAT1–4 are compared among themselves, and ~40% to 50% when NFAT1–4 are compared with NFAT5.

The primordial NFAT family member NFAT5/TonEBP is expressed ubiquitously in mammalian cells and regulates the response to hypertonic stress [4,7]. NFAT5 is also likely to be involved in regulating diverse other biological programs. Genetic analysis in *Drosophila* suggests that NFAT5 plays a role in the Ras signal transduction pathway, and this function may be conserved in evolution [8]. NFAT5 protein levels are increased by antigen receptor stimulation of T cells, suggesting a role for this protein in lymphocyte responses downstream of the TCR [9,10]. NFAT5 is also activated by α6β4 integrin stimulation of carcinoma cells, suggesting a role in tumor metastasis [11].

Except for NFAT2, which is the only family member expressed at a specific stage of development of cardiac valves [12,13], one or more calcium-regulated NFAT proteins are expressed redundantly in many cell types of the embryo and the adult [5,6]. Three structural features are common to all four calcium-regulated NFAT proteins: an N-terminal transactivation domain, a highly phosphorylated regulatory domain that binds and is dephosphorylated by the calcium/calmodulin-regulated phosphatase calcineurin, and a DNA-binding domain that is monomeric in solution, forms dimers on certain κB-like DNA elements, and interacts with Fos and Jun proteins on "composite" NFAT:AP-1 DNA elements [1–5].

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Structure and DNA-Binding

The NFAT family is evolutionarily related to the Rel/NFκB family [10,14,15]. The level of sequence identity between these two families is marginal (~17%) but the structural similarity is remarkable. The DNA-binding domains of all NFAT and NFκB/Rel family members have two domains, an N-terminal specificity domain (~180 amino acids) involved in making base-specific DNA contacts, and a C-terminal domain (~100 amino acids) involved in dimer formation [14,16,17]. Together these domains constitute the Rel homology region common to all members of the extended NFAT/NFκB/Rel family. Comparison of the structures of NFAT5/TonEBP and NFκB p50 dimers on DNA illustrates the striking structural similarity between the NFAT and NFκB families [15–17]. The C-terminal domains of NFAT5 and NFκB p50 utilize a similar interface to form the dimer contacts, but NFAT5 has an additional surface for dimer formation, which involves the N-terminal domain [15–17]. NFAT5 and Rel proteins are stable dimers in the absence of DNA, whereas the calcium-regulated NFAT proteins are monomeric [10,18]. Nevertheless, NFAT1–4 can also form dimers on certain κB-like sites, a finding now confirmed by crystallography of two NFAT1 dimer–DNA complexes [S. Harrison and L. Chen, personal communication]. An interesting point is that all NFAT proteins, including NFAT5, prefer monomeric binding sites on DNA, whereas Rel proteins bind loosely palindromic DNA elements [19].

Regulation

NFAT1–4

NFAT1–4 proteins are activated by ligand binding to a variety of cell-surface receptors [1–3,5,6]. The common feature of the receptors is their ability to activate phosphatidylinositol-specific phospholipase C (PLC), thereby inducing calcium influx across the plasma membrane. In the immune system, the ability of immunoreceptors (T- and B-cell antigen receptors, Fcε receptors on mast cells, and Fcγ receptors on NK cells and monocytes) to activate NFAT is well documented [1]. Stimulation of immune cells through these receptors activates a cascade of several tyrosine kinases, leading to tyrosine phosphorylation and activation of PLC-γ. In other cell types, NFAT activation has been shown to result from stimulation of G-protein-coupled receptors leading to PLCβ activation, or stimulation of receptor tyrosine kinases leading to PLCγ activation. This leads to PIP2 hydrolysis and generation of IP3, which by binding to the IP3 receptor and depleting intracellular endoplasmic reticulum (ER) calcium stores, initiates the process of store-operated calcium entry through the plasma membrane [20,21].

NFAT-dependent gene transcription is exquisitely sensitive to changes in intracellular calcium concentration ($[Ca^{2+}]_I$). Even in the continuous presence of stimulus, $[Ca^{2+}]_I$ levels may oscillate depending on specific parameters of receptor occupancy and desensitization [20]. $[Ca^{2+}]_I$ levels are thus modulated at two levels—amplitude and oscillation frequency—and NFAT activation is sensitive to both types of modulation [22–24]. This is well illustrated by T cells from two severe combined immunodeficiency patients with a primary defect in store-operated calcium entry [25]. When activated, these cells show a small $[Ca^{2+}]_I$ spike resulting from store depletion, but they lack the ability to sustain increased $[Ca^{2+}]_I$ levels for several hours as observed in wild-type T cells. The brief increase in $[Ca^{2+}]_I$ suffices for transient dephosphorylation and nuclear localization of NFAT, manifested by transcription of a small number of NFAT target genes [25,26]. However, optimal activation of NFAT-dependent genes requires sustained calcium/calcineurin signals [25,26], which are most effectively elicited by slow $[Ca^{2+}]_I$ oscillations or low sustained $[Ca^{2+}]_I$ increases [22–24]. The differing sensitivities of NFAT target genes to intracellular free calcium levels are likely to reflect the multiple configurations of NFAT sites in gene regulatory regions; for instance, the requirement for sustained activation could be either because NFAT itself needs to remain within a transcription complex for many hours, or because formation of a cooperative NFAT:AP-1 complex is important for gene transcription (*de novo* synthesis of Fos proteins is required for optimal AP-1 activation).

Interaction with calcineurin is central to the calcium responsiveness of NFAT1–4. The major calcineurin docking site on NFAT is located at the N terminus of the regulatory domain and has the consensus sequence PxIxIT [27]. Substitution of the PxIxIT sequence of NFAT1 with a higher affinity version obtained by peptide selection increases the basal $[Ca^{2+}]_I$ sensitivity of NFAT [28]. The surface of NFAT–calcineurin interaction is unlikely to be limited to the PxIxIT motif, however; a second interacting region is present at the C terminus of the regulatory domain [29,30]. By mass spectrometric analysis, NFAT1 isolated from resting cells was shown to contain at least 21 phosphoserine residues, of which 14 were located in characteristic conserved sequence motifs in the regulatory domain [31]. Following cell stimulation, 13 of these residues were dephosphorylated by calcineurin. Dephosphorylation of 5 phosphoserines in one of these conserved motifs (SRR-1) sufficed for exposure of a nuclear localization signal in the regulatory domain, while dephosphorylation of all 13 residues was required for masking of a nuclear export sequence, complete nuclear localization, and full transcriptional activity [31]. In NFAT2, dephosphorylation of the conserved SPxx motifs has been correlated with increased DNA binding [32]. It is interesting that the gene for an endogenous calcineurin inhibitor, MCIP1, is itself an NFAT target, implying the existence of a negative feedback loop that down-modulates NFAT activity [33].

When calcium entry is prevented or calcineurin activity is inhibited in stimulated cells, NFAT is rephosphorylated by NFAT kinases and rapidly leaves the nucleus. A variety of constitutive and inducible kinases, including casein kinase 1, casein kinase 2, GSK3, and the MAP kinases p38 and JNK, have been suggested as NFAT kinases ([32,34–40] and reviewed in [3]). Different NFAT proteins can be differentially

regulated, even in a single cell type under identical cell culture or stimulation conditions, a finding most likely explained by differential susceptibility of individual NFAT proteins to signal-activated kinases [41,42].

Mass spectrometric analysis indicated that in stimulated cells, NFAT1 becomes phosphorylated on a serine residue in its transactivation domain [31,43]. It remains to be determined if this modification is necessary for transcriptional function in the context of the full-length protein. Pim1, Cot, and protein kinase C zeta have been suggested as candidate kinases, because when overexpressed, they enhance reporter activity dependent on the NFAT transactivation domain [44–46]. However, as previously suggested in a similar study using calmodulin-dependent kinase II, such enhancement could arise from indirect effects on coactivator function rather than through direct NFAT phosphorylation [43].

NFAT5

The mechanism of NFAT5 regulation is not yet fully understood. In most cell types examined, NFAT5 is present in both the nucleus and the cytoplasm; however, in at least one subline of Jurkat T cells, NFAT5 is cytoplasmic and is translocated to the nucleus following hypertonic stimulation [7,19]. NFAT5 is basally phosphorylated in resting cells and hyperphosphorylated following stimulation [10], but it is not known how (or whether) phosphorylation contributes to its function. The kinases responsible for inducible NFAT5 phosphorylation have not been identified; in particular, NFAT5 does not appear to be regulated by p38, a kinase known to be responsive to osmotic stress (C. Lopez-Rodriguez and A. Rao, unpublished). Dimerization of NFAT5 is required for transcriptional activity; however, hypertonic stress does not regulate dimerization, since NFAT5 is already a dimer in resting cells [10]. Direct binding studies have not provided evidence for interaction of NFAT5 with any of the known IκBs, despite the fact that this protein conserves many IκB-interacting residues common to Rel proteins (C. Lopez-Rodriguez and A. Rao, unpublished). Potentially, the DNA binding or nuclear localization of NFAT5 could be regulated intramolecularly, by masking interactions with other protein domains, in a manner sensitive to phosphorylation. Alternatively, NFAT5 function might be regulated by cooperation with nuclear partner proteins whose expression or function was modulated in a signal-dependent manner.

Transcriptional Functions

All NFAT proteins contain intrinsic transactivation domains, and thus are bona fide transcription factors that can independently induce gene transcription. The large C-terminal region of NFAT5 contains a transactivation domain that functions in reporter assays when fused to the GAL4 DNA-binding domain [10]. By the same criterion, NFAT1 and NFAT4 possess two transactivation domains, at their N and C termini, respectively [47,48]; both are strongly acidic and contain a DELDF[S/K] sequence that may serve as a docking site for the SAGA histone acetyltransferase complex [49]. Similar motifs are found in the corresponding regions of NFAT3 and the constitutive isoforms of NFAT2. The transactivation domain of NFAT2 has surprisingly been mapped to the beginning of its regulatory domain, overlapping a sequence utilized as the major docking site for calcineurin [50]. Constitutively active NFAT1 and NFAT2, which mimic the corresponding dephosphorylated proteins, have been shown to induce or potentiate target gene expression in resting cells [31,51,52].

NFAT-Fos-Jun cooperation constitutes a major mechanism of NFAT-dependent gene transcription. This subject has been recently reviewed [4] and we will only briefly elaborate on it here. Whereas the residues required for Fos-Jun contact are almost completely conserved in the DNA-binding domains of all four calcium-regulated NFAT proteins [53], they are absent from NFAT5, suggesting that the ability to cooperate with Fos and Jun was a relatively late evolutionary development [19]. NFAT has also been shown to cooperate functionally with many other families of transcription factors including GATA [54,55], MEF2 [56], Maf [57], IRF4 [58], and PPARγ [59]. Unlike the NFAT:AP-1 interaction, which cannot be detected in solution, these interactions appear to be mediated by direct protein–protein contact and are not cooperative on DNA. In at least one case (the NFAT–GATA interaction), the surface of interaction of these other transcription factors with NFAT is known not to overlap the surface of NFAT:AP-1 interaction; a mutant NFAT1 protein engineered to lack completely the ability to cooperate with AP-1 [60] was as or more effective than wild-type NFAT1 in its ability to synergize functionally with GATA3 in a transient reporter assay in T cells [61].

The shortest isoform of NFAT2 (NFATc/A) is induced in a CsA-sensitive manner by NFAT itself, in a process suggested to constitute a positive autoregulatory loop [62]. This protein is generated through utilization of a distinct inducible promoter that is preferentially coupled to the most proximal polyadenylation site [63]. As a result, NFATc/A lacks the entire C-terminal domain and contains an alternate N-terminal domain that is not highly acidic and lacks the SAGA interaction sequence DELDF[S/K]. It is not clear whether this protein would be transcriptionally active in the absence of partner proteins such as AP-1.

Biological Programs Regulated by NFAT

NFAT1–4

The calcium-regulated NFAT proteins have been implicated in a variety of gene expression programs in diverse cell types and tissues. These include cardiac hypertrophy, slow- and fast-twitch fiber differentiation, cardiac valve development, vascular patterning during embryogenesis, chondrocyte development, and adipose differentiation among others. These developmental and differentiation processes have been recently reviewed and are not discussed further here [5,6].

Cooperation of NFAT proteins with members of the AP-1 family of transcription factors is known to play an important role in the establishment of a productive immune response. Activation of both transcription factors is achieved upon full stimulation of B and T cells through their antigen receptors and costimulatory molecules. Composite sites for NFAT and AP-1 have been described in promoters and enhancers of many cytokine genes [1]. Paradoxically, NFAT without AP-1 induces an unresponsive or tolerant state in lymphocytes [51]. This response is induced in T cells by TCR stimulation that is caused by stimulation through the TCR without engagement of costimulatory receptors. This results in increased $[Ca^{2+}]_I$ levels with minimal MAP kinase or IκB kinase activation, thereby leading to NFAT activation without appreciable AP-1 or NFκB activation. Under these conditions NFAT initiates a distinct gene expression profile associated with a profound block in TCR signaling [51]. Anergic T cells are unable to respond to a subsequent complete stimulation by producing IL-2 or initiating a proliferative response. Thus, NFAT proteins play a central role in the control of two opposite aspects of T-cell function by activating two different genetic programs depending on the presence or absence of AP-1 cooperation.

NFAT5

NFAT5 has been shown or suggested to play a role in several cellular responses including the osmoprotective response, the TCR response, Ras signaling, and tumor invasion. It was independently cloned in a yeast one-hybrid system as TonEBP (tonicity element binding protein) [7]. NFAT5 activates a large number of target genes implicated in osmoprotective responses, including those encoding aldose reductase, the betaine transporter, and the inositol transporter [64]. In *Drosophila,* NFAT5 has been implicated in modulating Ras signaling: NFAT5 overexpression affects Ras-1-dependent photoreceptor cell development, suppressing a rough-eye phenotype produced by activated Ras [8]. In carcinoma cells, NFAT5 activity is induced in response to α6β4 clustering and specific inhibition of NFAT5 results in reduced carcinoma invasion [11], suggesting a novel role for NFAT5 as an inducer of integrin-mediated tumor metastasis. In hyperosmotically stressed lymphocytes, NFAT5 up-regulates transcription of TNFα and LTβ and may play a role in organogenesis, apoptosis, and various pathological responses [10]. In human T cells, activation through the TCR increases NFAT5 levels and activates transcription from NFAT5-dependent reporters. The signal transduction pathways that induce NFAT5 in response to TCR engagement seem to be calcineurin dependent and, therefore, different from the mechanisms controlling NFAT5 activation in response to hyperosmotic stimuli [9,10]. These results suggest an additional function for NFAT5 as a mediator of responses downstream of the TCR.

Perspectives

Although a great deal has been discovered about the NFAT family in the 10 years since the founding family members were cloned, much still remains to be understood. It is clear that the individual family members can be independently regulated even in the same cell types, but the basis of this specificity is unclear. The genes regulated by NFAT1–4 in immune cells and by NFAT5 under hypertonic conditions in kidney cells are by large known, but the target genes for these proteins and their cell specific biological functions in other cell types remain to be identified.

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CHAPTER 289

Transcriptional Control through Regulated Nuclear Transport

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Introduction

A defining characteristic of eukaryotic cells is the compartmentalization of the genome into the nucleus, a membrane-bound subcellular organelle that is essentially impermeable to the passive diffusion of macromolecules. This spatial segregation of genomic DNA within the cell provides a means to control the expression of genome information that takes place during cell growth and differentiation through the regulated localization of transcriptional control proteins to the nucleus. The regulation of nuclear transport has thus been widely utilized in a variety of biological contexts to regulate transcription in a signal-dependent manner.

Regulated Nuclear Transport: Overview

The flow of information (i.e., proteins, RNA) into and out of the nucleus represents a dynamic balance between nuclear import and nuclear export. The signal-dependent localization of a protein to the cytoplasm or the nucleus can be altered by regulating either import or export, or by regulating both of these processes in a coordinated manner (Fig. 1). Transport occurs through interactions with soluble transport receptors of the karyopherin/importin family that interact or "dock" with components of the nuclear pore complex [1,2]. The interaction between a transport receptor and a target protein is mediated through specific nuclear localization (i.e., import) sequences (NLSs) or nuclear export sequences (NESs) present within the transported protein [3]. Directionality of transport is established in part by the differential interaction of transport receptors with the small GTPase, Ran, which is present in a GDP-bound form in the cytoplasm and a GTP-bound state in the nucleus [1].

Nuclear transport can be regulated by three distinct mechanisms (Fig. 2). First, the nuclear targeting signal itself, represented schematically as a black box in Fig. 2, can be directly modified to alter the binding affinity between the signal sequence and the relevant transport receptor. This is achieved by phosphorylation of residues typically located within or adjacent to the NLS or NES that are directly involved in the interaction with a transport receptor. One well-characterized example of direct phosphorylation influencing the interaction between an NLS or NES and a transport receptor is provided by the yeast Pho4 transcription factor, which translocates to the nucleus upon dephosphorylation in response to phosphate-free growth conditions. Phosphorylation within the Pho4 NLS inhibits interaction with the import receptor Pse1, whereas phosphorylation at other sites is necessary for interaction with the export receptor Msn5 in the nucleus [4–6].

The second and perhaps more common means of regulating nuclear transport is through regulation of protein–protein interactions, either intramolecular or intermolecular, which alter the accessibility of the transport signal sequence to the transport receptor. This strategy is often referred to as the *masking* or *unmasking* of the signal sequences. The interaction between the NFκB transcription factor and IκBα provides a structurally defined example of intermolecular masking in which association of IκBα blocks accessibility of the NFκB nuclear localization signals [7,8]. The induced degradation of IκBα that occurs upon receptor-dependent signaling results in the unmasking of these NLSs and subsequent nuclear translocation of NFκB.

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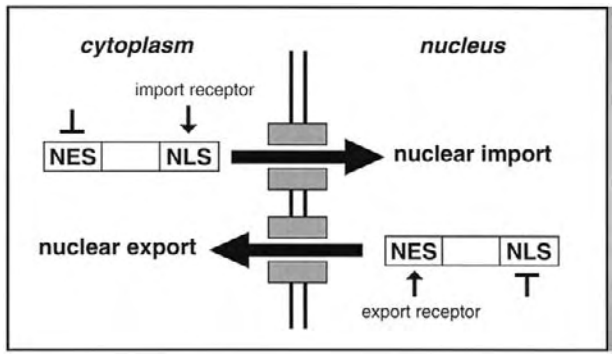


Figure 1 Dynamics of nuclear transport. Nuclear import results from association between a NLS and an import receptor or from the inhibition or disruption of the interaction between a NES and an export receptor. Conversely, nuclear export results from association between an NES and an export receptor or from the inhibition or disruption of the interaction between an NLS and an import receptor. Nuclear transport occurs through the nuclear pore complex. The coordinated regulation of both nuclear import and export provides a rapid, sensitive, and highly responsive means to dynamically translate intracellular signaling events into transcriptional responses in the nucleus.

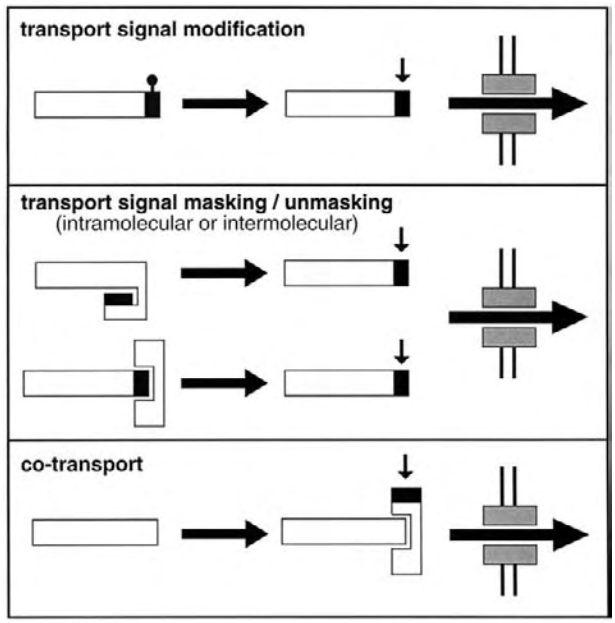


Figure 2 Mechanisms regulating nuclear transport. Nuclear import and export can be regulated by either direct modification of transport signals, masking or unmasking of transport signals through intramolecular or intermolecular protein interactions, or by interaction and cotransport with a carrier protein that contains a transport signal. The black box represents a transport signal. An arrow directed to the transport signal reflects accessibility and association with a transport receptor. The black circle represents protein phosphorylation. The direction of transport (import versus export) is not shown because the indicated mechanisms may affect either import (via NLS) or export (via NES).

The third strategy involves cotransport, whereby the import or export of a given protein is dictated by the interaction and cotransport with another protein that is itself differentially localized or expressed in a signal-dependent manner. Regulation of the NFκB transcription factor also provides an example of cotransport. The termination of signaling events that activate NFκB via IκB degradation results in reexpression of IκB. In addition to masking NLSs of NFκB and preventing nuclear import, IkBα also contains a functional NES that allows cotransport of NFκB out of the nucleus, thus providing a mechanism to actively terminate NFκB-dependent transcription upon cessation of upstream signaling [9–11].

Coordinate Regulation of Nuclear Import and Export: Calcium-Dependent Nuclear Localization of NFATc Transcription Factors

The coordinate regulation of both nuclear import and nuclear export in a signal-dependent manner is clearly illustrated in studies of the regulated nuclear transport of the NFATc family of transcription factors. NFATc transcription factors link calcium-dependent signaling to transcriptional responses that mediate a wide spectrum of biological processes, ranging from cardiac and vascular development to T-cell development and activation [12–14]. NFATc proteins localize to the cytosol in unstimulated cells and undergo rapid nuclear translocation in response to receptor-dependent increases in intracellular free calcium, which activate the calcium/calmodulin-dependent phosphatase calcineurin. In the absence of calcium-dependent signaling (i.e., at basal concentrations of cytoplasmic-free calcium), cytoplasmic NFATc proteins are constitutively and heavily phosphorylated, predominantly within a conserved regulatory domain consisting of a serine-rich region (SRR) and three SP repeat regions. The calcium-dependent activation of calcineurin results in NFATc dephosphorylation and nuclear localization.

Mutations of residues that are the target of calcineurin-dependent phosphatase activity result in constitutive, calcium-independent nuclear localization mediated by functionally defined NLSs [15–17]. Similarly, export of NFATc proteins from the nucleus requires phosphorylation-dependent interaction between the Crm1 export receptor and variably defined NESs, mutation of which results in impaired nuclear export [17–20]. The state of NFATc protein phosphorylation thus dictates the accessibility of both NLSs and NESs through phosphorylation-dependent alterations in protein conformation, hence, representing a mechanism of signal-dependent intramolecular masking and unmasking of nuclear transport signals [15,17].

Several kinases have been identified that are capable of phosphorylating NFATc proteins and functionally regulating the balance of nuclear import/export by antagonizing calcineurin-dependent phosphatase activity, thereby effecting NFATc-dependent transcription. These include glycogen synthase kinase 3, casein kinase I, JNK, ERK, p38, and casein kinase II [16,21–24]. Thus, in addition to calcium-dependent activation of calcineurin, signal-dependent alterations in NFATc kinase activities may also influence NFATc-dependent transcriptional responses by altering the dynamic balance between NFATc nuclear import and export. Calcineurin and NFATc protein kinases also form stable complexes with

NFATc proteins [20,24,25], providing an additional means of regulating the state of NFATc protein phosphorylation and the accessibility of transport sequences. The coordinated, calcium-dependent regulation of both the nuclear import and export of NFATc proteins thus provides a rapid, sensitive, and highly responsive mechanism through which the frequency and duration of receptor-dependent calcium signals can be differentially translated into appropriate transcriptional responses [26–28].

Regulated Nuclear Transport of Non-DNA-Binding Transcriptional Regulatory Proteins

Although regulated nuclear transport of site-specific DNA-binding transcription factors represents an important means of regulating transcription of those target genes that contain appropriate DNA-binding sites, more recent studies have demonstrated that non-DNA-binding transcriptional regulatory proteins that influence chromatin structure are also subject to signal-dependent nuclear transport, thus revealing a novel perspective on signal-dependent transcriptional control mechanisms. The epigenetic modification of histones, proteins that function to package DNA into chromatin, represents an important means of regulating transcription. Histone acetylation is a particularly important modification regulated by a dynamic balance between histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity, with histone acetylases typically associated with active transcription and histone deacetylases associated with repressed transcription [29]. The expression of muscle-specific genes that are induced during myocyte differentiation is controlled by the MyoD and MEF2 family of transcription factors [30]. The class II histone deacetylases HDAC4 and HDAC5 bind to MEF2 and repress MEF2-dependent transcription [31–33]. Myogenic signals induce not only the dissociation of HDACs from MEF2 but also the export of the HDACs from the nucleus [34,35]. Export of HDAC5 from the nucleus is dependent on phosphorylation at Ser-259 and Ser-498 and mediated by activation of the calcium/calmodulin-dependent protein kinase (CaMK) pathway, specifically CaMKI and CaMKIV [34,35]. Alanine substitutions at these positions result in the constitutive nuclear localization of HDAC5 and inhibition of myocyte differentiation [35,36].

The regulated nuclear export of HDAC4 and HDAC5 involves the phosphorylation-dependent association with the intracellular chaperone protein 14-3-3 [36–38]. Phosphorylation-site mutants that constitutively localize to the nucleus also fail to interact with 14-3-3, suggesting that binding of 14-3-3 is necessary for nuclear export [36]. The recent identification of an NES present at the extreme carboxy terminus of HDAC5 (also present in HDAC4 and HDAC7) capable of mediating phosphorylation-dependent nuclear export suggests a model of intramolecular masking in which amino-terminal sequences mask the carboxy-terminal NES [39]. Thus, induction of calcium-dependent signaling activates CaMK, which phosphorylates HDAC5 at Ser-259 and Ser-498, allowing the association of 14-3-3 and unmasking of the carboxy-terminal NES. 14-3-3 has also been shown to decrease the affinity of HDAC4 for the import receptor importin α [37], suggesting that phosphorylation-dependent association of 14-3-3 may regulate HDAC nuclear transport by simultaneously masking an NLS and unmasking an NES.

Nuclear export of HDAC5 may not be essential for activation of MEF2-dependent transcription given that a HDAC5 nuclear export mutant that is competent to bind 14-3-3 continues to exhibit CaMK-dependent activation of MEF2 [36]. Thus, the phosphorylation-dependent binding of 14-3-3 to HDAC5 resulting in the dissociation of MEF2 and HDAC5 appears to be sufficient to allow activation of MEF2-dependent transcription independent of the nuclear export of HDAC. What then is the function of HDAC nuclear export? As suggested by McKinsey et al. [36], localization of HDACs out of the nucleus may allow for a more sustained activation of MEF2-dependent transcription. Alternatively, perhaps the signal-dependent nuclear export of HDACs, by altering the balance between acetylase and deacetylase activity in the nucleus, results in a more global reduction in transcriptional thresholds that facilitates the optimal execution of subsequent steps in a contingent program of transcriptional responses regulating cellular differentiation.

Conclusion

The regulated nuclear transport of DNA-binding transcription factors as well as non-DNA-binding transcriptional regulatory proteins represents an important means of regulating transcription in response to receptor-mediated signaling events. Both nuclear import and export can be regulated by either direct modification of transport signals, masking or unmasking of transport signals through intramolecular or intermolecular protein interactions, or by interaction and cotransport with a carrier protein that contains a transport signal. The coordinated regulation of both nuclear import and export by various combinations of these mechanisms provides a rapid, sensitive, and highly responsive means to dynamically translate intracellular signaling events into transcriptional responses in the nucleus.

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CHAPTER 290

Proteasome/Ubiquitination

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Protein Degradation and the Ubiquitin/Proteasome System

Although most cellular proteins are long lived, a large class exists of normally short-lived proteins. Within this class one finds mainly regulatory factors, of which transcription factors constitute the largest group. One explanation for the short half-life of many regulatory factors is that it enables the rapid modulation of the steady-state concentration of the protein: The levels of constitutively short-lived proteins will respond much faster to changes in their rate of synthesis than that of long-lived ones. Additionally, many factors, rather than being constitutively short lived, can be conditionally stabilized or degraded in response to various stimuli. The question of how extracellular stimuli ultimately can determine the stability of specific transcription factors is central to the understanding of many signaling pathways.

The ubiquitin/proteasome pathway is the principal cellular system for selective protein degradation of normally short-lived proteins and of damaged or unfolded proteins (see [1] for a more extensive review). The ubiquitin system covalently ligates the conserved, 76-amino-acid protein ubiquitin to target proteins by creating an isopeptide bond linking the terminal carboxyl group of the ubiquitin polypeptide to an ε amino group of an internal lysine of the target polypeptide (or, occasionally, to the α amino group of the target polypeptide chain). An internal lysine residue of the first ubiquitin adduct can then serve as acceptor for an additional ubiquitin moiety, eventually yielding a cross-linked polyubiquitin chain that can contain tens of molecules. The polyubiquitin chain serves as a tag for recognition and destruction of the target protein by the 26S proteasome, a large, multicatalytic cytoplasmic protease. The ubiquitination reaction requires a number of enzymes and recognition factors that act sequentially: the ubiquitin-activating enzyme, or E1; an ubiquitin-conjugating enzyme, or E2; and an ubiquitin/protein ligase, or E3. Ubiquitin is initially conjugated in an ATP-requiring reaction to E1 via a thiolester bond, then transferred via a transesterification reaction to a cysteine residue in the active site of the E2, which finally transfers the activated ubiquitin moiety to an amino group of the target protein (Fig. 1). The E3—or ubiquitin/protein ligase—is responsible for substrate recognition; it serves to bring together the E2 and the substrate in a single complex, thereby allowing ubiquitination to occur. Ubiquitin ligases form a heterogeneous group of proteins that appears to consist of two main subgroups: RING finger domain-containing ligases (the larger group) and HECT domain-containing ligases. With the HECT domain ligases, the catalytic cascade of ubiquitination includes an additional transthiolation step in which the activated ubiquitin is transferred from the E2 to a cysteine residue on the E3 prior to its conjugation to the target.

The main site of regulation of the various ubiquitination reactions is at the level of the ubiquitin ligase–substrate interaction, which can be modulated either by modification of the substrate, or by modulation of ubiquitin ligase activity. In the next sections, we review pathways in which the ubiquitin system plays a role in the regulation of transcription factor activity in response to extracellular signals.

Regulation of Ubiquitination by Substrate Modification

Stimulation of Ubiquitination by Substrate Phosphorylation

Modification of proteins by the addition of phosphate groups can modulate their function in a multitude of ways, including by enabling interactions with other proteins. In the event that such an interaction occurs with a proteolytic system,

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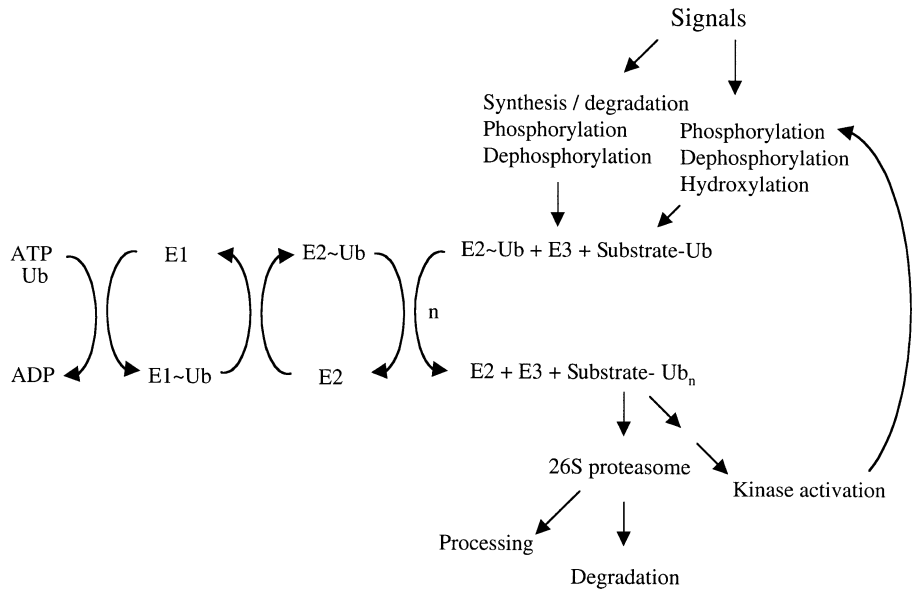


Figure 1 Schematic representation of the ubiquitination reaction cycle. Known points of regulation of the ubiquitin system by signaling pathways are indicated. See text for details.

phosphorylation will result in degradation of the protein. A number of ubiquitin ligases were described that require phosphorylation of the substrate prior to ubiquitination. The known ubiquitin ligases requiring prior phosphorylation of the substrate are primarily of the SCF class. SCF ubiquitin ligases form a complex of at least four proteins: the three components that were initially isolated, which gave the complex its name (SCF stands for *S*kp1, *C*dc53 or *C*ullin, and *F*-box protein [2,3]), and a subsequently identified RING finger domain-containing component, Rbx1 or Roc1 (reviewed in [4]). The F-box protein is the variable component of the complex. Given that SCF complexes with different F-box proteins exhibit different substrate affinities, the F-box proteins are thought to carry the substrate recognition function of the complex (see [5] for a review). The SCF complex containing a specific F-box protein is marked with a superscript, for example, SCF^{CDC4}.

Several transcription factors are known to date to be regulated by SCF-mediated ubiquitination, including Gcn4 [6] and Met4 [7] in yeast, and NFκB [8], β-catenin [9], ATF4 [10], and E2-F1 [11] in mammalian cells. Some of these factors were shown to be regulated at the level of protein stability by signaling pathways that modulate the phosphorylation state of the substrate, and therefore recognition by the SCF complex.

IκBα

One of the most thoroughly investigated signaling pathways is that of NFκB activation (reviewed in [8]). IκBα is an inhibitor of NFκB, which normally sequesters it in the cytoplasm. Upon stimulation of the pathway, IκBα becomes phosphorylated at two specific serine residues, whereupon it becomes a substrate for ubiquitination by the $SCF^{\beta\text{-}TrCP}$ complex. Interestingly, ubiquitination plays additional roles in the NFκB pathway: at the level of processing of the NFκB precursor, p105 (see later section), and at the level of activation of the IκBα kinase (see later section).

Gcn4

Another example of phosphorylation being required for degradation is that of the yeast transcription factor Gcn4. Gcn4 degradation depends on SCF^{CDC4} and on phosphorylation at a single specific site by a cyclin-dependent kinase, Pho85 [6] in conjunction with the Pho85 cyclin Pc15 [12], or, alternatively, on phosphorylation on an undefined site by another cyclin-dependent kinase, Srb10 [13]. Regulation of Gcn4 turnover by the availability of nutrients or, more generally, by the protein biosynthetic capacity of the cell, is mediated by regulation of Pho85 activity [6,12].

β-Catenin

As a last example, turnover of the mammalian transcriptional coactivator β-catenin depends on phosphorylation by glycogen synthase kinase 3β (GSK3β), which leads to ubiquitination of β-catenin by $SCF^{\beta\text{-}TrCP}$ (reviewed in [9]). Activation of the Wnt pathway leads to inhibition of the kinase and, consequently, to stabilization of β-catenin. Interestingly, another pathway of β-catenin destruction was recently identified that also depends on an SCF complex, but in this case the interaction with the substrate is phosphorylation independent and regulation occurs at the level of the ubiquitination complex (see later section).

Inhibition of Ubiquitination by Substrate Phosphorylation: p53

The tumor suppressor protein p53 is regulated at several levels, including degradation by the ubiquitin system. p53 Ubiquitination requires mdm2, a RING finger-containing ubiquitin ligase [14]. Genotoxic stress leads to phosphorylation of p53 at several serine residues, including serine 20.

Phosphorylation of serine 20 was found to stabilize the protein, probably by preventing its interaction with mdm2 [15]. Thus, in the case of p53, phosphorylation exerts a stabilizing effect on the protein.

Stimulation of Ubiquitination by Substrate Hydroxylation: HIF-1α

Although phosphorylation is the most common protein modification, other types of modification could, in principle, regulate the interaction between a protein and the ubiquitin system. The global transcriptional regulator of the hypoxic response, HIF-1α, provides an example of regulation by an alternative type of modification. HIF-1α is rapidly degraded under normoxic conditions, but stabilized under hypoxic conditions. Degradation of HIF-1α depends on ubiquitination by an ubiquitin ligase complex, the VBC complex (*V*on Hippel-Lindau protein–Elongin *B*–and Elongin *C*), which resembles the SCF complex in general architecture, and which contains the Von Hippel-Lindau tumor suppressor protein as a substrate recognition component [16–18]. However, rather than phosphorylation, hydroxylation of a specific proline residue in HIF-1α was recently found to be responsible for the interaction of the protein with the VBC complex [19,20]. This hydroxylation occurs only under normoxic conditions, thereby explaining the dependence of HIF-1α degradation on the oxygen concentration. A specific proline hydroxylase, the activity of which is directly regulated by the oxygen concentration, is responsible for the modification of HIF-1α [21,22]. This pathway thus represents a case where a very short signaling cascade links the stimulus (oxygen concentration) to the response (HIF-1α degradation).

Regulation of Ubiquitin Ligase Activity

Signaling pathways can modulate degradation of proteins by modification of the substrate, as shown earlier. In principle, the same effect could be achieved by modification of the ubiquitination system in response to extracellular signals. The ubiquitin/protein ligase, being the substrate recognition component of the ubiquitin system, would *a priori* be the preferred site of regulation. Regulation of the ubiquitin ligase is best established in the case of the E3 anaphase promoting complex/cyclosome (APC/C), which is responsible for the degradation of the regulators that govern, among other processes, the various mitotic transitions. Regulation of APC/C activity by signals emanating from the cell cycle machinery is essential for orderly cell cycle progression. However, it is not known whether the APC/C also responds to extracellular signals (reviewed in [23,24]).

F-Box Protein Stability

Activity of SCF-type ligases can be modulated at the level of regulation of the F-box component, the substrate recognition component of the complex. Many F-box proteins were found to be intrinsically unstable [25] and, therefore, could be tightly regulated at the level of their synthesis. The transcription factor E2F-1 accumulates in the late G_1 phase and is rapidly degraded in the S phase, following ubiquitination by the SCF^{SKP2} complex. Regulation of E2F-1 degradation was shown to be due to the cell-cycle-dependent synthesis of the F-box protein SKP2 [11]. Another example is that of the yeast transcription factor Met4, which is regulated by the SCF^{MET30} ubiquitin ligase [7]. Met30 is transcriptionally regulated by Met4 [7], but it is also regulated at the level of protein stability by the availability of methionine, so that in the absence of methionine, Met30 is destabilized and disappears from the cell, leading to increased Met4 activity [26]. Here again, regulation appears to be exerted by the concentration of an intracellular metabolite, rather than by extracellular signals.

Regulation of SCF Complex Activity by NEDD8

The recent convergence of several lines of investigation raises the possibility that SCF activity may be subject to direct regulation by cellular signaling pathways. First, the Cullin subunit of the SCF was found to be subject to modification by the addition of an ubiquitin-like protein, Rub1 (in yeast)/NEDD8 (in mammalians) to a specific lysine residue. This modification (henceforth "neddylation") appears to activate the SCF [27]; it is essential for SCF activity in fission yeast [28], although not in budding yeast [29]. The enzymatic machinery of neddylation, which consists of dedicated, Rub1/NEDD8-specific E1 and E2 enzymes, has been characterized, but it is not known whether it is subject to regulation [30]. A protein complex that contains a deneddylation activity, the COP9 signalosome, was also identified [31]. The COP9 signalosome is a conserved protein complex first identified in plants, where it plays a role in photomorphogenesis [32]. The signalosome complex is structurally similar to a part (the "lid") of the regulatory subcomplex (19S) of the 26S proteasome [33]. Although the functional significance of this homology is not yet clear, the signalosome and the "lid" subcomplex of the proteasome include homologous subunits, Jab1 and Rpn11, that appear to carry the catalytic activity of the deneddylation and deubiquitination activities of the respective complexes [34,35]. In mammalian cells, the NEDD8 pathway was recently shown to participate in the p105 processing pathway by affecting the activity of the $SCF^{\beta-TrCP}$ ubiquitination complex [36]. In *Arabidopsis*, COP9 activity modulates the activity of at least one specific ubiquitination complex, SCF^{TIR1} [31], previously implicated in the cellular response to the plant hormone auxin. Thus, the deneddylation function of the signalosome might connect extracellular signals (in the case of *Arabidopsis*, light) to cellular responses via modulation of the activity of the ubiquitin system.

Protein Processing by the Ubiquitin System

The p50 subunit of the transcription factor NFκB is generated by proteolytic processing of a 105-kDa precursor, p105.

Processing is mediated by the proteasome, in what constituted the first instance of partial degradation of an ubiquitinated protein [38]. The proteasome degrades the C-terminal half of the precursor, but arrests degradation and releases the mature, N-terminal half because of the presence of a "stop-transfer" signal of ill-defined nature in the middle of the precursor [39]. The only salient feature of the signal is that it is rich in glycine residues; how such a sequence would inhibit complete degradation of a protein is unclear, but it is notable that a similar glycine-rich region is found in the viral protein EBNA-1, where in contrast to p105, it confers complete protection of the protein against degradation by the proteasome [34]. p105 Processing can occur in two modes at least, constitutive and stimulated. The constitutive mode probably depends on signals near the glycine-rich region and on an undefined ubiquitin ligase [39]. The second, stimulated mode of processing, depends on phosphorylation of residues in the C terminus of p105 by the IκB kinase. The phosphorylated precursor is then recognized and ubiquitinated by $SCF^{\beta\text{-}TrCP}$, and processed or degraded by the proteasome [40,41]. This second mode ensures that upon activation of the NFκB pathway, not only are NFκB molecules formerly sequestered in the cytosol recruited to the nucleus upon degradation of IκB, but new NFκB molecules are rapidly generated.

The yeast transcription factors Spt23 and Mga2 present another example of processing by the ubiquitin/proteasome system [42]. These two proteins are required for expression of *OLE1*, encoding a fatty acid desaturase essential for the synthesis of the monounsaturated fatty acids, palmitoleic and oleic acid. Regulated synthesis of these compounds is essential for the maintenance of proper membrane fluidity. Spt23 and Mga2 are normally membrane bound due to a C-terminal transmembrane domain. The N-terminal domain of the transcription factor is released from its membrane anchor via ubiquitination and selective degradation of the C-terminal domain by the proteasome. This processing is inhibited by addition of unsaturated fatty acids to the medium. The simplest hypothesis is that in this case, the signal for processing of the precursor is not extracellular, but rather consists of variations in membrane fluidity or thickness that depend on the ratio of unsaturated to saturated fatty acids.

Modulation of Kinase Activity by Ubiquitination

The ubiquitin system is best known for its role in protein degradation; however, examples are emerging of nondestructive roles for ubiquitin. In signal transduction, a recent example for such a nondestructive role is the function of ubiquitination in the activation of the IκB kinase (IKK) [43]. The NFκB pathway is activated by proinflammatory factors such as interleukin 1 that bind to their cognate receptors and ultimately lead to phosphorylation and degradation of IκB, thereby releasing NFκB to the nucleus [8].

One of the first proteins in the NFκB pathway is TRAF6, a signal transducer that links receptor activation to activation of IKK. TRAF6 activation depends on a complex of two ubiquitin conjugating enzymes, Ubc13/Uev1A, that catalyze the polyubiquitination of TRAF6 following receptor activation and (probably) TRAF6 oligomerization [43]. IKK activation depends on phosphorylation by a complex that includes the kinase TAK1. TAK1, in turn, is activated by binding to activated TRAF6. The fact that polyubiquitinated TRAF6 escapes destruction is probably due to the nature of the ubiquitin chain: Whereas "classical" ubiquitin chains are cross-linked via lysine 48 of ubiquitin, the Ubc13/Uev1A ubiquitin conjugating enzymes catalyze the formation of a lysine 63-cross-linked chain, which is probably not recognized by the proteasome. The mechanistic details of how this polyubiquitination of TRAF6 activates TAK1, however, remain obscure.

Conclusion

In the interplay between cellular signal transduction and the ubiquitin system, the latter was often thought of as lying near the bottom of the signaling cascade, in the role of executioner of phosphorylated proteins, merely carrying out the verdict of the kinases. However, not only have we seen instances where the ubiquitination complex is itself the recipient of the signal, but in one case so far, that of the NFκB pathway, the ubiquitin system can even interfere with signaling by directly modulating the activity of kinases. It is likely that the NFκB pathway, which exemplifies the versatility of the ubiquitin system, will serve as a useful paradigm for the role of the ubiquitin system in other signal transduction pathways.

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CHAPTER 291

Fluorescence Resonance Energy Transfer Microscopy and Nuclear Signaling

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Introduction

Signal transduction pathways are typically represented as linear, connect-the-dot diagrams that terminate within the cell nucleus. The dots represent different protein complexes, each functioning sequentially to convey information that the cell will interpret into changing patterns of gene expression. Understanding how the cell assembles these signaling protein complexes is critically important to unraveling disease processes, and to the design of therapeutic strategies. This knowledge is being gained through the combination of biochemical, genetic, and molecular approaches. Importantly, these *in vitro* approaches are now being complemented by noninvasive techniques that allow direct visualization of dynamic protein interactions in their natural environment within the living cell.

Monitoring the social interactions of proteins inside the cell became possible with the cloning of fluorescent proteins (FP) from marine organisms [1,2]. Extensive mutagenesis of the jellyfish green fluorescent protein (GFP) and the cloning of a second FP from coral have yielded genetically encoded tags that emit light from the blue to red range of the visible spectrum [3,4]. These different color protein tags can be used in combinations that are distinguishable by multispectral imaging [4–7]. Because the FPs have no intrinsic intracellular targeting, they usually adopt the subcellular localization of the linked protein. Therefore, the intracellular distribution of several different labeled proteins relative to one another can be monitored in real time in the same living cell, and overlap in their distributions can be determined. The colocalization of the labeled proteins can be an indication that the proteins are assembled in common signaling complexes.

Unfortunately, the diffraction of light limits the resolution of the microscope to approximately 0.2 μm (2000 Å), and objects that are closer together than this will appear as a single object. For perspective, the basal transcription factor IID is an ensemble of three different interacting proteins forming a complex approximately 200 Å across. This complex was visualized *in vitro* by electron microscopy [8], but falls well below the detection limit of the light microscope (Fig. 1). Therefore, considerable distances may actually separate proteins that appear to be colocalized by fluorescence microscopy, and detecting the physical interactions between proteins within a signaling complex would appear to be beyond the reach of the light microscope.

FRET Microscopy Improves Spatial Resolution

Fortunately, it is possible to achieve this degree of spatial resolution by conventional light microscopy by using the technique of fluorescence resonance energy transfer (FRET) microscopy (Fig. 1). FRET has been used as a spectroscopic technique for many years [9,10], but the emergence of the genetically encoded FP tags has dramatically increased the utility of FRET microscopy of living cells [11]. FRET is the radiationless transfer of energy from a donor fluorophore to a nearby acceptor fluorophore resulting in sensitized

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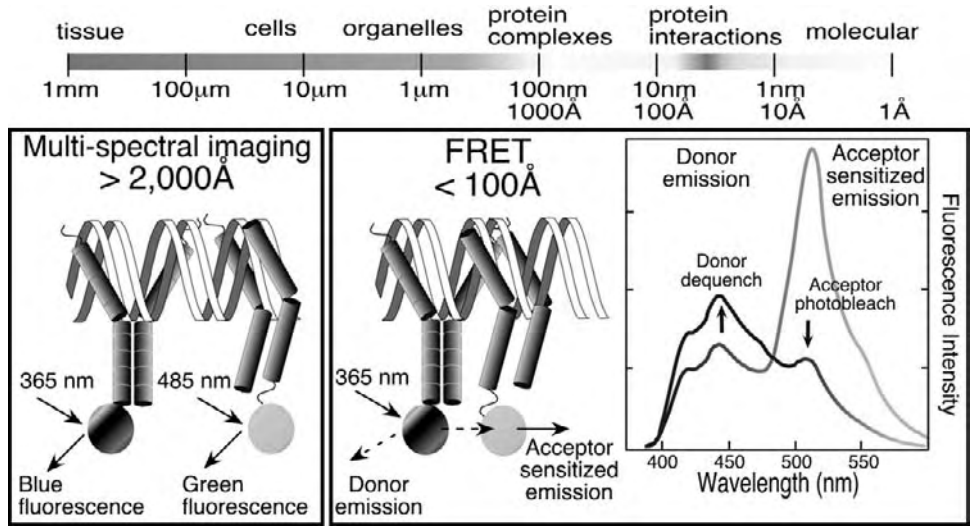


Figure 1 The optical resolution of the microscope is limited to approximately 2000 Å, but this resolution can be improved 50-fold by using FRET microscopy. The detection of sensitized fluorescence emission from acceptor fluorophores upon illumination of the donors can indicate that the tagged proteins are less than 100 Å apart. When energy transfer occurs, the donor fluorescence signal is quenched. Thus, FRET can also be measured by detecting the dequenching of the donor following acceptor photobleaching.

fluorescence emission from the acceptor (Fig. 1). Because the efficiency of the energy transfer drops precipitously as the inverse of the sixth power of the distance separating the donor and acceptor fluorophores, FRET is limited to distances of less than 100 Å. Therefore, the detection of sensitized fluorescence emission from acceptor fluorophores upon illumination of the donors can provide strong evidence for the physical interaction of the tagged proteins.

Limitations of FRET Microscopy

In practice, the detection of FRET signals from living cells expressing independent donor- and acceptor-tagged proteins is difficult because of limited control over the relative amounts of the expressed proteins in any given cell. This produces a highly variable spectral background signal, contributed by both the donor and acceptor fluorophores, from which weak FRET signals must be extracted [12]. Importantly when energy transfer occurs, the donor fluorescence signal is quenched because of the direct transfer of excitation energy to neighboring acceptors (Fig. 1). By measuring donor dequenching following acceptor photobleaching, FRET signals can be mapped to precise subcellular locations [13]. Here, we demonstrate how acceptor photobleaching FRET microscopy can detect the localized interactions of the transcription factor C/EBPα in specific subnuclear domains in the living pituitary cell.

The C/EBP family members are determinants of cell differentiation, and they regulate the expression of genes involved in energy metabolism. The basic region-leucine zipper of C/EBPα forms dimers through contacts in the leucine zipper and binds to specific DNA elements via the basic region. Our studies show that GFP-C/EBPα localizes to regions pericentromeric chromatin in pituitary GHFT1-5 and preadipocyte 3T3-L1 mouse cell lines. This pattern of subnuclear localization is identical to the location of endogenous C/EBPα in differentiated mouse adipocyte cells [14]. In Fig. 2, we show FRET signals originating from these subnuclear sites occupied by C/EBPα tagged with the yellowish (YFP) and blue (BFP) color variants. This fluorescent probe combination was selected for three reasons. First, despite its low quantum yield and sensitivity to photobleaching, the BFP variant used here provides a sufficient signal from the nucleus, where there is a low autofluorescence background. The much higher autofluorescence signal in the cytoplasm makes the BFP variant a poor choice for studies outside the nucleus. Second, the overlap of the BFP emission and YFP excitation spectra is suitable for energy transfer, but this pair has a reduced spectral background when compared to the CFP/YFP combination commonly used for these types of studies [13]. Third, the YFP variant is sensitive to photobleaching [13,15], making it a good choice for acceptor photobleaching FRET.

Detecting C/EBPα Intersections

The results shown in Fig. 2 illustrate the application of acceptor photobleaching FRET to detect the physical association of C/EBPα proteins in specific subnuclear domains. A GHFT1-5 cell coexpressing YFP- and BFP-CEBPα was selected and a reference image was acquired using the YFP filter set (for details, see [13]). Images were then acquired using the FRET filter set (donor excitation, acceptor emission) and the donor filter set (donor excitation, donor emission) at the same focal plane and under identical conditions (Figs. 2B and C, prebleach). The acceptor (YFP-CEBPα) was then

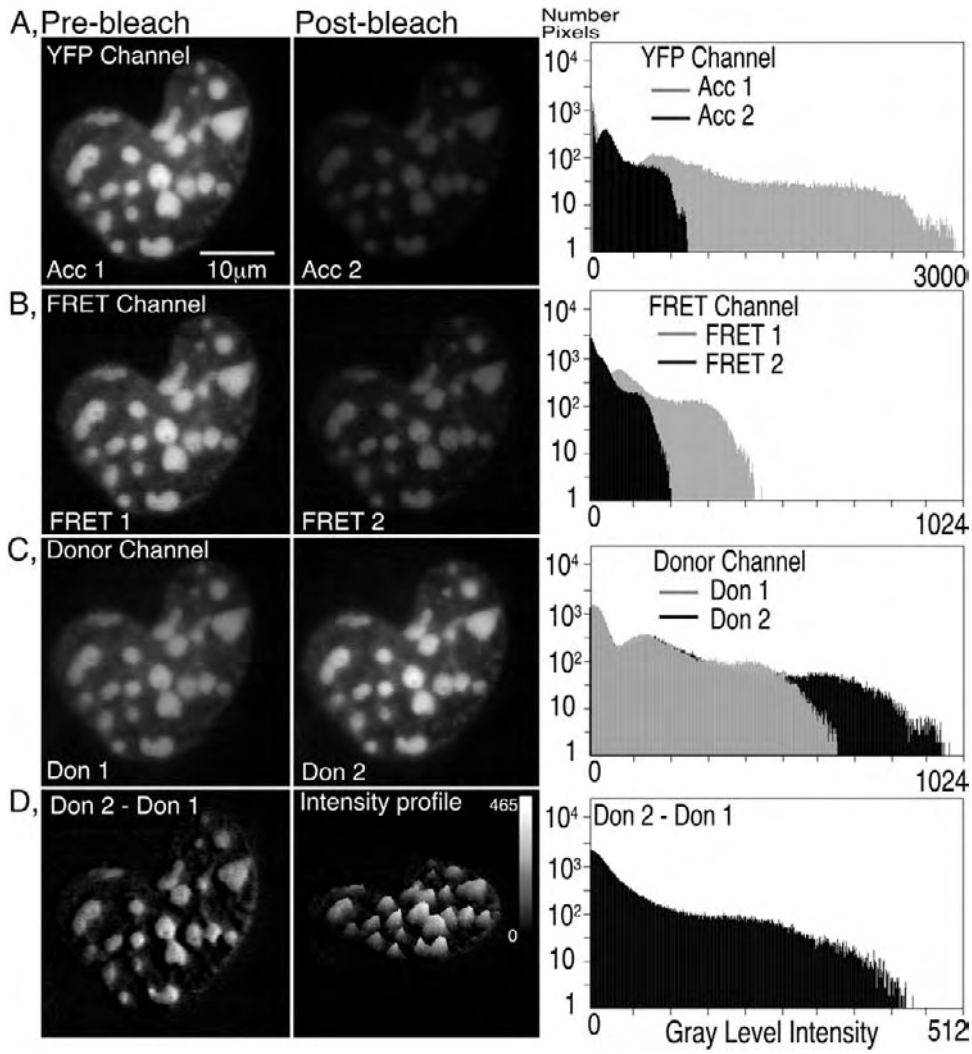


Figure 2 Acceptor photobleaching FRET microscopy of a pituitary GHFT1-5 cell expressing BFP- and YFP-C/EBPα. *Prebleach panels:* (A) The reference image showing subnuclear foci occupied by YFP-C/EBPα (Acc 1); bar = 10 μm. (B) Acceptor emission with donor excitation (FRET 1) and (C) the donor emission (Don 1) obtained under identical conditions. *Postbleach panels:* (A) YFP signal after 5-min bleach period obtained using the same conditions as for the first image. (B) The effect of selective acceptor photobleaching on the FRET signal (FRET 2) and (C) the donor fluorescence intensity (Don 2). (D, left panel) The dequenching of the donor was quantified by subtracting the Don 1 digital image from the Don 2 image, and (D, right panel) The subnuclear location where FRET occurred was mapped in the intensity profile; the calibration bar indicates the gray-level intensity.

photobleached by exposure to 500-nm excitation light for 5 min. A second YFP reference image was then acquired under identical conditions (Fig. 2A, postbleach), showing that the acceptor signal was reduced by approximately 70% (see histogram, Fig. 2A). This selective acceptor photobleaching abolished the FRET signal and revealed the contribution of the donor spectral background signal to the FRET signal (see histogram, Fig. 2B). In contrast, the donor fluorescence intensity was increased following acceptor photobleaching because of donor dequenching (Don 2, Fig. 2C, compare histograms). The donor dequenching was quantified by digital subtraction of the Don 1 image from the Don 2 image (Fig. 2D, left), and the subnuclear location of the dequenched donor signal was mapped by plotting the intensity profile (Fig. 2D, right). Together, the demonstration of a FRET signal above the expected spectral cross-talk background and the observed increased donor signal after acceptor photobleaching provide strong evidence for the physical association of these proteins at discrete subnuclear sites in these living pituitary cells.

Concluding Remarks

The detection of FRET in the living cell is limited by the accuracy of quantifying the intensity and position of the fluorescence signals in space. Accuracy is most severely compromised when measuring low signal intensities near the background noise of the system. Therefore, microscope systems used for FRET studies must be optimized to increase

sensitivity and reduce noise. The FRET efficiency will be improved by selecting donor and acceptor fluorophores with substantial spectral overlap, but the cost of the improved efficiency is an increased spectral cross-talk background from which FRET signals must be extracted. The FRET signal can also be improved by optimizing the ratio of the expressed donor- and acceptor-tagged proteins to favor productive interactions. In this regard, however, artifacts can arise from fusion protein overexpression, and control experiments confirming the normal function of the labeled proteins are essential. Further, there are many potential reasons FRET may not be detected from fusion protein partners that interact, including competition by endogenous proteins for the tagged proteins or incorrect orientation of the donor or acceptor fluorophore relative to one another. Therefore, the failure to detect FRET from a pair of labeled proteins carries no intrinsic information regarding the association of the proteins.

Finally, the mobility of higher order protein complexes within the three-dimensional space of the living cell interior represents a major problem for intensity-based measurements of sensitized acceptor emission and donor dequenching. The example shown here is a dimerized transcription factor that localizes to discrete and relatively immobile structures in the cell nucleus. Detecting FRET from signaling protein complexes that are highly mobile requires a temporal resolution that is difficult to achieve using intensity-based measurements. The technique of fluorescence lifetime imaging (FLIM) microscopy, however, can achieve both the spatial and temporal resolution necessary to detect highly dynamic protein interactions. FLIM measures the nanosecond duration of the excited state of a fluorophore, and when combined with FRET, this approach can provide direct evidence for dynamic protein interactions in four dimensions [16,17].

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CHAPTER 292

The Mammalian Circadian Timing System

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Introduction

Many aspects of mammalian physiology follow daily oscillations controlled by a circadian pacemaker. Clock-regulated processes include sleep–wake cycles, heartbeat, blood pressure, body temperature, most functions of the digestive tract, renal physiology, liver metabolism, acuity of sensorial systems, and many endocrine functions [1]. Remarkably, this clock produces such daily oscillations indefinitely in the absence of external time cues such as changes in light intensity or temperature. Nevertheless, it can measure a 24-hour period only approximately, and thus it has to be readjusted every day by a separate input pathway in order to remain in resonance with geophysical time [2].

The central mammalian pacemaker resides in the suprachiasmatic nuclei (SCN) of the hypothalamus and drives all known overt circadian rhythms in physiology and behavior [3]. The photoperiod is the major *Zeitgeber* (timing cue) for the SCN clock, and SCN neurons receive photic information directly via the retino-hypothalamic tract [4]. The oscillations generated in SCN neurons are translated into overt circadian rhythms via mechanisms that are as yet poorly understood. Surprisingly, circadian oscillators with a molecular makeup similar to that of the SCN oscillator are also operative in most peripheral body cells, but these peripheral clocks dampen after a few days if they do not receive periodic phase-resetting inputs from the SCN pacemaker [5,6].

In this chapter we briefly summarize recently acquired information about the molecular circuitry generating daily oscillations, the input pathways by which light synchronizes these oscillations, and the output pathways by which the SCN clock governs circadian fluctuations in physiology and behavior. Most of the observations described here have been made in the mouse; however, the major conclusions are likely to apply to most mammalian organisms.

The Molecular Oscillator

During the past few years, seven mammalian clock genes have been isolated and studied by genetic and biochemical approaches: *Clock*, *Bmal1*/*Mop3*, *Cry1*, *Cry2*, *Per1*, *Per2*, and *CK1*ε. With the exception of *Clock*, which was identified and cloned via a forward genetics approach in the mouse, all mammalian pacemaker genes were identified by their sequence homology with known *Drosophila* clock genes (see reviews in [7,8]).

Circadian oscillations are generated by feedback loops in gene expression that involve both transcriptional and post-transcriptional mechanisms (Fig. 1). The two PAS domain helix–loop–helix transcription factors CLOCK and BMAL1 (also termed MOP3 in some publications) heterodimerize and bind to E-box sequences to activate transcription of the *Per* and *Cry* genes [9,10]. PER and CRY proteins are translocated into the nucleus, where they repress transcription

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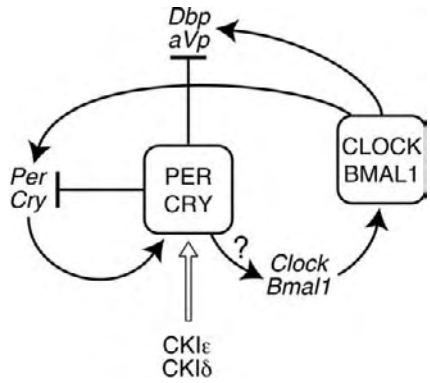


Figure 1 Simplified model of the mammalian circadian oscillator. At the heart of the oscillator is a multi-protein complex composed of period (PER) and cryptochrome (CRY) proteins. Transcription of the genes encoding these proteins is enhanced by CLOCK:BMAL1 heterodimers. When the CRY/PER proteins reach a critical threshold concentration, these proteins enter into the nucleus and repress transcription of their own genes by a yet unknown mechanism. Concomitantly, they provoke an anticyclic accumulation of CLOCK and BMAL1 proteins, probably via an indirect mechanism (?) (see note added in proof and reference 12). Upon decay of the CRY and PER proteins, transcription of the *Per* and *Cry* genes is reestablished, and a new cycle can occur. Casein kinase Iε and δ (CKIε, CKIδ) affect the nuclear translocation and the stability of the CRY/PER multi-protein complex by specific phosphorylations. The transcription of some clock-controlled genes, such as albumin D-element binding protein (*Dbp*) or arginine vasopressin (*aVp*), is regulated by the same mechanism as *Per* and *Cry* genes.

of their own genes as multisubunit complexes. Once the concentration of PER:CRY complexes falls below the critical threshold required for autorepression, *Per* and *Cry* transcription can resume (see review in [2]). This transcriptional feedback loop is assisted by several post-transcriptional control mechanisms [11]. For example, PER1, PER2, BMAL1, and CLOCK undergo robust daily cycles in protein phosphorylation, and these posttranslational modifications may affect their stability and function. CK1ε and CK1δ are among the protein kinases that phosphorylate PER and BMAL1 proteins, and the existence of protein complexes containing PER1, CRY2, CLOCK/BMAL1, CK1ε, and CK1δ was demonstrated by coimmunoprecipitation [11].

In liver, all of the clock genes specifying transcriptional regulatory components are transcribed in a circadian fashion, although the amplitudes of the various mRNA accumulation cycles vary greatly between genes (from less than 2-fold for *Cry2* to more than 20-fold for *Bmal1* and *Per2* [11]; J. A. Ripperger and U. Schibler, unpublished results). The circadian accumulation of *Cry* and *Per* mRNA is roughly in antiphase with that of *Bmal1* and *Clock* mRNA, suggesting that the rhythmic expression of positively and negatively acting clock components is governed by different mechanisms. Indeed, while CLOCK and BMAL1 stimulate the transcription of *Cry* and *Per* genes [9], they down-regulate transcription of their own genes via an indirect mechanism [12].

Photic Entrainment of the Central Pacemaker

SCN neurons receive photic information from the retina through the retino-hypothalamic tract. Glutamate is the major neurotransmitter used in this synaptic communication [13,14]. Surprisingly, rare light-sensitive ganglion cells in the inner retina layer, rather than the classical rods and cones in the outer retina layer, could be the photoreceptor cells required for the photic entrainment of the circadian pacemaker. These ganglion cells express the photopigment melanopsin and establish direct synaptic connections with SCN neurons as well as with other brain regions [15,16]; however, a disruption of the melanopsin gene will be needed to adequately assess its candidacy for the locus that encodes the circadian photopigment (see note added in proof and references 41 and 42). Light resets the phase of circadian rhythms in a highly gated manner. Thus, in nocturnal laboratory rodents kept in constant darkness, only light pulses delivered during the subjective night can elicit phase shifts. Furthermore, exposure to light during the first half of the subjective night provokes phase delays, whereas exposure during the second half of the subjective night causes phase advances [17].

Light pulses capable of phase shifting also elicit a transient activation of immediate early genes. These include *Per1*, *Per2*, and about a dozen other genes encoding transcriptional regulatory proteins (e.g., *cFos*, and *JunB*) [18]. cAMP serves as a second messenger both in the photic activation of immediate early gene transcription and in phase shifting ([19] and references therein). Increased cAMP levels activate the protein kinase PKA, which in turn phosphorylates the transcription factor CREB at serine residues S133 and S142 ([19] and references therein). Phosphorylated CREB then stimulates the transcription of immediate early genes, such as *Per1* and *c-Fos*. Light also provokes extensive histone H3 phosphorylation in the nuclei of SCN neurons, a phenomenon that is usually associated with changes in chromatin structure [20]. Whether these changes are causally related to immediate early genes has not yet been determined.

Outputs of the SCN Pacemaker

Most probably, the SCN controls overt circadian rhythms in behavior and physiology through neuronal and humoral outputs. In fact, SCN neurons project into various other brain centers (see reviews in [21,22]). These projections may affect circadian outputs directly through electrical signaling or via the rhythmic secretions of hormones of the hypothalamic-pituitary gland axis and other endocrine glands (e.g., the pineal gland). Some of these oscillating hormones, especially those of the hypothalamus-pituitary gland axis like glucocorticoids and thyroid hormones, may serve as phase-resetting cues for oscillators in non-neuronal cell types (see later sections). Cyclically secreted

SCN-derived neuropeptides such as arginine vasopressin and vasoactive intestinal peptide (VIP) may also participate in the regulation of rhythmic behavior and physiology [23,24].

Transforming growth factor-α (TGF-α), which signals through epidermal growth factor receptors, has recently been identified as a key regulator in the control of circadian locomotor activity. TGF-α is rhythmically expressed in SCN neurons, and constant infusion of this cytokine into the third ventricle abolishes circadian sleep–wake cycles in hamsters [25].

Outputs via Subsidiary Clocks

A second facet of the SCN's regulation of circadian physiology involves peripheral clocks. Circadian oscillators of a molecular makeup similar to that of SCN neurons exist in most peripheral tissues. Nevertheless, circadian gene expression in these tissues is not maintained in SCN-lesioned animals [26]. Hence, in intact animals, ongoing oscillations in peripheral organs depend on periodic inputs from the SCN. In immortalized tissue culture cells, circadian transcriptional oscillations can also be achieved by "jump starting" the cells with a serum shock or with substances that activate known signaling pathways. These involve tyrosine receptor kinases, G-protein-coupled receptors, nuclear hormone receptors (e.g., the glucocorticoid and retinoic acid receptors), protein kinases (e.g., PKA, PKC, and MAPK), and Ca^{2+} channels [27–31]. It is noteworthy that the immediate-early genes activated by these pathways before circadian gene expression is initiated include the same genes that are light induced in SCN neurons. This observation suggests that phase shifting by light in the SCN and signaling to the periphery by the SCN could share similar elements.

A similar conservation may exist in the direct regulation of output genes by the molecular clock. E-box sequences, similar to those used by CLOCK and BMAL1 proteins to activate *Per* gene transcription (see earlier section), are probably also important for the circadian expression of output genes such as DBP or arginine vasopressin [32,33]. Hence, these genes may be directly regulated by peripheral clock components in their cognate tissues.

Synchronization and Functions of Peripheral Oscillators: Time and Food

A functional SCN is required for the maintenance of circadian oscillations in peripheral cell types [6,26]. The output pathways by which the SCN synchronizes peripheral clocks are still poorly understood, but they are likely to be complex, given the panoply of signaling pathways that can affect circadian gene expression in peripheral cell types (see earlier section). Important clues on the phase entrainment of peripheral clocks have recently come from restricted feeding experiments [34,35]. Although feeding time does not affect the phase of the SCN pacemaker, it is the dominant *Zeitgeber* for many peripheral oscillators. Hence, the SCN may control the phase of peripheral timekeepers simply by determining the activity phase—and thus feeding time. Food entrainment may involve hormones related to metabolism, such as glucocorticoids [36] or metabolite-induced changes in the NAD(P)H/NAD(P)+ redox potential [37].

The dominant role of feeding time for peripheral phase resetting suggests that the anticipation of food uptake and processing may be a major purpose of peripheral clocks, at least in tissues such as the liver. This conjecture is strongly supported by recently published transcriptome profiling studies [38]. Indeed, a large fraction of cycling liver transcripts encodes proteins that are related to pathways implicated in sugar and fat metabolism, redox potential, and the detoxification of potentially harmful substances that may be present in the food.

Conclusions and Perspectives

Figure 2 summarizes our current view of the mammalian circadian timing system. Although impressive progress has been made in the analysis of all its aspects, much remains to be done. For example, the definitive identification of melanopsin as the key circadian photopigment will require genetic studies (see note added in proof and references 41 and 42). In addition, the presynaptic and postsynaptic light-signaling components downstream of melanopsin will have to be characterized. The possible role of mammalian cryptochrome as an additional circadian photopigment still remains a matter of debate, and compelling genetic evidence for or against such a function is still lacking. Considerable efforts will also have to be invested in the biochemical dissection of the mechanisms by which CRY and PER proteins repress CLOCK:BMAL1 mediated gene expression. Furthermore, studies aimed at the signaling pathways by which the SCN governs overt rhythms in physiology and behavior are still in their infancy. Finally, and perhaps most surprisingly, irrefutable experimental evidence supporting a strong benefit of circadian clocks is still scarce. Two studies, in which the survival of arrhythmic SCN-lesioned chipmunks and antelope ground squirrels in natural habitats have been recoded, showed only moderate advantages for the intact control animals [39,40]. Further studies conducted under conditions in which the survival of a species depends on predator avoidance, competition for mating partners and food, and resistance to extreme environmental variations will be required to evaluate the selective advantage that the circadian timing system may provide to its owners.

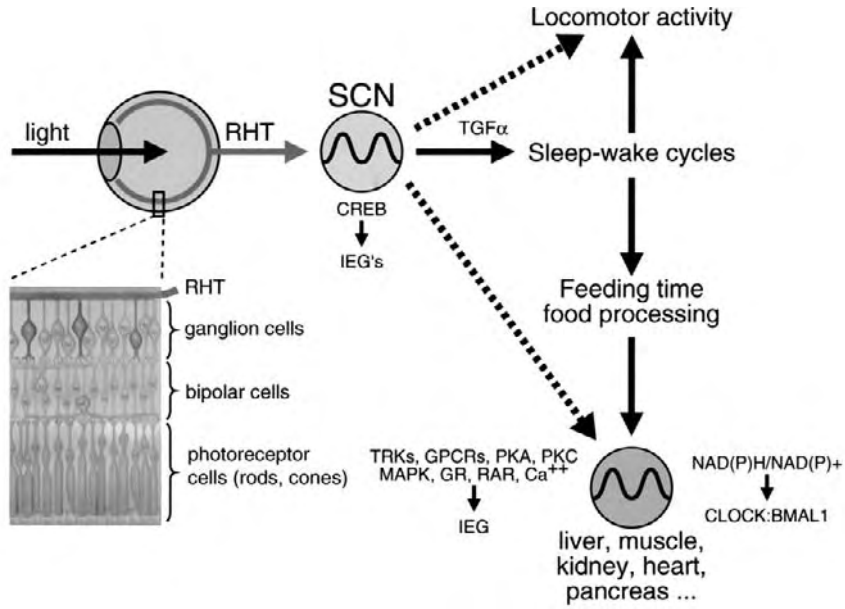


Figure 2 Phase-entrainment of central and peripheral circadian oscillators. Recent evidence suggests that photic cues are perceived by specific, melanopsin-expressing ganglion cells within the eye's retina. This light information is transmitted to the SCN via the retino-hypothalamic tract (RHT) and provokes the transcriptional stimulation of immediate early genes (IEG's, see text) via activation of the transcription factor CREB. Immediate early gene expression is thought to change the phase of the SCN pacemaker. In addition, specific diffusible molecules are secreted by SCN neurons to regulate physiological processes. One of these, TGFα, appears to regulate sleep-wake cycles. In turn, these cycles may affect locomotor activity and food uptake. Peripheral oscillators may be linked to the SCN directly via specific signaling cascades activating tyrosine receptor kinases (TRKs), G-protein coupled receptors (GPCRs), nuclear hormone receptors like the glucocorticoid (GR) and retinoic acid receptors (RAR), protein kinases like PKA, PKC, and MAPK, and Ca^{2+} channels. Alternatively, the SCN may control the phase of peripheral oscillators indirectly via food uptake and/or processing. The metabolism of nutrients may affect the redox-potential (the ratio of NADH to NAD+) within the cells, which in turn may regulate the DNA-binding activity of CLOCK:BMAL1 heterodimers.

Notes Added in Proof

Two important issues that had been unanswered (or unpublished) at the time of writing have been solved during the preparation of this book. First, the orphan nuclear receptor REV-ERBα has been identified as the transcriptional regulator connecting the positive and negative limbs of the circadian oscillator (see Reference 12). Second, genetic loss-of-function studies clearly revealed that melanopsin plays an important role in the photic entrainment of the mouse circadian clock. Thus, mice deficient in the conventional rod and cone photoreceptor cells and without a functional melanopsin allele are unable to light-entrain the phase of their circadian oscillator (see references 41 and 42).

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CHAPTER 293

Protein Arginine Methylation

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Introduction

Posttranslational modification of proteins is a hallmark of signal transduction, allowing cells to alter existing proteins to react rapidly to changes and events in their extracellular environment. Protein phosphorylation on serine, threonine, or tyrosine residues as a mechanism of intracellular signal transduction has been extensively studied. In contrast, the role of protein methylation at lysine, arginine, or histidine residues is much less understood. The subject of this chapter, methylation of arginine residues, was discovered more than 30 years ago, but has only recently received appreciation as a novel mechanism of signal transduction, transcriptional regulation, and protein sorting. As more arginine methylated substrates are discovered, and as novel functions of arginine methylation are reported at a very rapid pace, it appears that this protein modification may be of equal importance to protein phosphorylation in the regulation of cellular functions. This chapter summarizes the current knowledge on protein arginine methyltransferases and the function of arginine methylation in signal transduction.

Arginine Methylation and Arginine-Methyltransferases

The transfer of a methyl group from S-adenosylmethionine (SAM) onto arginine can occur on either or both of the guanidino nitrogen atoms. As such, arginine methylation, which is restricted to eukaryotic cells, can be found in three distinct forms—N^G-monomethylarginine, N^GN^G (asymmetric) dimethylarginine, and $N^GN'^G$ (symmetric) dimethylarginine (Fig. 1)—frequently in the context of RGG tripeptides [1,2].

Two classes of protein arginine methyltransferases have been characterized and classified based on the symmetry of their reaction products. Both, type I and type II enzymes generate N^G-monomethylarginine. However, whereas type I protein arginine methyltransferases account for the formation of asymmetric N^G,N^G-dimethylarginine, type II enzymes catalyze the formation of symmetric N^G,N'^G-dimethylarginine (Fig. 1). The best conserved region within this enzyme family is the SAM-interacting methyltransferase motif, which is closely related to that found in other methyltransferases that use SAM as a methyl donor [3].

Five related enzymes that catalyze asymmetric arginine methylation have thus far been identified. The majority of type I arginine methyltransferase activity in eukaryotic cells appears to be accounted for by PRMT1 and its functional yeast homolog Hmt1/Rmt1 [4]. These enzymes contain only a few residues outside the methyltransferase core domain, and their enzymatic activity appears to correlate with dimer formation. In contrast, PRMT2 contains an N-terminal SH3 domain, whereas PRMT3 harbors an N-terminal zinc-finger motif that modulates its substrate specificity [5]. The crystal structures of the conserved core regions of Hmt1 and human PRMT3 revealed significant structural similarity between these enzymes [6]. PRMT4 (Carm1) contains both N- and C-terminal extensions to the methyltransferase core region [7]. The recently identified PRMT6 resembles PRMT1 most closely in that it is comprised solely of the catalytic core without any additional domains [8]. PRMT5 (JBP), which was identified as a Janus kinase binding protein, is the only arginine methyltransferase identified to date that has type II enzyme activity [9,10]. As such, it is capable of catalyzing the symmetric arginine methylation of myelin basic protein, which was one of the first arginine-methylated proteins identified. In addition, two other symmetrically dimethylated RNA-binding proteins, spliceosomal snRNP proteins SmD1 and SmD3, can function as substrates for PRMT5 *in vitro* [11].

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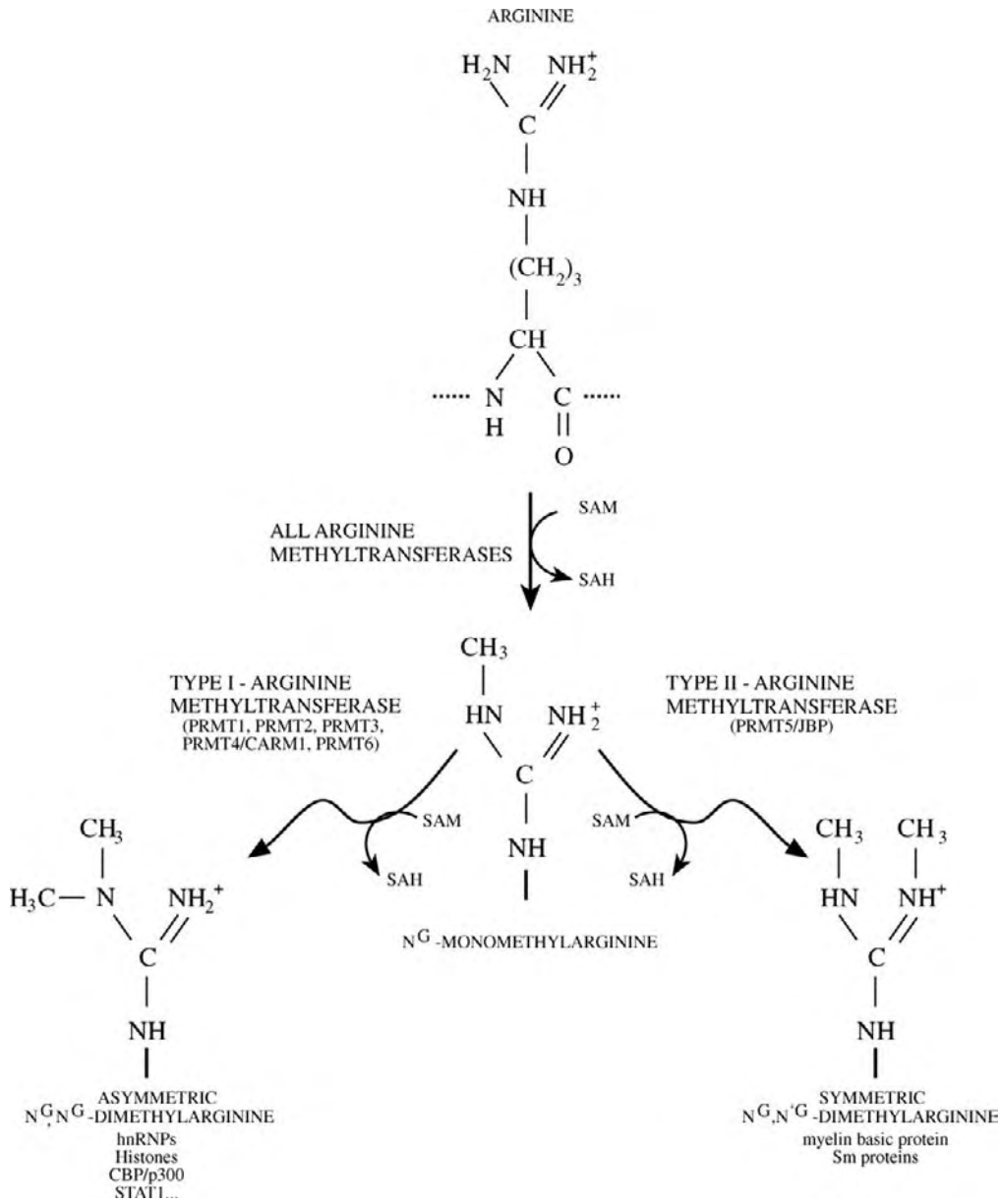


Figure 1 Protein arginine methyltransferases use SAM as methyl donor to catalyze mono- and dimethylation of the guanidino nitrogen atoms, generating SAH in the process.

Function of Arginine Methylation

Addition of methyl groups to the guanidino nitrogen atoms of arginine residues increases steric hindrance and reduces the ability to form hydrogen bonds without altering the overall charge of the amino acid. This suggests a role for methylation in the modulation of intra- or intermolecular interactions. Although the heterogeneous nuclear ribonucleoproteins (hnRNPs) involved in nucleocytoplasmic RNA transport comprise the majority of dimethylarginine containing proteins in the nucleus, their methylation appears to have no effect on their affinity for their respective RNA targets [12]. In contrast, several recent studies have illustrated an important function of arginine methylation in the regulation of protein–protein interactions. Methylation of Sam68, a proline-rich src-kinase substrate known to interact with WW- or SH3-domain containing signaling proteins, decreases its affinity for SH3 domains, but does not alter binding to WW-motif containing proteins [13]. Similarly, arginine methylation of the STAT1 (signal transducer and activator of transcription) transcription factor decreases its affinity for its inhibitor PIAS1 (protein inhibitor of activated STAT 1). In the absence of STAT1 arginine methylation, PIAS1 associates with STAT1 and prevents its binding to DNA, thereby modulating interferon induced gene transcription [14]. A positive modulatory effect of arginine methylation on protein–protein interaction is observed with the type II arginine methyltransferase substrates SmD1 and SmD3, which require methylation for efficient binding to the spinal muscular atrophy gene product, SMN [11,15].

Role of Arginine Methylation in Signal Transduction

Very little is still known about the regulation of enzymatic activity and the biological functions of arginine methyltransferases. PRMT1, the first mammalian enzyme, was discovered through its physical interaction with the immediate-early proteins TIS21 and the related BTG1, which have the ability to modulate the activity and substrate specificity of PRMT1 [16]. Deletion of PRMT1 in mice through gene targeting resulted in early embryonic lethality, demonstrating an important role for the enzyme in mammalian development. Interestingly, PRMT1 deficiency does not cause lethality on a cellular level [17]. PRMT1 has been implicated in intracellular signaling by its ability to bind the cytoplasmic domain of the type I interferon receptor [18] and to methylate an arginine residue conserved between the N-terminal regions of all mammalian STAT proteins. Indeed, methylation of the STAT1 transcription factor was shown to be essential for interferon α/β-mediated transcriptional induction [14].

Arginine methylation of histones H2A and H3 [7], as well as the transcriptional coactivators CBP/p300 [19], supports a model in which protein methylation contributes to chromatin remodeling and initiation of transcription [20]. PRMT4 or *c*oactivator-*a*ssociated a*r*ginine *m*ethyltransferase (Carm1) cooperates with p160 family coactivators to stimulate transcriptional activation by nuclear receptors [7]. Furthermore, PRMT4 interacts with myocyte enhancer factor 2C (MEF2C) to promote myocyte differentiation [21].

PRMT5 or Jak-binding protein 1 (JBP1) participates in the assembly of a large arginine methyltransferase complex that targets several spliceosomal Sm proteins of the snRNPs [22]. Direct interaction of survival of motor neurons (SMN), the spinal muscular atrophy gene product, with the arginine-rich domain of the Sm proteins to form functional snRNP core particles increased upon symmetric dimethylation of the Sm proteins. Interestingly, anti-Sm autoantibodies detected in lupus erythematosus patients specifically recognize symmetric arginine-dimethylated Sm proteins [11]. Autoimmune responses against myelin basic protein (MBP), the only other protein known to be symmetrically dimethylated on arginine residues, account for the development of multiple sclerosis. These observations raise the possibility that posttranslational modification by arginine methylation plays an important role in the development of autoimmune disorders.

With the number of identified arginine methyltransferases and arginine methylated proteins rapidly increasing, the question remains whether this modification is similarly reversible as phosphorylation. Thus far, no arginine demethylase activity has been reported. Although it is possible that this modification persists over the lifetime of the protein, other enzymatic reactions such as protein arginine diimination could in theory catalyze the removal of methylated nitrogens.

Much additional work is still required to elucidate the regulation of arginine methylation and its role in cellular function. Arginine methylation has just recently begun to be appreciated as a posttranslational modification that contributes significantly to signal transduction, protein sorting, transcriptional regulation, chromatin remodeling, and perhaps disease progression.

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CHAPTER 294

Transcriptional Activity of Notch and CSL Proteins

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Introduction

The Notch receptor controls diverse cell fate decisions developmentally and functions in a variety of processes in adults (for reviews, see [1–8]). Because the Notch intracellular domain (ICD) was shown to translocate to the nucleus following receptor activation and cleavage [9,10], its transcriptional activity has been actively investigated. In this capacity, ICD interacts with its DNA binding partner and alters its function from that of a repressor to an activator (for reviews, see [11,12]). This protein is known variously as Su(H) (for *Su*ppressor of *H*airless), CBF1, RBP-J, and LAG-1, depending on the species, and all orthologs are collectively referred to as CSL proteins [for *C*BF1, *Su(H)*, and *L*ag-1]. [For simplicity, we use the designation Su(H) in this review.] Here we present a summary of the biochemical functions of Su(H), ICD, and proteins participating in the Notch transcriptional complex, followed by evidence supporting models of Notch transcriptional activity *in vivo*. Although Su(H)-independent pathways of Notch signaling have been described [13–17], the transcriptional role of Notch has been examined largely in relationship to Su(H); therefore we address Notch activity only in that context.

Components of the Notch Transcriptional Complex

Architecture of the Notch ICD

The domain structure of the Notch ICD is shown in Fig. 1, which uses the 110-kDa *Xenopus* Notch1 ICD as a model. The number of Notch subtypes varies from organism to organism, that is, *Drosophila* expresses one receptor subtype, *Caenorhabditis elegans* two, and vertebrates four (for a review, see [18]). Data derived from several species and receptor subtypes suggests that all Notch intracellular domains exhibit the conserved structural motifs shown in Fig. 1 and that these domains mediate common functions.

The notable exception to this rule is the activity of the Notch3 ICD, which is in some contexts a poor transcriptional activator and antagonistic to Notch1 ICD [19]. The N-terminal RAM domain of ICD interacts directly with Su(H) and is required for high-affinity binding to ICD *in vitro* [20]. Su(H) also binds weakly *in vitro* to the ankyrin repeats immediately downstream of the RAM domain [21]. All Notch ICDs contain six ankyrin repeats; however, structural studies of *Drosophila* ICD indicate a motif resembling a seventh [22]; human Notch1 mutant in this region binds Su(H) but is nonfunctional, suggesting that the motif is required for transactivation [23]. Immediately downstream of the ankyrin repeats is a conserved region known both as the ICD TAD (for transactivation domain), containing one of the two nuclear localization signals [9] and sequences required for transactivation; this region is discussed in detail later. The ICD C terminus contains a glutamine-rich opa repeat domain and a C-terminal PEST domain associated with protein turnover [24] (see later discussion).

Notch Signaling Antagonizes Su(H)-Mediated Repression

Notch ICD does not bind DNA but activates transcription through interaction with Su(H), which binds to the motif YGTGRGAA *in vitro* [225]. Sites identical or highly

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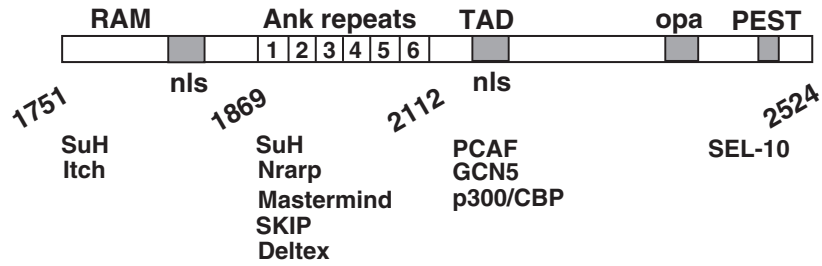


Figure 1 Domain structure of the 773 amino acid intracellular domain (ICD) of *Xenopus laevis* Notch1. Structural motifs shown are conserved in most Notch proteins, and numbering corresponds to amino acid residues of the unprocessed Notch receptor. Interacting proteins are listed below (for their functions see Table I). In some cases, namely Itch and SEL-10, potential interaction domains are fairly broad. Itch interacts with constructs of ICD containing the RAM domain and ankyrin repeats, while SEL-10 interacts with the C-terminus of ICD. SuH protein interacts with both the RAM domain and the reiterated ankyrin repeats, and both regions are required for Notch function. Six ankyrin repeats are shown, although there is some evidence for the existence of a seventh (see text). Sequences required for transactivation, designated the TAD, lie C-terminal to the ankyrin repeats and as indicated are known to bind several histone acetylases. Downstream from the TAD is a glutamine-rich domain designated opa and a PEST domain, which may be involved in protein turnover. ICD has two nuclear localization signals (nls): one C-terminal to the RAM domain and the other in the TAD.

homologous to this site (designated here as high-affinity sites) have been mapped in Notch-responsive promoters *in vivo* [26–31], as have divergent sites [32]. In the absence of Notch signaling, Su(H) has been shown to be a transcriptional repressor, by blocking interactions between TFIIA and TAF110 of TFIID on a viral promoter [33], but more generally by recruiting corepressors containing histone deacetylase (HDAC) activity. In vertebrates, Su(H) interacts with the corepressors SMRT [34], CIR [35], and a third repressor KyoT2 [36], and antagonizes Notch activation of Su(H)-dependent reporters in transient transfection assays. In *Drosophila*, Hairless, a nuclear protein that binds Su(H), genetically antagonizes Notch signaling in *Drosophila* [37–39] and mediates Su(H) repressor functions by acting as an adaptor protein between Su(H) and the corepressors CtBP [12,40] and Groucho [12,41]. Together, all of these corepressors can recruit both Class I and Class II HDACs (for a review, see [42]).

The implication of these findings is that in the absence of Notch signaling Su(H) acts as a local repressor; biochemical evidence for this hypothesis comes from chromatin immunoprecipitation assays showing that Su(H) binds to target promoters in the absence of Notch signaling [43]. *In vivo* support for a Su(H) repressor function comes from further analysis of both artificial enhancers and enhancers derived from Notch target genes, as described later [29,44].

Notch Signaling Derepresses Su(H) and Activates Transcription

Significantly, the physical interaction of corepressors and ICD with Su(H) is mutually exclusive, suggesting that Notch signaling leads to changes in gene expression in part by derepression. However, a large body of evidence also indicates that ICD activates transcription by recruiting additional proteins that contain histone acetyltransferase (HAT) activity. One line of evidence suggests that these HAT-containing proteins are recruited by directly binding to sites on ICD; specifically, to a TAD located in a 200-amino-acid stretch downstream of the ankyrin repeats (see Fig. 1) [45–47]. Deleting or mutating this region in ICD attenuates Notch-mediated transcription in frog embryos [438], *in vitro* [49], in cell culture models [46], and in *in vivo* models of ICD-mediated oncogenesis [50]. This region has been shown to bind the HAT-containing proteins, PCAF and GCN5, in both mouse Notch1 and *Drosophila* ICD, while a similar region in mouse Notch1 has been shown to bind the HAT-containing coactivator, p300/CBP.

Another line of evidence suggests that HAT-containing proteins are recruited indirectly by ICD through interactions with large, glutamine-rich nuclear proteins referred to here as the Mastermind (mam) proteins [49,51–53]. Loss-of-function analysis of the mam proteins in *Drosophila* [54], *C. elegans* (where it is known as Lag-3) [51], and vertebrates [49,55] suggests that these proteins are required for Notch signaling and the activation of Notch target genes. The mam proteins form a tight ternary complex with Su(H) and the ankyrin repeats of ICD [51–53] and this complex appear to potentiate ICD transcriptional activity in transient transfection assays [52,53], suggesting that mam is a Notch coactivator. Indeed, when assayed *in vitro* on chromatin templates, ICD requires mam to initiate transcription [49]. Activity in this assay requires a region of mam between amino acids 74 and 301 that binds tightly to CBP/p300; disrupting this binding markedly decreases the ability of ICD to initiate transcription *in vitro* and to promote the expression of Notch target genes *in vivo* [49]. CBP/p300 binds to mam under conditions that are more stringent than those that allow binding to ICD, suggesting that mam is the key element in recruiting HAT-containing proteins to the Notch transcriptional complex [49].

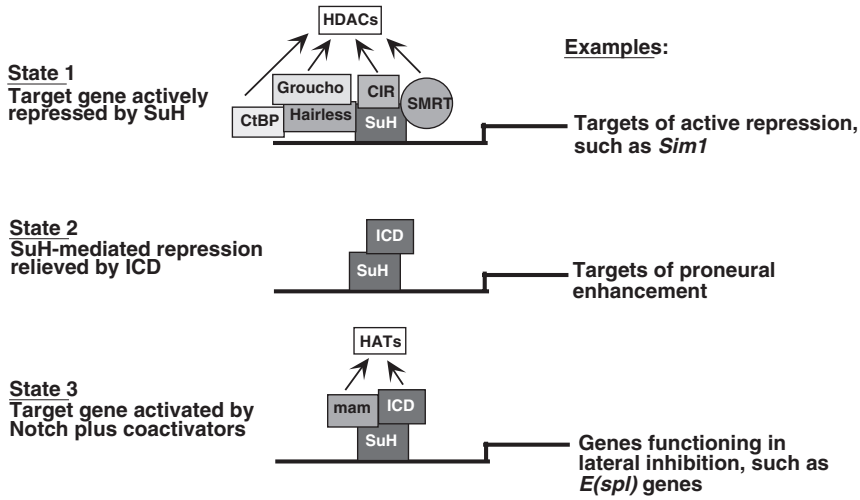


Figure 2 The SuH/ICD transcriptional switch can exist in several states. In State 1, SuH functions to actively repress transcription by interacting with several factors, such as the SMRT/NCor, CIR, CtBP, and Groucho co-repressors. In Drosophila, both CtBP and Groucho interact with SuH through the adaptor protein Hairless. For the sake of simplicity not all of the numerous proteins interacting with SuH in a repressive state are depicted. All of these co-repressors function to recruit HDACs, suggesting that chromatin remodeling occurs in the course of changes in Notch signaling. Active repression has been demonstrated in Drosophila not only on artificial enhancers but in vivo as being required for regulation of expression of *sim1*. State 3 represents the opposite activity; through interaction with ICD, co-repressors and accompanying HDACs are dismissed from SuH, and several HATs (See Figure 1 and Table 1) are recruited to the promoter through ICD and its dedicated co-activator mastermind (mam). Transcriptional activation by Notch is thought to be required for processes such as lateral inhibition and is best represented by upregulation of *E(spl)* genes in Drosophila. Evidence for an intermediate state (State 2), in which repression is off but activation through Notch is not required, comes from studies of eye development in SuH-files, which lack both SuH-mediated repression and Notch signaling. These studies indicate that relief from SuH-mediated repression is sufficient to activate, or "enhance", expression of putative targets, such as proneural genes, which function in differentiation.

The current model suggests that the Su(H)/ICD repressor/activator switch is mediated by HDAC/HAT recruitment, respectively, and strongly suggests that changes in the acetylation state of nucleosomes occurs in the course of Notch signaling [56]. This model posits several states in which Su(H) can exist, thus regulating gene expression in an ICD-regulated manner. In the absence of ICD, Su(H) acts as a local transcriptional repressor by recruiting HDAC-containing corepressors (state 1; Fig. 2). By associating with Su(H), ICD relieves this repression and may weakly recruit coactivators (state 2). The combination of Su(H) and ICD recruits additional coactivators such as mam, resulting in strong transcriptional activation (state 3).

This model will certainly undergo revision as various components of the Notch transcriptional complex are revealed. For example, we already know that ICD is more than a tether for mam because its TAD acts cooperatively with mam to promote transcription both *in vivo* and *in vitro*. How this TAD acts is unknown. In addition, mam contains a second TAD within the carboxy terminus that is required for Notch transcription *in vivo* but not *in vitro* [49], suggesting interactions with yet another group of transcriptional cofactors.

Additional Proteins Interacting with Su(H)/ICD

A number of proteins have been identified that interact with ICD and/or Su(H) (see Table I). In some cases, it remains unclear whether these proteins function in transcription per se, or whether they are regulatory subunits that modulate the activity of the transcriptional complex.

NRARP

Nrarp (for *N*otch *r*egulated *a*nkyrin *r*epeat *p*rotein) is expressed in many cases where Notch signaling regulates cell fate in the vertebrate embryo, and its expression is dependent on Notch signaling [57–59]. Nrarp binds to ICD and Su(H) but only as part of a ternary complex, interacting with the ICD ankyrin repeats. On the one hand, Nrarp promotes Notch transcription in transient transfection assays presumably by promoting the formation of a complex between ICD and Su(H). On the other hand, when misexpressed in frog embryos, Nrarp blocks Notch signaling and promotes the loss of ICD protein, suggesting that it inhibits Notch signaling by destabilizing ICD. Further genetic analysis is required to determine whether Nrarp is primarily a positive or negative factor in the Notch transcriptional complex, and whether it is required for Notch signaling in some or all cases *in vivo*.

SKIP

The v-*ski*-*i*nteracting *p*rotein SKIP interacts physically with both Su(H)/corepressor complexes, through interaction with both Su(H) and SMRT, and with ICD, through the fourth

Table I Proteins Interacting with ICD/Su(H) or Su(H)[a]

Interactor	Function	References
Interactors with ICD/Su(H)		
Mastermind	Coactivator	51–53
Nrarp	Unknown; mediates ICD degradation	57–59
SKIP	Unknown; potential coactivator	60
Deltex	Unknown; may antagonize activators	63–66
GCN5, PCAF	HATs; coactivators	46
CBP/p300	HAT; coactivator	47
Itch	E3 ligase; ICD degradation	83
SEL-10	E3 ligase; ICD degradation	86
Interactors with Su(H)		
SKIP	Unknown	60
SMRT/NCoR	Corepressor	34
CIR	Corepressor	35
KyoT2	Corepressor	36
CtBP	Corepressor	12, 40
Hairless	Adaptor; interacts with corepressors	37–39
TFIIA, TFIID	Coactivators; antagonized by Su(H)	33

[a]These proteins interact with either ICD/Su(H) or Su(H) alone in a manner suggesting they modulate or regulate transcriptional activity.

ankyrin repeat [60]. When expressed as Gal4 fusion proteins, human and *Drosophila* SKIP repress transcription. However, evidence that SKIP's primary role is to facilitate ICD transcription is more compelling. First, SKIP potentiates Su(H)-dependent ICD activity in transient transfection assays. Furthermore, antisense SKIP can rescue a block in muscle cell differentiation promoted by ICD [60], suggesting that SKIP is required for ICD function in this context. Finally, SKIP interacts with and transcriptionally coactivates several other proteins, including viral transactivators [60], nuclear receptors [61], and Smads [62].

DELTEX

Found in flies and vertebrates, Deltex proteins interact through their N terminus with the ICD ankyrin repeats [63]. Deltex proteins also contain a proline-rich domain and a C-terminal ring-finger motif, which promotes oligomerization [64]. What role Deltex plays in Notch signaling is currently unclear; furthermore, assigning a role for Deltex in transcription is speculative, because it is predominantly cytoplasmic [14,65] and also plays a role in Su(H)-independent Notch pathways [16,66]. On the one hand, genetic screens in *Drosophila* indicate that Deltex positively regulates Notch [67]. However, in functional assays Deltex proteins [66,68,69] can block ICD/Su(H) transcriptional activity, an activity requiring the ICD TAD and PEST sequences [69]. Similarly, Deltex inhibits transactivation of Gal4 reporters by both ICD and full-length mam fused to Gal4 [69]. This study, taken together with evidence that both ICD [47] and mam [49] interact with p300/CBP and that Deltex inhibits p300/CBP activity [66], suggests that in some contexts Deltex may negatively regulate Su(H)-dependent Notch activity by interfering with p300/CBP recruitment.

Modification of ICD

Notch ICD, like several transcription factors, is modified posttranslationally, primarily by phosphorylation but also by ubiquitination. Although ICD modification is likely to have transcriptional consequences, those consequences are not well understood at this time.

PHOSPHORYLATION

Following receptor activation and cleavage ICD becomes hyperphosphorylated [70–73]. Biochemical analysis of phosphorylation of Notch1 indicates that it occurs in two kinetically separable steps, in which ICD is phosphorylated in the cytoplasm and then hyperphosphorylated in the nucleus [74]. Although correlated with receptor activation, the mechanism and role of ICD phosphorylation remain unclear. Highly phosphorylated forms of *Drosophila* ICD immunoprecipitate with Su(H), suggesting that phosphorylation does not preclude inclusion in a transcriptional complex [70,72]; however, dephosphorylated ICD also immunoprecipitates with Su(H), indicating that ICD's phosphorylation state is unlikely to regulate complex formation.

It is not known whether phosphorylation of ICD is required for Su(H)-dependent transcription per se. Differential phosphorylation in response to growth factors alters Notch2 ICD function, although the transcriptional consequences

are unknown [75]. In labeling experiments, much of the phosphate incorporated by ICD maps to the region required for transactivation [72], suggesting a transcriptional role, although the C-terminal half of mouse Notch1 ICD, containing both the TAD and PEST sequences, incorporates phosphate at multiple sites [72].

The activity of the coactivator mam suggests that phosphorylation regulates ICD turnover. Mam promotes hyperphosphorylation of *Xenopus* ICD in a Su(H)-dependent manner, an event correlating with loss of ICD protein from cell extracts [49]. Truncated ICD containing the TAD but lacking the C-terminal PEST domain is neither hyperphosphorylated nor destabilized in the presence of mam; likewise, full-length ICD is not hyperphosphorylated and destabilized by mam truncations containing only the N terminus and N-terminal TAD. Given that truncated forms of ICD initiate transcription in the presence of mam [49], these observations support the following model: that ICD/Su(H)/mam interaction favors recruitment of a kinase to catalyze the nuclear phosphorylation of sequences in the ICD C terminus, and that such hyperphosphorylation may function in a downstream transcriptional event such as elongation, in ICD turnover, or in both. Some ubiquitin ligases such as SEL-10 target phosphorylated ICD substrates for degradation (see later discussion); although loss of ICD protein from cell extracts accompanies mam-mediated hyperphosphorylation, it is not known whether a kinase activity potentially recruited by mam is required for ICD recognition by SEL-10.

An opposite role for phosphorylation in regulating ICD turnover comes from reports that the serine kinase GSKβ (Shaggy) phosphorylates the Notch1 ICD *in vitro* and thereby enhances the stability of ICD protein [76]. *In vivo* cells lacking GSKβ activity exhibit less extensively phosphorylated forms of ICD, which is attributable to either direct or indirect interaction with GSKβ [76]. Given that ICD is so highly phosphorylated, these data and the activity of mam indicate that phosphorylation, which is likely mediated by several kinases, plays multiple roles in regulating ICD stability and potentially transcriptional activity.

UBIQUITINATION

Ubiquitination not only marks proteins for degradation but is required for activation of some transcriptional factors (for a review, see [77]). Numerous studies indicate a role for ubiquitination in the turnover of proteins in the Notch pathway [74,78–84]; furthermore, disabling the proteasome stabilizes misexpressed ICD in both fly embryos and cultured cells [81,85]. As yet, however, there is no evidence for an activating role of ubiquitination in Notch transcription, although the observation of paradoxical degradation/activation activity by proteins such as Nrarp and mam suggests that ICD activation could be linked to turnover.

The F-box E3 ligase, SEL-10, was first identified as a suppressor of hypomorphic Notch alleles in *C. elegans* genetic screens [86]. Vertebrate Sel-10 homologs promote the ubiquitination of ICD, preferentially recognizing hyperphosphorylated forms of ICD [74,81,82]. Interaction with SEL-10 requires sequences downstream of the ICD ankyrin repeats, the domain shown to be highly phosphorylated as noted earlier [72]. Ubiquitination by SEL-10 apparently leads to turnover given that dominant-negative forms of SEL-10 stabilize ICD protein [81]. Dominant-negative SEL-10 potentiates ICD activity on Su(H)-dependent reporters [81,74], while full-length SEL-10 reduces transcriptional activity [82]. These observations coupled with genetic evidence suggest that SEL-10-mediated ubiquitination does not activate Notch transcription but primarily acts to promote ICD turnover.

A HECT-type ubiquitin ligase known in flies as Suppressor of Deltex (Su(dx)) and in mouse as Itch also ubiquitinates Notch ICD [83]. Although genetic evidence indicates that Su(dx) negatively regulates Notch signaling [87], the phenotype of $Itch^{-/-}$ mice, which exhibit inflammation and immune defects [88,89], does not immediately suggest a role in Notch signaling. Therefore the relevance of Itch to Notch signaling *in vivo* in vertebrates remains unclear.

Notch Transcriptional Activity *In Vivo*

Biochemical analysis of the Notch transcriptional complex has revealed the mechanism by which ICD converts Su(H) from a transcriptional repressor to an activator. We next consider how this transcriptional complex alters gene expression during Notch signaling based on *in vivo* analysis of Notch-responsive enhancers. This analysis has been primarily pioneered in *Drosophila* where a large number of Notch-responsive enhancers have been characterized molecularly. One issue addressed by these studies is whether ICD regulates transcription by simply promoting derepression of Su(H), or whether it also promotes transcriptional activation by recruiting coactivators. These two possibilities can be distinguished genetically in *Drosophila* because they predict that Su(H) loss-of-function mutants will exhibit increased Notch signaling phenotypes in the former case, but decreased Notch signaling phenotypes in the latter. We review here cases in which Notch acts by derepression, although even in these cases, Notch signaling also acts by switching Su(H) to a transcriptional activator.

A second issue addressed here is how Notch activates expression of targets in a context-dependent fashion. For example, Notch signaling directly regulates related genes that encode bHLH repressors in the *Enhancer of Split* locus even though these genes differ dramatically in both their spatial and temporal expression patterns during development. This finding implies that specificity in Notch signaling is derived from other factors, which act in combination with ICD. We discuss here support for such a combinatorial mechanism, which has been observed in *Drosophila* on both native and artificial Notch-responsive enhancers. Finally, we conclude with a brief discussion of vertebrate Notch response elements and enhancers, some of which reflect the molecular organization and/or activities seen in flies. Table II provides a summary of Notch response elements including bona fide Notch enhancers.

Table II Notch Responsive Enhancers/Promoters[a]

Target gene	Function	References
Dm Pax2	Enhancer	30
Dm m4	Enhancer	29
Dm E(spl)mγ	Enhancer	28
Dm E(spl)m8	Enhancer	95, 99
Dm sim	Enhancer	44, 96
Dm yan	Enhancer	100
Dm vestigial	Enhancer	31
Xenopus hairy2	Enhancer	26
Human, mouse *pre-TCRα*	Enhancer	101
Mouse *Lunatic fringe*	Enhancer	97, 98
Mouse *HES1*	Response element	102
Mouse *HES5*	Response element	103
Mouse *HES7*	Response element	104
Mouse *HERP1*	Response element	105
Mouse *HRT2*	Response element	106
Mouse, human *Hey1*	Response element	107
Human *NFkappaB2*	Response element	108
Human *ERBB-2*	Response element	109
Human *IL-6*	Response element	110, 111
Mouse *p21*	Response element	43
Rat, human *Cyclin D1*	Binding Site	112
Human β-globin LCR	Binding Site	32

[a]Function is defined as follows: enhancer, tissue specific enhancer responsive to Su(H)-dependent Notch signaling *in vivo*; response element, genomic fragment from a likely Notch target gene containing Su(H) binding sites and responsive to ICD activation, Su(H)-mediated repression, or both in cultured cells; binding site, element DNA binding Su(H) *in vitro* and derived from genomic DNA flanking a potential Notch and/or Su(H) target gene.

Repression by Su(H)

The role of Notch in relieving Su(H)-mediated repression is supported by the analysis of the *Drosophila singleminded* (*sim*) gene, which is expressed in a single row of cells in the embryonic ventral mesectoderm. High levels of *sim* expression within this single row of cells require Su(H)-dependent Notch signaling [44]. However, expression of *sim* in Su(H) null mutants is not only reduced, as one would predict, but also ectopic, suggesting that in the absence of Notch signaling Su(H) represses *sim* expression in cells adjacent to the stripe. This possibility was tested by characterizing sequences in the *sim* enhancer required for proper transgene expression. The *sim* enhancer contains 10 Su(H) sites, 6 of which are high-affinity sites, and several in proximity to binding sites for two other positive regulators of *sim* expression, Dorsal and Twist, and to binding sites for a negative regulator, Snail [44]. When all 10 Su(H) sites are mutated, the enhancer no longer drives high levels of expression at the midline but is also expressed ectopically in adjacent cells in a manner analogous to the *sim* expression pattern in Su(H) null flies. These data suggest strongly that Su(H) not only mediates activation of target gene expression in areas of Notch signaling, but also repression of genes outside this area where Notch signaling is silent.

Transcriptional repression mediated by Su(H) is also revealed by analysis of a synthetic enhancer containing different combinations of binding sites for the bHLH protein Grainyhead and for Su(H) [90]. In general, reporters driven by Grainyhead sites alone are expressed widely and at moderate levels. When Grainyhead sites are combined with Su(H) sites, expression of the enhancer is inhibited in tissues where Notch signaling is absent, suggesting that Su(H) without ICD represses the activity of Grainyhead. However, transgene expression driven by the same enhancer is robust in regions where Notch signaling and Grainyhead expression coincide, suggesting that ICD not only depresses Su(H) but cooperatively interacts with Grainyhead to activate transcription. These findings support those observed with the *sim* enhancer, revealing a role of Su(H) mediated repression in preventing ectopic expression of Notch target genes.

Importantly, in both of these examples, mutating Su(H) binding sites, or genetically removing Su(H), is insufficient to drive high levels of Notch target gene expression in the normal pattern, indicating that transcriptional activation by ICD is also needed. The only case in which Notch signaling has been proposed to act solely by promoting derepression is in a process known as *proneural enhancement*, in which Notch signaling is required to promote proneural gene expression in the developing eye [91]. Notch signaling in this case can be phenocopied by removing Su(H) genetically, indicating that Notch is required only for derepression [91]. However, this interpretation may be premature until more is known about whether or not this regulation is direct instead of a complex outcome of Su(H) regulation of other genes.

ICD Activates Target Genes in Combination with Other Activators

In the majority of Notch-responsive enhancers examined thus far, Notch signaling in combination with Su(H) is required for high levels of tissue-specific expression. Significantly, data from these *in vivo* studies also indicate that many target genes are fairly refractory to signaling by Notch alone. For example, transgenic flies expressing reporters driven by multimerized Su(H) sites exhibit little transgene expression in *Drosophila* larval tissues, even in areas positive for Notch signaling, suggesting that target activation *in vivo* requires input from Su(H)/ICD in combination with other factors [11,12,92]. Analysis of this combinatorial input in a number of different enhancers reveals synergistic transcriptional interactions among ICD/Su(H) and a variety of different transcriptional activators.

Drosophila m4

An extensively characterized family of Notch target genes in *Drosophila* is clustered within the *E(spl)* complex; these genes are closely related and likely arose by recent

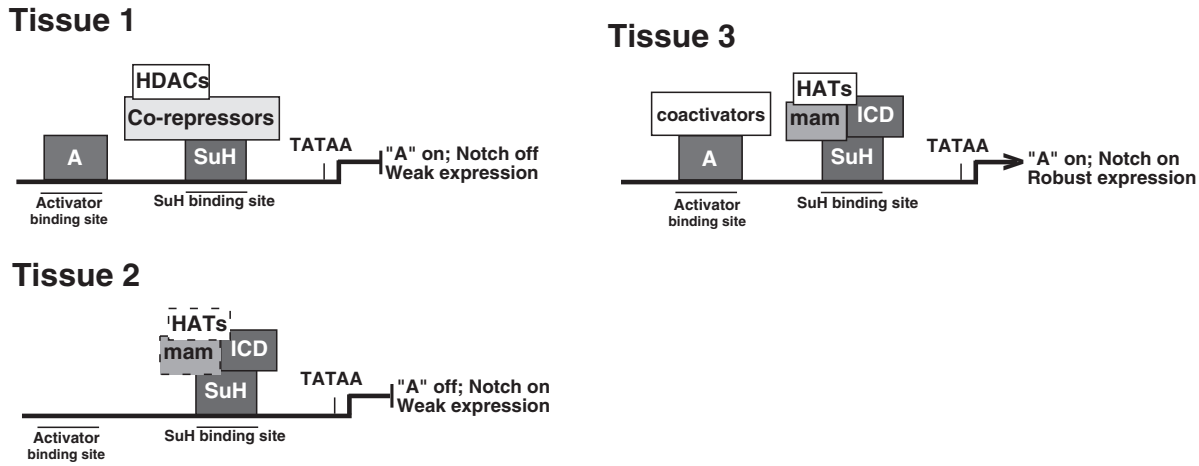


Figure 3 Hypothetical Notch responsive enhancer requiring cooperative interactions. Interactions on this element are shown in three different tissues: Tissue 1, in which Notch signaling is off, and Tissues 2 and 3, in which Notch signaling is on. For simplicity one heterologous activator (A) is depicted, although interaction with SuH/ICD by more than one cooperating activator is not precluded (see text). Likewise, SuH could bind to several sites in the enhancer. In Tissue 1 the target gene is either not expressed or expressed very weakly because potential activity mediated by A bound to the enhancer is repressed by corepressors interacting with SuH. Despite the interaction of ICD with SuH in Tissue 2, the absence of heterologous activators prevents cooperative interaction required for robust activation of transcription in vivo. Whether the co-activator mastermind (mam) or HATs interact with ICD in this condition is unknown; therefore, those potential interactions are represented by dashed lines. Robust activation of transcription can only occur in Tissue 3, where Notch signaling is on, heterologous activator proteins are bound to the enhancer, and coactivators functioning cooperatively are likely recruited by both. Such a model based on cooperativity does not rule out hypothetical activation states represented in figure 2; for example, proneural enhancement could occur in Tissue 1 if Notch signaling relieved SuH-mediated repression, allowing A and accompanying co-activators to activate transcription.

gene duplication [29,93,94]. The response of these genes to Notch signaling depends on Su(H) binding sites, many of which are found in a so-called paired arrangement consisting of two inverted Su(H) binding sites, separated by 30 bp and containing a conserved hexamer motif (GAAAGT). Mutational analysis of the enhancers for several *E(spl)* genes, including *m4* and *mγ*, shows that both the Su(H) binding sites and the hexamer are required for tissue-specific gene expression. However, the *m4* and *mγ* enhancers, as well as those of other *E(spl)* complex genes, also contain E-box motifs recognized by proneural bHLH proteins in proximity to both paired and isolated Su(H) binding sites [28,29,95]. Mutating these sites also results in a marked reduction in enhancer activity [29]. Enhancer activity is similarly reduced in flies mutant in either candidate proneural proteins or Su(H), indicating further that both proteins cooperate to promote expression of these Notch target genes [28,29,94]. A model for a Notch enhancer exhibiting such cooperative activity is shown in Fig. 3.

DROSOPHILA PAX2

Combinatorial activation by Notch has also been demonstrated by analyzing the *Drosophila* eye-specific enhancer of the gene encoding the transcription factor Pax2 (DPax2). This ~350-bp enhancer accounts for the *DPax2* expression in cone cell precursors in the larval eye disc and contains binding sites for three potential effectors; namely, three binding sites for the runt-domain transcription factor Lozenge, six Ets factor binding sites, and eight consensus Su(H) binding sites. Many of these sites are clustered within 100 base pairs of one another [30]. Disabling all three pathways independently indicates that Lozenge binding, EGF signaling (required to activate the Ets factor Pnt), and Notch signaling through the Su(H) sites are all required for eye-specific expression of *DPax2*. The marked loss in enhancer activity seen when one pathway is disrupted suggests that the activity of Lozenge, Pnt, and ICD/Su(H) is not only combinatorial but synergistic.

DROSOPHILA SIM

Cooperativity between Su(H)/ICD and the transcription factor Dorsal has also been demonstrated on the same *sim* enhancer element described earlier [96]. Just as ICD is required for high levels of *sim* expression [44], disabling mutations in Dorsal block *sim* expression in the mesectodermal stripe [96]. Ectopic expression of high levels of activated Notch rescues *sim* expression in Dorsal mutant embryos but very weakly; however, high expression of ectopic ICD in fly embryos expressing low levels of Dorsal robustly induces *sim* expression, indicating cooperation between ICD and Dorsal. This finding is remarkable given that the *sim* enhancer, like the eye-specific *DPax2* enhancer, contains many Su(H) binding sites, most of them high affinity and likely functional, and shows that without Dorsal activity even high levels of ectopic ICD are insufficient to activate transcription through these sites. Clearly, low levels of ICD and Dorsal pack more transcriptional punch than high levels of ICD alone, either due to architecture of the enhancer, a requirement for ICD/Dorsal cooperativity, or both.

Vertebrate Notch Response Elements

Compared to *Drosophila*, relatively little is known about enhancers that drive the expression of vertebrate Notch target genes. Nonetheless, likely targets of Notch signaling have been identified by isolating flanking sequences of vertebrate homologs of genes regulated by *Drosophila* Notch, such as those that encode the bHLH repressors in the Enhancer of Split Complex (*E(spl)-C*). One such mouse gene, called *HES1*, is likely regulated by Notch in part, and the sequence upstream of the start of transcription contains high-affinity, paired Su(H) binding sites similar to that found in the *Drosophila E(spl)-C* genes. In transient transfection assays, this element responds to activated Notch. However, whether these binding sites constitute part of a Notch-responsive *HES1* enhancer element *in vivo* has not been determined.

One characterized Notch-responsive enhancer in vertebrates is a *Xenopus* gene called *hairy2*, which encodes a homolog of the bHLH encoded in the ESPL-C [26]. *Hairy2* is expressed in the frog neural plate and transiently in a narrow anterior stripe of the presomitic mesoderm (PSM) immediately prior to formation of a somite. Reminiscent of the *m4* enhancer and *HES1* proximal promoter, the *hairy2* proximal promoter contains paired Su(H) binding sites flanking a hexameric motif. Frogs transgenic for a reporter driven from this element express the transgene in a pattern recapitulating *hairy2* expression in the neural plate and PSM. Mutation of either Su(H) site or the hexamer alone abolishes the PSM stripe pattern of transgene expression. At first glance, the fact that Su(H) consensus sites in the 90-bp element function as a tissue-specific *hairy2* enhancer seems to argue against the cooperativity model, because consensus binding sites for activators other than ICD/Su(H) are not apparent based on sequence analysis of the region. Nonetheless, unidentified factors binding to the hexameric element or elsewhere could cooperate with or be required for Su(H)/ICD activation of this enhancer.

A direct Notch target in vertebrates that is not a *HES/E(spl)* gene is the gene encoding the glycosyltransferase Lunatic fringe (Lfng), which modifies and modulates the activity of the Notch receptor. In the mouse and chick presomitic mesoderm, the expression of Lfng oscillates periodically in synchrony with somite formation [113], and a 110-bp upstream element from the *Lfng* 5′ flanking sequences containing both E-box motifs and a single Su(H) binding site drives oscillating expression of a reporter gene in transgenic mice [97,98]. Mutation of either the E boxes [97] or the Su(H) binding site [98] disrupts the cyclic expression pattern of the reporter in the presomitic mesoderm, strongly suggesting that input from both E-box recognizing proteins and Notch signaling is required to up-regulate periodic *Lfng* expression. As is the case with *sim*, *DPax2*, and a number of *E(spl)* genes noted earlier, these observations strongly suggest that in vertebrates as well as *Drosophila* Notch signaling functions in a combinatorial fashion with other transcription factors to up-regulate target genes.

Conclusion

It is clear from the study of Notch signaling in development that Notch transcription is often extremely dynamic as well as versatile in the activation of targets. In this chapter, we have reviewed recent literature analyzing the biochemical activities that underlie Notch transcription, with the hope that themes would emerge to account for such behavior. One such theme is that a multiplicity of cofactors is associated with the Notch transcriptional complex. Some of these cofactors such as mam are required for ICD-mediated transcription in many if not most cases. Others such as SKIP, Nrarp, HATs, and modifying activities such as kinases may be required for transcriptional activity *in vivo* but also may play important regulatory roles in both turning on and turning off the transcriptional complex. A second theme is that Notch seems to function as an on/off switch in that it converts Su(H) from a repressor into an activator. This switch presumably accounts for the large dynamic range over which Notch regulates expression of its targets during development. A third theme is that the up-regulation of Notch target genes is in many cases the result of combinatorial activity between ICD/Su(H) and other transcription factors. This feature of Notch transcription presumably accounts for the diversity of Notch target genes in different developmental contexts. Future studies will need to combine an evaluation of biochemical activities associated with Notch transcription *in vitro* with the analysis of Notch target genes *in vivo* to account for both dynamic and versatile activity.

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CHAPTER 295

The β-Catenin: LEF/TCF Signaling Complex: Bigger and Busier than Before

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Introduction

The Wnt/Wingless pathways regulate genes that control cell fate, proliferation, and apoptosis, as well as tissue and organ identity in many organisms. The best characterized Wnt pathway targets the armadillo-repeat protein, β-catenin (β-cat), which functions as a dedicated coactivator of the LEF-1/TCF HMG enhancer-binding proteins. In this pathway, Wnt signaling through the Frizzled:LRP (lipoprotein-related protein)-5/8 receptors inactivates the APC:Axin:GSK3 complex that would otherwise phosphorylate β-cat, marking it for degradation through the ubiquitin-proteasome machinery. The newly stabilized hypophosphorylated β-cat enters the nucleus and interacts with LEF/TCF proteins to induce downstream target genes in a manner dependent on a strong transcription activation domain located at the C terminus of β-cat.

Interestingly, recent genetic and molecular studies in *Drosophila* reveal that nuclear Wnt transcription complexes also incorporate at least two other coactivators: Legless (human Bcl9) and a PHD-domain protein termed Pygopus. Both of these factors appear to be required universally for the induction of Wnt target genes, and function in a Wnt/Wg pathway-specific manner. The different components of the Wnt enhancer complex are likely to control many different steps in transcription, from the initial binding of the complex to chromatin, leading to activation and the subsequent disassembly of the complex or its reassembly with corepressors to return to the repressed state. Importantly, the failure to properly control β-cat underlies the oncogenic activation of the Wnt transcription pathway in colon cancers, melanomas, and other human cancers arising from mutations in β-cat or the Axin:APC complex. In this chapter we examine recent advances in understanding β-cat regulation and its function as a dedicated transcriptional coactivator in Wnt signaling.

Regulated Proteolytic Turnover of β-Cat

The level of free cytosolic β-cat is tightly controlled by phosphorylation-directed ubiquitination and proteolysis carried out by several different destruction pathways, only one of which is sensitive to Wnt signaling. The Wnt-responsive destruction pathway is outlined schematically in Fig. 1 (for recent reviews, see [1–5]). As part of a multiprotein scaffolding complex that contains the adenomatous polyposis coli (APC) tumor suppressor and Axin, β-cat undergoes a series of sequential N-terminal phosphorylation events that are essential for its recognition by the F-box β-TrCP ubiquitin ligase complex (see review in [6]). First, phosphorylation by casein kinase 1 (CK1α or CK1ε) at Ser-45 in the amino terminus [7,8] primes β-cat for sequential phosphorylation at residues 41, 37, and 33 within the amino terminal "destruction box" motif (D-**S**33-G-I-H-**S**37-G-A-T-**T**41-T-A-P-**S**45-L-S) by glycogen synthase kinase 3β (GSK3β). The β-TrCP ubiquitin ligase specifically recognizes S33- and S37-phosphorylated β-cat for ubiquitination [9]. In support of this two-kinase mechanism, oncogenic mutations in β-cat have been described at both the CK1 and GSK3β-phosphorylation sites [5,6]. However, important questions remain as to whether CK1α or CK1ε is the relevant isoform,

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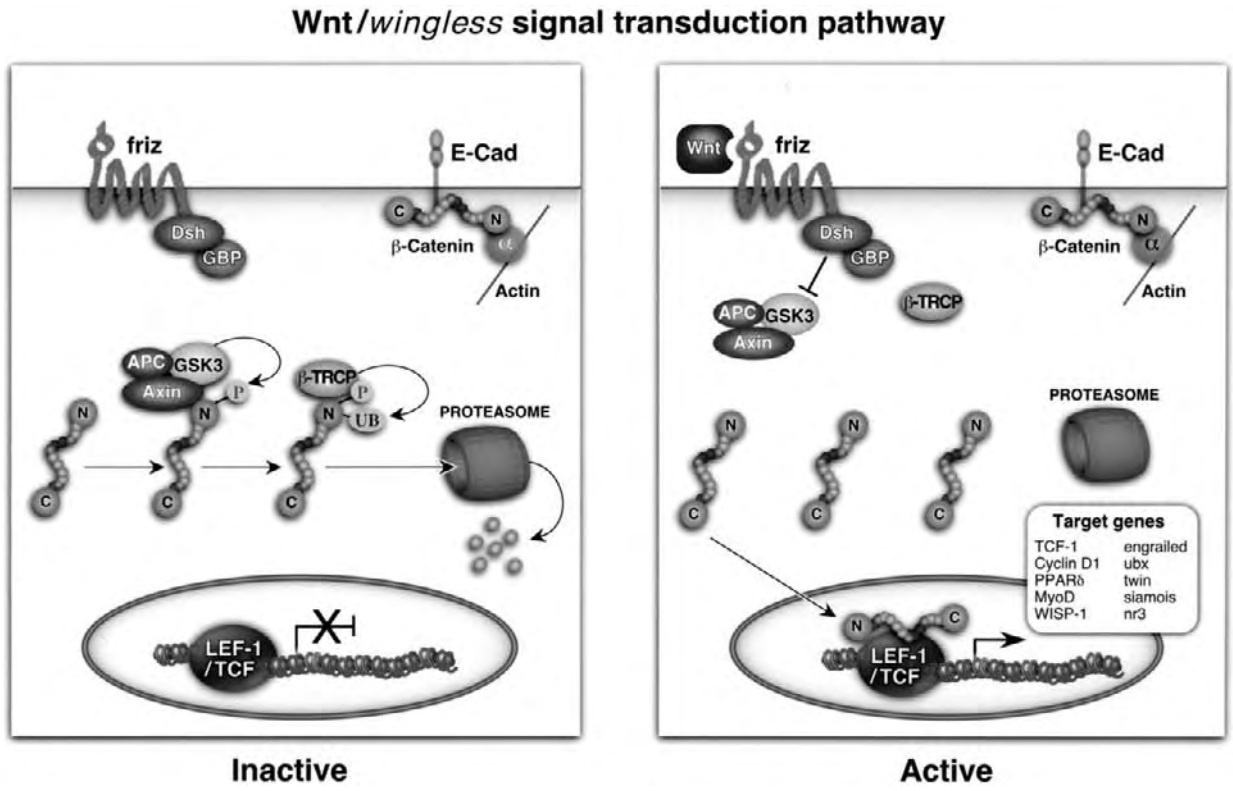


Figure 1 Overview of the canonical Wnt/Wg signaling pathway. A fraction of β-cat is tethered to the plasma membrane by E-cadherin and α-actin. Cytoplasmic free β-cat is rapidly phosphorylated by CK1 and GSK3β in the multiple-subunit Axin:APC complex, ubiquitinated by β-TrCP, and degraded by the ubiquitin-dependent proteosome degradation pathway (Inactive). The Wnt class of secretory glycoproteins binds to the Frizzled transmembrane receptors, which signals through Disheveled to inhibit the GSK3β phosphorylation. The stabilized and underphosphorylated β-cat enters the nucleus, binds to LEF1/TCF proteins, and activates the transcription from target genes (Active).

and how this protein kinase may become recruited to the Axin: APC complex. For example, CK1 has been reported to interact directly with Axin [7], or to be recruited to Axin through the ankyrin repeat protein Diversin [10], and alternative scenarios not involving Axin have also been suggested [6].

The central event in Wnt signaling is the inactivation of the APC:Axin scaffolding complex upon binding of Wnt ligands to the Frizzled/LRP coreceptor complex. The Disheveled (Dsh) protein plays a key intermediary role at this step, moving to the membrane to bind Axin and inhibit the activity of the complex. At present it is not clear whether the complex actually disassembles, or even if GSK3β is the direct target for inactivation within the complex. Binding of Dsh to GBP/Frat-1 might displace GSK3β from the complex or, alternatively, the mechanism may involve the Dsh-associated PP2C or Axin:APC-associated PP2A enzymes. Interestingly, phosphorylation at S45 is not affected by Wnt signaling, and S45-phosphorylated β-cat can enter the nucleus [7,8], indicating that β-cat can associate with the Axin:APC complex and undergo phosphorylation by CK1 during Wnt signaling, but fail to undergo the step that couples CK1 with GSK3β.

Additional mechanisms also contribute to the rapid proteolytic degradation of free β-cat in cells. Unexpectedly, a recent study found that presenilin (PS1) can also function to couple S45-phosphorylated forms of β-cat with GSK-3β, thereby modifying β-cat as a substrate for degradation [11]. In this case, phosphorylation at S45 is thought to be carried out by protein kinase A (PKA). Loss of presenilin enhances β-cat stability *in vivo* and disrupts phosphorylation by GSK3β, and activation of PKA by forskolin strongly enhances phosphorylation at S45 *in vivo* and *in vitro* [11]. In addition, the p53-inducible F-box protein Siah-1 can regulate nuclear β-cat levels in DNA-damaged cells (reviewed in [12]). However, neither of these destruction pathways is subject to regulation by Wnt signaling. Rather, these pathways seem to serve as alternative mechanisms to control β-cat molecules that escape the Axin:APC axis.

The subcellular distribution of the various regulatory components also plays a role in the dynamics of Wnt signaling. APC, like β-cat, is present in the nucleus and cytoplasm, and can actively shuttle between the two compartments (for recent reviews, see [2,13,14]). Phosphorylation by CK1ε may enhance binding of β-cat to LEF/TCF [7], whereas phosphorylation by GSK3β inhibits its transcriptional activity *in vivo* and *in vitro* [15,16]. Although most models indicate that degradation occurs in the cytoplasm, the β-TrCP ubiquitin ligase complex is predominantly nuclear. Nuclear β-TrCP exists in a stable complex with hnRNP-U, but dissociates in the presence of high-affinity substrates [17]. Thus β-TrCP must shuttle between compartments to ubiquitinate β-cat in the cytoplasm. In addition, β-cat may resemble other short-lived activators and undergo phosphorylation and ubiquitination

in the nucleus as a consequence of transcriptional activation (for a review, see ref [18]). One possibility is that the differently modified forms of β-cat may arise separately in the nuclear and cytoplasmic compartments, yet undergo a similar proteolytic fate in the cytoplasm.

Regulation of the Wnt-Assembled Enhancer Complex in the Nucleus

Upon Wnt signaling, β-cat enters the nucleus and associates with LEF-1/TCF proteins (lymphocyte enhancer factor/T-cell factor; LEF-1, TCF1, TCF3, TCF4) (reviewed in [19,20]). These factors are monomeric high mobility group (HMG) sequence-specific DNA-binding proteins, frequently classified as architectural owing to their ability to bend DNA and recognize highly distorted DNA sequences. The LEF-1/TCF proteins are transcriptionally inactive on their own, but activate transcription strongly in association with β-cat. In T cells, the LEF/TCF proteins synergize with other T-cell factors to activate specific T-cell enhancers, but function independently of β-cat in this context [19,20].

In the absence of Wnt signaling, the LEF-1/TCF proteins repress Wnt gene expression through assembly with corepressors such as Groucho or the C-terminal binding protein (CtBP) [21–23], both of which recruit histone deacetylases to maintain chromatin in a repressed state (Fig. 2). Osa-containing Brahma chromatin remodeling complexes are also required for repression of Wnt target genes *in vivo* [24]. Interestingly, different LEF-1/TCF gene products and spliced isoforms appear to play dedicated roles in activation, inhibition, or repression of Wnt target genes [22,25,26]. For example, TCF3 functions as a potent repressor of Wnt signaling, even though it retains the ability to bind β-cat, potentially reflecting a higher intrinsic affinity for the CtBP corepressor [26]. The LEF/TCF proteins are also differentially regulated by Wnt signaling in transformed cells. Activation of the Wnt pathway up-regulates the expression of LEF-1 without affecting transcription from an adjacent promoter that directs expression of a truncated LEF-1 protein termed ΔNLEF. The ΔNLEF protein is unable to bind β-cat, and functions as a feedback inhibitor of Wnt signaling *in vivo* [27,28]. Therefore, the relative levels of different LEF/TCF gene products or isoforms can significantly affect the level of expression of Wnt target genes.

The activity of the Wnt enhancer components in the nucleus is also subject to regulation by posttranslational modifications and small regulatory peptides. For example, LEF-1 was recently shown to undergo modification by small ubiquitin-like modifier (SUMO) peptides, which causes it to become sequestered in an inactive form within PML/ND10 nuclear bodies [29]. Phosphorylation of LEF-1/TCF proteins by mitogen-activated protein kinases has been shown to block binding to DNA and may also affect proteolysis (reviewed in [2–4]). A small peptide inhibitor, ICAT, interacts directly with β-cat ARM repeats and is a strong and selective inhibitor of LEF-1: β-cat activity *in vivo* [30] and *in vitro* [16,30,31].

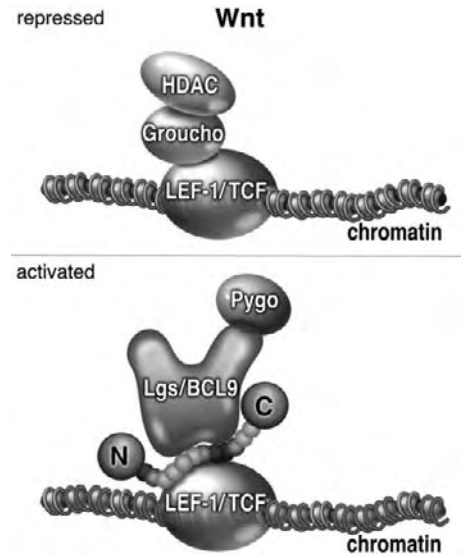


Figure 2 Repression of Wnt target genes is mediated by LEF/TCF isoforms that preferentially bind or function with Groucho or CtBP corepressors, and involves the recruitment of HDACs (top panel) as well as chromatin remodeling complexes (not shown). Upon signaling through the Wnt pathway, β-catenin assembles with LEF/TCF proteins and supplies a strong constitutive activation domain. The complex that forms on chromatin may also include Legless (human BCL9) and Pygopus [41–44].

ICAT can block the ability of β-cat to interact with LEF-1 [30,31] and can also interfere with transcriptional activation subsequent to binding of the complex to chromatin, potentially through interfering with the recruitment of transcriptional coactivators to the LEF-1:β-cat complex [16]. The ability of LEF-1/TCF to bind DNA can also be disrupted by the inhibitor of myogenic basic helix–loop–helix proteins, I-mfa [32], however this regulator, unlike ICAT, is not specific to the Wnt pathway.

Upon binding to LEF/TCF proteins, β-cat supplies a strong activation domain located within the C terminus of the protein. Although the specific target for the activation domain has not yet been identified, putative coactivators for β-cat include CBP/p300 [33–36], Brg-1-containing SWI/SNF chromatin remodeling complexes [37], pontin 52 [38], and the TATA-binding protein, TBP [39]. The central core of the β-cat molecule contains 12 armadillo (ARM) repeats that mediate mutually exclusive binding interactions with LEF/TCF, E-cadherin, APC, and other proteins required for Wnt signaling [40]. *In vitro*, recombinant β-cat interacts specifically with LEF-1 and the two proteins bind in a highly cooperative manner to nucleosomal templates [16]. In addition, β-cat strongly enhances transcription by LEF-1 in a chromatin-dependent manner on a minimal enhancer composed of reiterated LEF/TCF binding sites *in vitro.* An extended region of the C terminus of β-cat, including ARM repeats 11–12, appears to constitute the activation domain *in vitro*, and transcription *in vitro* is potently and selectively inhibited by a fragment of the CT-ARM domain, as well as by the physiological inhibitor ICAT. Although CBP/p300 is a positive coactivator of LEF-1:β-cat activity *in vivo* and *in vitro* and

has been reported to interact directly with β-cat [34], nuclear p300 does not interact directly with β-cat in pull-down experiments, and, moreover, the purified LEF-1:β-cat complex is insufficient to direct p300-mediated histone acetylation on nucleosomal templates *in vitro* (R. Landry and K. A. Jones, unpublished data). Moreover, although binding of LEF-1:β-cat to chromatin requires ATP-dependent chromatin remodeling, purified SWI/SNF is unable to carry out this step alone *in vitro* [16]. Consequently the identity and ordered recruitment of specific coactivators by the β-cat transactivation domain remain to be clearly established.

Enter Pygopus and Legless (hBcl9)

Among the most important and exciting advances in the past year were the identification of Legless (Lgs) and Pygopus (Pygo) as two new essential nuclear components of the Wnt/Wg signaling pathway [41–44]. Basler and colleagues [41] identified Lgs as a genetic suppressor of an eye phenotype that arises from high levels of Wg signaling in *Drosophila*. Molecular cloning of Lgs revealed it to be a homolog of the Bcl-9 oncogene, which was originally identified by the translocation t(1;14) (q21;q32) from a patient with precursor-B-cell acute lymphoblastic leukemia, and has been implicated to play a role in the development of non-Hodgkin's lymphomas. Pygo was identified by several groups genetically as a suppressor of an activated ARM phenotype in flies [42–44], and was independently uncovered by the Basler group [41] in a two-hybrid screen for proteins that interact with Lgs. Mutant fly embryos that lack either Lgs or Pygo generate phenotypes that resemble loss of wingless. The functions of Pygo and Lgs appear to be largely specific to the Wg signaling pathway in flies, and both proteins are universally required in Wg-responsive cells and tissues throughout development [41–44]. Thus a Wnt/Wg signal cannot be transmitted in the absence of either protein *in vivo*.

Further genetic analyses revealed that both Lgs and Pygo function downstream of ARM in flies. Although nuclear levels of ARM/β-cat are somewhat reduced in cells lacking Pygo, the primary defect appears to be a loss in transcriptional activation mediated through the β-cat:LEF/TCF protein complex. Interestingly, Pygo is a strong activator of β-cat:LEF/TCF activity in transient expression experiments, whereas Lgs is comparatively inactive [41,42]. Therefore, Pygo may be more limiting than Lgs in cells or, alternatively, the normal function of Lgs may be effectively bypassed when Pygo is expressed at very high levels in the cell.

Pygopus is a large nuclear protein that contains an N-terminal nuclear localization sequence and C-terminal plant homeotic domain (PHD) motif, also known as a leukemia-associated protein (LAP) domain [41–44]. The PHD domain is essential for Pygo function, although its role in the transactivation mechanism remains unclear. PHD domains coordinate zinc through a C4HC3 motif that is distantly related to the RING and FYVE finger domains. PHD domains are frequently found in chromatin-binding proteins; however, no specific conserved function has been ascribed to this motif. Interestingly, a PHD-containing protein, Pf1, appears to link Groucho/TLE corepressor proteins with the Sin3A histone deacetylase complex [45]. The PHD domain of Pf1 is critical for its function, but does not mediate binding to Sin3A, and its role is unclear. Similarly, the PHD domain of Cti6 is required for switching of coactivator and corepressor complexes, but does not mediate protein–protein interactions between the two complexes [46]. The PHD domain of the unrelated MEKK1 protein functions enzymatically as an E3 ubiquitin ligase required for the proteolytic turnover of ERK1 [47], whereas the PHD domain of p300 is required for the structural integrity of the histone acetyltransferase domain [48].

In the model proposed by Basler *et al.* [41], the PHD domain of Pygo mediates a direct interaction with Lgs. Moreover, the N-terminal half of Lgs was reported to interact both with Pygo and with ARM/β-cat, through interactions involving the HD1 and HD2 domains of Lgs, respectively (Fig. 3). A mutant form of Lgs protein lacking HD2 functions as a strong dominant-negative inhibitor of Wg signaling *in vivo*. These observations indicate that Pygo may be tethered to the β-cat:LEF/TCF complex through binding to Lgs [41]. In this scenario, Pygo is postulated to stimulate transcription through an activation domain (NHD) located at the amino terminus of the protein [41]. An alternative possibility, proposed by Bienz *et al.* [42], suggests that the PHD domain in Pygo facilitates the binding or transcription activation function of the β-cat:LEF/TCF complex on chromatin. The Bienz group [42] suggests that Pygo may enter the complex indirectly or through binding to TCF proteins, and they further indicate that the conserved amino terminal sequences of Pygo most likely constitute a nuclear localization sequence. Consequently, the exact details concerning the function and mode of recruitment of Lgs and Pygo to the β-cat:LEF-1 complex remain, for the moment, open to debate. Taken together, these recent findings constitute a major advance in the field, particularly because they radically alter previous notions about the composition of the multiple-subunit enhancer complex that assembles on chromatin in response to Wnt signaling. The discovery of Lgs and Pygo also provides exciting new targets for anticancer drugs that may be able to selectively block Wnt signaling at target genes. Looking back at these remarkable accomplishments, we can anticipate that further mechanistic studies of Lgs, Pygo, and β-cat will continue to lead the field in interesting and unanticipated new directions.

Perspectives

We have presented a summary of the current understanding of the key regulated events in the Wnt/Wg pathway. One of the key challenges for the future will be to characterize biochemically the different multiple-subunit complexes that engage β-catenin, from the Axin:APC complex

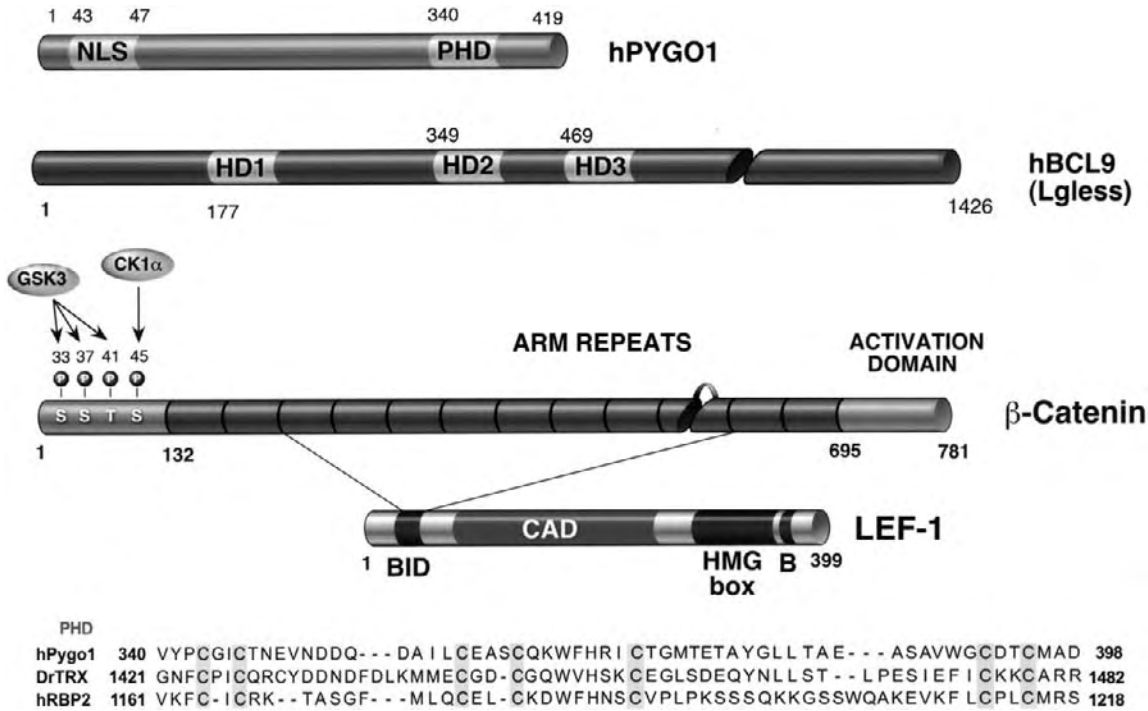


Figure 3 Domain structures of the regulatory components of the Wnt enhancer complex. The LEF-1/TCF proteins mediate sequence-specific binding to DNA through their HMG domain, and recruit β-catenin through the N-terminal β-cat interaction domain (BID). LEF-1 recognizes Armadillo (ARM) repeats 3–10, and structural studies indicate that the interaction does not significantly alter the conformation of β-cat [40]. Emerging studies indicate a critical role for the nuclear Pygo and Lgs in Wnt-directed transcription; however, it is not clear how these proteins are recruited to the β-cat:LEF/TCF complex. A comparative alignment of the critical PHD finger domain of human Pygopus 1 with *Drosophila* Trithorax and human RBP2 is shown schematically at the bottom. Sequences were aligned by the Clustal V method of multiple sequence alignment using DNASTAR.

to the nuclear shuttling complexes and the functional transcription complexes that assemble on Wnt target genes. Biochemical studies of the functions of these various regulatory proteins would also contribute to a deeper understanding of the dynamics of Wnt-initiated transcription. Analyses of the ordered recruitment of regulatory components by chromatin immunoprecipitation and other approaches will also reveal important facets of the dynamics of activation and repression. Continued efforts to build on the outstanding structural information already available for β-catenin and some of the components of the Wnt pathway will define the critical protein contacts in detail and are essential for efforts to develop specific chemical inhibitors of Wnt signaling. It will be interesting to learn whether these regulatory subunits are modified posttranslationally, and how the turnover of β-cat is regulated in the nucleus. Clearly many more surprises await in the future for those interested in understanding the mechanism of the Wnt/Wg regulatory network.

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CHAPTER 296

Cubitus Interruptus

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Introduction

A striking example of multiple signaling pathways eliciting their effects through the regulation of a single transcription factor is seen in the coregulation of embryonic segmental patterning and limb development in Drosophila by the *hedgehog* (*hh*) and protein kinase A (PKA) signal transduction pathways. The Hh signaling cascade activates a series of genes through the transcription factor Cubitus interruptus (Ci). The PKA signaling pathway suppresses the expression of *hh*-responsive genes in cells that do not receive a Hh signal by phosphorylating the 155-kDa form of Ci and targeting it for proteolysis to a 75-kDa repressor form (Ci[rep]). Paradoxically, PKA can further activate Ci-mediated transcription in cells that do receive a Hh signal. Thus both the activation and repression of *hh*-responsive transcription is modulated by the PKA signaling pathway.

Protein Structure and Expression Patterns of Ci

A summary of the Ci functional domains is illustrated in Fig. 1. Both the *ci* gene and its vertebrate homologs *Gli1–3*, encode proteins with five highly homologous, unique, and tandemly repeated zinc fingers that bind DNA through a consensus DNA-binding site [1]. The crystal structure of the Gli1 zinc fingers bound to the DNA-binding site shows that zinc finger 1 does not contact the DNA, whereas the remaining four fingers wrap around the DNA, with fingers 4 and 5 making most of the base contact with the DNA [2]. This conformation might explain the higher homology shared by zinc fingers 3 through 5 among the members in the Gli gene family.

The N terminus of Ci has a repressor function; expression of Ci that is truncated C-terminal to the zinc-finger region in the anterior compartment of the fly embryonic segments causes the down-regulation of *ci* target genes [3]. Ci deleted for the N terminus can function as a transcriptional activator of *ci* target genes when introduced into Drosophila embryos [3]. Mutations in Ci that delete amino acids 611–760 result in proteins that cannot be proteolyzed in response to PKA phosphorylation [4,5]. Likewise, the region between amino acids 675 and 860 is required to maintain Ci in the cytoplasm [6].

Expression of the *ci* gene is zygotic and has a complex pattern during development that includes all embryonic tissues [7]. For this chapter, it is important to note that coincident with the onset of ectodermal segmentation, both the message and the protein are restricted to the anterior compartment of each segment by the *engrailed* (*en*) gene, resulting in 15 broad metamerically repeating stripes. Just prior to germ band shortening, the protein and message expression patterns no longer coincide. Although transcripts are still uniformly distributed throughout the anterior compartment of each segment, the stripe of Ci 155 within each segment is graded such that higher levels of protein are detected in the rows of cells adjacent to the Hh-producing cells of the posterior compartment. Lower levels are seen in the middle of the Ci expressing stripe. This pattern of expression also exists in imaginal disks where *ci* is uniformly transcribed in the entire anterior compartment and higher levels of CI 155 are detected along the anterior/posterior (A/P) boundary.

Regulation of Ci by Hedgehog

Genetic epistasis experiments and cell culture experiments from a variety of laboratories have provided substantial evidence that *hh* signaling establishes A/P polarity in the

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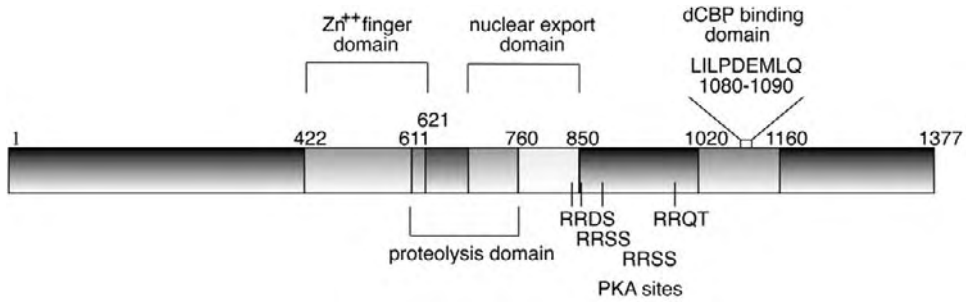


Figure 1 Cubitus interruptus.

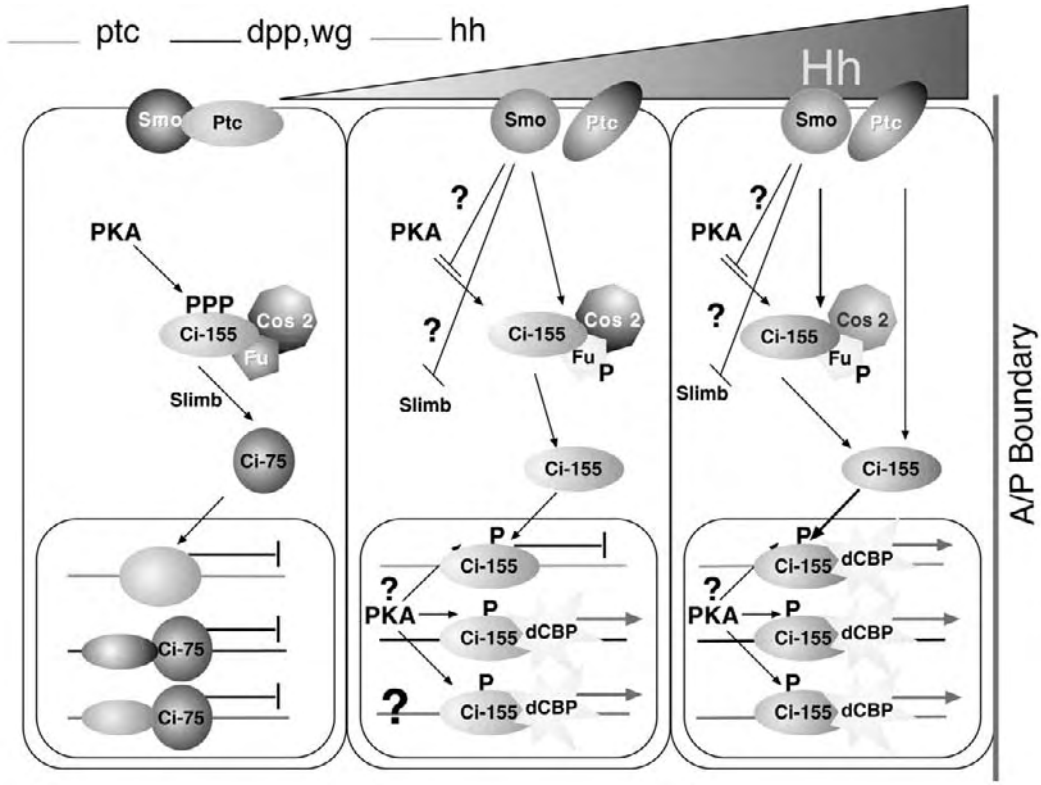


Figure 2 Hedgehog and PKA signaling regulate Ci activity. (A color representation of this figure is available on the CD version of the *Handbook of Cell Signaling*.)

embryonic segments and the developing wing primordia by regulating the transcriptional activity of Ci [3,8–10]. A model summarizing this work is illustrated in Fig. 2. The evidence to date shows that the transmembrane protein Ptc is the receptor for Hh and, in the absence of Hh binding, inhibits the *hh*-signaling pathway [11,12]. The binding of Hh to Ptc relieves the inhibition of Ptc on Smo, a protein that spans the bilayer seven times and the positive transducer of *hh* signaling [13,14]. Hh signaling antagonizes the proteolysis of Ci 155 and converts it to Ci[act], an activator of transcription, in the anterior cells abutting the A/P boundary [4–6]. In the anterior cells outside the range of Hh activity, proteolysis of the 155-kDa Ci generates the 75-kDa repressor, Ci[rep], by cleaving the sequences C-terminal to zinc-finger domain [4–6]. Antibodies that detect both Ci 155 and Ci[rep] stain the entire anterior compartment of the embryonic segments and imaginal disks [4]. At the cellular level, the antibody staining is distributed evenly in both the nucleus and cytoplasm. In contrast, Ci 155 resides primarily in the cytoplasm. The levels are very low in the cells of the anterior compartment that do not receive a *hh* signal, and very high along the A/P boundary that contacts the *hh*-producing cells [7].

The mechanism involved in the *hh* regulation of full-length Ci levels is currently under intense investigation. It has been shown that a domain C-terminal to the zinc-finger domain and N-terminal to the PKA phosphorylation sites is responsible for the retention of the Ci 155 in the cytoplasm [15]. In the absence of Hh signaling, a complex containing the Cos2, Fu, and Su(fu) proteins sequesters Ci in the cytoplasm. In response to Hh signaling, Ci appears to be released from this complex and is free to shuttle in and out of the nucleus [6,15–18]. The integrity of the complex appears critical to the retention of

Ci in the cytoplasm. Either Fu or Su(fu) can be removed without releasing Ci, but if both are missing, Ci is no longer sequestered [19]. The Fu protein plays two opposing roles in this process. The regulatory domain is in part responsible for retaining Ci in the cytoplasm, whereas the kinase function is required for the release of Ci in the presence of Su(fu) [19]. The Ci protein contains both a nuclear localization site (NLS) and a nuclear export signal, both of which account for its shuttling [15,20]. Processing of Ci 155 into the Ci 75 repressor removes the nuclear export signal but not the NLS, resulting in the stable accumulation of Ci 75 in the nucleus.

Regulation of Ci by PKA

PKA negatively regulates *ci* activity in cells that do not receive an *hh* signal by targeting Ci 155 for proteolysis to Ci[rep] [21–23]. Loss of PKA function or inhibition of PKA activity in these cells increases the levels of Ci 155 in both embryos and disks and results in the misregulation of some *hh*-responsive genes [24–28]. Ci has four consensus PKA phosphorylation sites in its C terminus that can be phosphorylated by PKA (Fig. 1). Substitution of the serine with alanine in any one of the first three PKA sites inhibits Ci proteolysis and increases the levels of Ci 155, suggesting that Ci is the direct target of PKA regulation. Recent evidence suggests that phosphorylation of Ci by PKA is a prerequisite for subsequent phosphorylation of Ci by glycogen synthase kinase 3(GSK3) and casein kinase 1 (CK1) and that all three phosphorylation events target Ci for proteolysis to Ci[rep] [29,30]. The *Drosophila slimb* gene is thought to mediate Ci proteolysis and encodes an F-box/WD40-repeat protein that is related to the yeast protein cdc4p, which is involved in targeting cell cycle regulators to the ubiquitination-mediated degradation pathway [31].

Although PKA promotes the generation of Ci[rep] and is thus a negative regulator of *hh*-responsive gene activation, it is an activator of Ci[act] in cells that receive an Hh signal [18,32]. It is not known how the *hh* signal transduction cascade inhibits the phosphorylation or proteolysis of Ci 155 in Hh-stimulated cells, but it is clear that the suppression of proteolysis is not sufficient to activate Ci 155 for all of its target genes [5,20]. For example, Ci that is mutant for the proteolytic cleavage site remains full length but cannot transactivate the Ci target gene *ptc* in the wing disk without an Hh signal. Ci that is mutant for the three PKA sites is a poor activator of *ptc* expression in the wing disk. However, in embryos, Ci that is mutant for the three PKA sites can activate the Ci target gene *wg* and is independent of Hh signaling [22]. Thus, PKA is required to activate Ci[act] for *ptc* expression, whereas Ci 155 is sufficient for *wg* expression in embryos.

Ci Transcriptional Regulation

The Ci target genes identified to date include the following: *wg*, a morphogen that signals to the cells posterior to the A/P boundary and stimulates *hh* expression; *decapentaplegic* (*dpp*), a long-range signaling morphogen of the TGF-β family; and *ptc*, a component of a negative feedback loop and *hh* itself. The context of the Ci target gene promoters defines overlapping but nonidentical sets of targets for Ci[rep] and Ci[act] even though they bind to and regulate the same consensus sequence [33]. Thus, although Ci[rep] and Ci 155 regulate both *wg* and *dpp*, Ci[rep] regulates *hh* and Ci[act] regulates *ptc* [5]. Furthermore, the different Ci targets are sensitive to the level of Hh signaling. Ci[act] is only found in the cells that receive the highest levels of Hh signaling. In the wing imaginal disk, where the targets of Hh signal transduction have been studied in most detail, this occurs in anterior cells directly adjacent to the compartment boundary. These boundary cells have high-level *ptc* expression, late anterior compartment expression of *en*, and activation of *collier*. On the other hand, expression of *dpp*, which is regulated by Ci 155 and Ci[rep], is observed in a much broader stripe. Why do certain targets require Ci[act] while others respond to Ci 155? This is most likely a consequence of the other components assembled on the particular enhancers.

The interaction of Ci with other transcription factors is also responsible for regional and tissue-specific responses to Hh signal transduction. For example the regulation of *dpp* imaginal disk expression is remarkably complex. The continuous *dpp* stripe is actually composed of a series of subelements, each of which is regulated by a distinct enhancer [34]. The *HO* enhancer directs *dpp* expression in the wing pouch because it pairs binding of Ci with that of Arm/dTCF and Vg/Sd [35]. The combined action of these factors is responsible for the final expression pattern. For efficient transactivation, Ci binding must be coupled with the binding of other factors [36]. This makes very good sense, because Hh signaling is utilized in many different contexts and must elicit distinct responses in different tissues. Ci's requirement for additional bound factors may be a consequence of its shuttling out of the nucleus. Even in cells receiving high-level Hh signaling, the nuclear concentration of Ci is low. Therefore, high-level occupancy of an enhancer by Ci may only occur when a prebound factor is present to facilitate Ci binding. Alternatively, it is known that activation of transcription requires the recruitment of multiple components. It is possible that binding of Ci brings certain set of factors to the promoter while the regional and tissue-specific transcription factors recruit complementary components. One coactivator that Ci 155 requires to activate *wg* is Drosophila CREB-binding protein, dCBP [37]. The binding domain for dCBP is in the activation domain and it is thought that Ci[rep] is a repressor, at least in part, because it cannot recruit dCBP to the promoter. However, it is not known whether Ci[act] or all Ci-activated promoter complexes require dCBP for their activity.

A number of questions regarding Ci regulation remain. How does Hh signaling antagonize the proteolysis of Ci? Does it inhibit the different kinases or does it activate a phosphatase? What are the biochemical events that control the release of Ci? Do they involve modification of Ci or the other components of the complex? Finally, what are the

specific interactions of Ci[rep], Ci 155, and Ci[act] at enhancer complexes, and how do these interactions lead to distinct, Hh-signaling responses? Further studies will certainly bring us closer to an understanding of the intricacies involved in the regulation of development by signal transduction cascades.

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CHAPTER 297

The Smads

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Introduction

The transforming growth factor ß (TGF-ß) superfamily of ligands regulates a broad range of biological functions in both developing embryos and adult organisms, and the mechanisms by which these ligands regulate cell-type-specific effects on transcription has been a central problem in TGF-ß biology. The Smads were identified in the mid-1990s as a family of intracellular transducers of TGF-ß signals, and the study of how Smads mediate transcriptional regulation by TGF-ßs has begun to reveal how specificity is achieved for the broad range of biological activities of these ligands (for reviews, see [1–4]).

The founding member of the Smad family, MAD, was identified in *Drosophila* as a modifier of the effects of *dpp*, a *Drosophila* bone morphogenetic protein (BMP) homolog [5]. A related set of genes, Sma1, 2, and 3, were identified as required for signaling by a TGF-ß homolog in *Caenorhabditis elegans* [6], and both homology-based searches and functional screens in vertebrates subsequently identified vertebrate MAD/Sma homologs (reviewed in [7]), defining a broadly conserved family, renamed Smads [8], as transducers of TGF-ß signals.

Families: R-Smads, Co-Smads, and I-Smads

In both vertebrates and invertebrates, the Smads fall into three functional categories (reviewed in [9]): (1) the receptor-regulated R-Smads, which act downstream of specific subsets of TGF-ß superfamily ligands and are directly phosphorylated by Type I receptors; (2) the co-Smads, which are not pathway specific, but which interact with R-Smads in response to ligand stimulation and are required for R-Smad signaling; and (3) the inhibitory I-Smads, which are often induced as early responses to TGF-ß stimuli and which appear to act primarily as feedback inhibitors of signaling. A total of eight Smads have been identified in vertebrates (reviewed in [9]): Smads 1, 2, 3, 5, and 8 are R-Smads, Smad4 (and the closely related Smad10/Smad4ß, to date identified only in frogs) are co-Smads, and Smads 6 and 7 are I-Smads. All the Smads share two distinct regions of homology, an N-terminal MH1 domain and a C-terminal MH2 domain, separated by a linker that varies in size among the different Smad family members. The MH2 of Smad4 crystallizes as a trimer, and biochemical data indicate that Smads can homo-oligomerize via the MH2 domain *in vivo* in the presence or absence of ligand stimulation (reviewed in [10]). The extent to which Smads homo-oligomerize *in vivo* may differ for each Smad and has not been fully worked out [10].

Smad Oligomerization and Regulation by Receptors

Following the binding of TGF-ß ligand to specific type I and type II transmembrane receptors, signaling is initiated when the type I receptor phosphorylates an R-Smad at a conserved C-terminal SSXS motif (Fig. 1) (reviewed in [7]; see also the chapter by J. Wrana in this volume). Smad2 and Smad3 are activated downstream of activin, TGF-ß, and nodal family ligands, whereas Smad1, Smad5, and Smad8 are activated primarily downstream of BMP ligands. The interaction of Smad2 and Smad3 with their upstream type I receptors is mediated by a coupling protein, SARA [11]; whether a comparable Smad-receptor coupling protein exists for the BMP-Smad1/5/8 signaling pathway is not known.

Following phosphorylation by the type I receptor, the R-Smads heterodimerize with the co-Smad Smad4 [12,13] (Fig. 1). Whether the resulting complex is a simple heterodimer or a higher order oligomer as a result of homomeric

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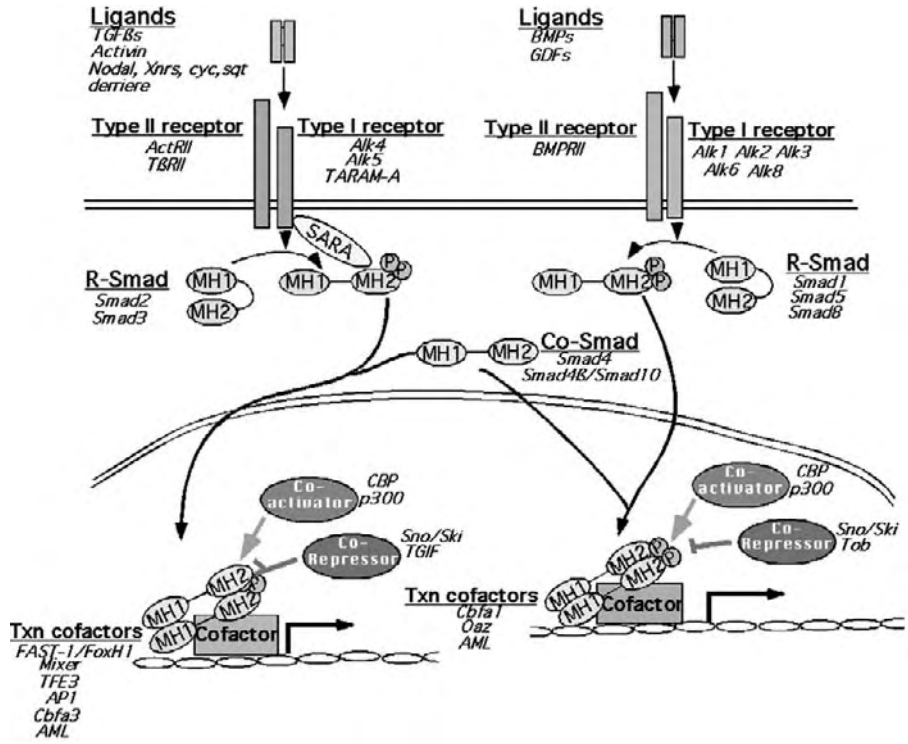


Figure 1 Basics of Smad signaling. The general pathway for transcriptional regulation by Smads is shown. Specific examples of vertebrate components of the Smad pathway are indicated in italics. These examples are not intended to be exhaustive and do not include invertebrate components of Smad pathways. For reviews of invertebrate Smad signaling, see [59,60].

interactions among the Smad MH2 domains (see earlier discussion) is not clear (reviewed in Shi, 2001[10]). The activated R-Smad/Smad4 complex then translocates to the nucleus. This nuclear translocation may be driven by two distinct properties of the R-Smads: (1) a canonical nuclear localization signal (NLS) in the MH1 domain that is unmasked following receptor phosphorylation at the C terminus [14] and (2) nuclear localization of the MH2 domain independent of a canonical NLS, which is inhibited in the unstimulated state by association (in the case of Smad2 and Smad3) with membrane bound SARA [15]. Smad4 is not required for nuclear localization, and in fact contains a nuclear export signal (NES) that may serve to rapidly remove Smad4 from the nucleus following dissociation from the R-Smads [16].

Transcriptional Regulation by Smads

Following translocation to the nucleus, the R-Smad/Smad4 complex can interact with DNA by means of two distinct mechanisms: (1) direct, site-specific binding of DNA by the MH1 domain [17] and (2) interaction with additional site-specific transcription factors [18,19] (Fig. 1). R-Smads and co-Smads preferentially recognize the motif GTCT, although binding to other target sites, particularly GC-rich sequences, has also been reported [10]. Although tandem repeats of the GTCT motif are sufficient to mediate TGF-ß-regulated transcriptional reporter activation, and these motifs are present in naturally occurring TGF-ß responsive promoters, recognition of this 4-bp motif by Smads does not seem sufficient to account for highly specific patterns of transcription in response to TGF-ßs *in vivo*, or for the differing biological effects of activation of different R-Smad pathways. This specificity in transcriptional responsiveness may be achieved primarily by interaction of the R-Smad/Smad4 complex with additional site-specific DNA-binding proteins. Interactions of R-Smad complexes with a wide variety of transcription factors have now been reported [2,3]. Many of these transcription factors are cell type specific, and many interact preferentially with either Smad2/Smad3 [18,20] or with Smad1 [21], providing an explanation for both ligand-specific and cell-type-specific transcriptional responses to TGF-ß superfamily ligands. Binding of the R-Smad/Smad4 complex and additional transcription factors to DNA is often cooperative, further enhancing the specificity and affinity of target site recognition [22–24]. Smad binding to transcription factors is often mediated through the MH2 domain; in one of the best characterized examples of Smad–transcription factor binding, a short α-helical region in the MH2 domain of Smad2 was shown to be a necessary and sufficient determinant of specificity in the binding of Smad2, but not Smad1, to the transcription factor FAST-1 (FoxH1) [25]. In other cases, however, regions outside the MH2 domain have also been shown to participate in the interaction of R-Smads with additional transcription factors.

Once bound to DNA, complexes of Smads and additional transcription factors regulate transcriptional activation by a

number of mechanisms. R-Smads have been shown to interact with the histone acetyltransferase (HAT) p300 [22,26–28]. Additional transcriptional coactivators, which may act at least in part as bridges linking Smads and HATs, have also been shown to interact with both R-Smads and co-Smads, although their specific mechanism of action is not known [29,30]. In addition to binding coactivators, several examples have been reported in which Smads may activate transcription by displacing or sequestering transcriptional inhibitors (reviewed in [19]).

Although mechanisms of transcriptional activation have been the major focus of study of Smad function, several transcriptional corepressors have also been shown to interact with Smads and to be recruited to specific promoters by DNA-bound Smad complexes (reviewed in [31]). While interaction with corepressors may be a general mechanism for terminating or opposing Smad transcriptional activation, it is also possible that complex-specific interaction of Smads with corepressors results in the ligand-regulated inhibition of transcription at specific promoters. Specificity determinants that distinguish coactivator and corepressor interactions in different Smad–transcription factor complexes remain to be worked out, but may be an important component of understanding the full range of Smad effects on transcription.

Down-Regulation and Cross-Regulation of Smads

Various mechanisms have been identified by which Smad signaling can be down-regulated, including induction of I-Smads, specific protein degradative pathways, and kinase-mediated inhibition by other signaling pathways. Expression of I-Smads is often induced by TGF-ß superfamily signals, suggesting that they function as negative feedback components (reviewed in [32]). I-smads can act by functionally sequestering receptors or Smad4, or by targeting receptors for degradation, thus inhibiting TGF-ß signals at several distinct steps.

Specific degradation of R-Smads is regulated by a family of ubiquitin E3 ligases called Smurfs, which interact with R-Smads via the linker region that lies between the MH1 and MH2 domains [33]. Ubiquitin-mediated degradation of R-Smads may be either ligand independent or stimulated by TGF-ß ligands [33–37], whether there are additional points of regulation of ubiquitin/Smurf-mediated degradation of Smads remains to be elucidated.

In addition to the receptor-catalyzed activating phosphorylations at the C termini of the R-Smads, Smads are regulated by phosphorylation at additional sites. Best characterized are phosphorylations by MAP kinase in the linker domains of Smad1 and Smad2, which can inhibit Smad translocation to the nucleus and therefore inhibit signaling [38,39]. These MAP kinase catalyzed phosphorylations provide a mechanism by which receptor tyrosine kinase signaling can antagonize TGF-ß superfamily signals. Ca/CaM-dependent kinase phosphorylation of the Smad2 linker domain has also been reported to inhibit signaling without inhibiting nuclear translocation; the mechanism of this inhibition is not clear [40]. In addition to regulation by kinases, Smads can also stably interact with components of other signaling pathways, including LEF-1 [41], which acts downstream of wnt signals, and the estrogen receptor [42]. While it is not yet clear how these interactions are used *in vivo*, they do provide potential mechanisms for the integration of multiple signals in the determination of cell fate and behavior.

Function *In Vivo*: Gain of Function, Loss of Function

Both loss-of-function and gain-of-function experiments *in vivo* have confirmed a crucial role for Smads in TGF-ß signaling during a variety of biological processes [43–46]. Ectopic expression of Smad1 and Smad2 in frog embryos recapitulates the developmental effects of BMPs and nodals, respectively [47–49]. Conversely, the loss of Smad5 function in zebrafish phenocopies the loss of BMP ligands in early dorsal-ventral patterning [50], and Smad2 and Smad4 knock-outs in mice have severe developmental defects at gastrulation consistent with proposed roles for TGF-ß superfamily signals in early patterning [51–54]. Interestingly, however, cells derived from embryos lacking Smad4 do not show a complete loss of TGF-ß signaling, indicating that some biological effects of TGF-ßs are mediated independent of Smads in these cells [55].

Because of the complex roles TGF-ß signals play in different cell types, the simple association of phenotypes resulting from the loss of Smad function and known effects of TGF-ß ligands becomes more problematic as development progresses. Functional redundancy among different Smads also complicates the interpretation of Smad knock-out phenotypes [56]. Compound Smad knock-outs, and the substitution of one Smad for another at endogenous loci, will ultimately be necessary to sort out the distinctive role of each Smad *in vivo*. Antibodies directed against the C-terminally phosphorylated, activated forms of the R-Smads provide an additional tool for investigating exactly where and when Smads function *in vivo* [57].

While Smads have emerged as key mediators of TGF-ß signals, a growing body of data indicate that TGF-ßs utilize Smad-independent as well as Smad-dependent signaling pathways in the regulation of multiple biological processes (reviewed in [3,58]), and the relative contribution of each pathway to specific processes *in vivo* remains to be determined. The identification and characterization of the Smads has provided a coherent framework for understanding how specificity is generated in the wide range of biological actions of TGF-ßs. Future work will flesh out the full range of transcriptional targets for Smad interaction, the mechanisms and significance of Smad interaction with other signaling pathways, and the relationship between Smad-dependent and Smad-independent signals in mediating TGF-ß action.

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SECTION C

Damage/Stress Responses

Albert J. Fornace, Jr., Editor

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CHAPTER 298

Complexity of Stress Signaling and Responses

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Introduction: A Variety of Stresses

Life has evolved in an environment fraught with potential dangers. Organisms have survived at least in part by evolving complex responses to meet the challenges posed by their environments. Many different kinds of stress can be encountered by a cell or organism. One important category of stressors is genotoxic agents that cause damage to DNA. These include ultraviolet and ionizing radiations as well as many chemical mutagens and carcinogens. Organisms must also defend against a myriad of physical stresses acting mainly through mechanisms other than DNA damage. Such stresses may include shear stress, wounding, infection, nutrient deprivation, osmotic stress, hypoxia, and heat shock. These last two stresses are discussed in detail in chapters by Koong and Giaccia, and Rick Morimoto, respectively, in this volume.

The cellular strategies used to combat these assaults on equilibrium are equally diverse, but relatively well conserved through evolution (Table I). Much has been learned from the study of lower organisms including bacteria, yeast, and *Drosophila* as discussed in later chapters by Walker, Demple, Bennett and Resnick, Muzi-Falconi, and Abrams. In all organisms, sensor systems are needed to detect stress and its resultant damage. Signals are then exchanged between different cellular compartments, and even between cells, resulting in changes in expression and function of specific transcripts and proteins (Fig. 1). This in turn impacts various cellular processes such as cell cycle progression, DNA repair, and activation of the apoptotic program, ultimately resulting in either recovery and repair or in death. This chapter attempts to provide an overview of these interwoven signaling pathways and processes, many of which will be presented in greater detail in the chapters that follow.

Origin of Signals

The Nucleus

Damaged DNA has long been recognized as originating a stress signal. For instance, tracts of single-stranded DNA at damaged sites in *Escherichia coli* are coated by the RecA protein, which initiates the bacterial SOS response to be discussed in detail in the chapter by Walker. In mammalian cells, double-strand breaks are bound by DNA-PK, a complex of the DNA-PKcs (catalytic subunit) and the Ku70-Ku80 heterodimer. DNA-PK binding to broken DNA ends may initiate signaling to the cellular apoptosis machinery [1]. The ATM kinase has also been shown to bind to broken DNA ends [2,3] and in combination with various cofactors may initiate a DNA damage signal (reviewed in [4]). Activation of ATM results in phosphorylation of a number of downstream targets involved in signal transduction, including p53, Chk1, Chk2, Brca1, and Mdm2 [5–8]. Signaling through ATM is discussed further in the chapter by Lavin *et al.*, whereas the cascades of events triggered specifically by double-strand breaks are covered in chapters by Bennett and Resnick and by Jackson and Bradbury.

Single-strand breaks and damaged bases in DNA also result from many types of genotoxic stress. When a damaged site cannot be bypassed by the polymerase, stalled replication forks can result. This in turn initiates a stress signal to prevent initiation of further DNA synthesis and to arrest cell cycle progression (reviewed in [9,10]), possibly signaling through ATM and Chk2 [11].

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Table I Examples of Conserved Signaling Pathways Activated by Different Stress Stimuli

Stress	Signaling pathways	Drosophilla homologs	S. cerevisiae
Ionizing Radiation	ATM, CHK2	Mei-41	Mec1, Tel1, Rad53
	DNA-PKcs	Mei-41	Tor1
UV Radiation	ATM, CHK2	Mei-41	Mec1, Tel1
	ATR, CHK1	Mei-41, grp	Esr1
	p38	p38b-P1	Hog1
UPR	PEK/PERK	Dmpek (E2K3)	Gcn2
	IRE1, ATF6, JNK	Bsk	Ire1
	HSF1	Hsf	Hsf1
Hypoxia	HIF1A	Sima	Swi1
	VHL	d-VHL	
Heat Shock	HSF1	Hsf	Hsf1
	Raf-1/ERK	Draf-1	Bck1

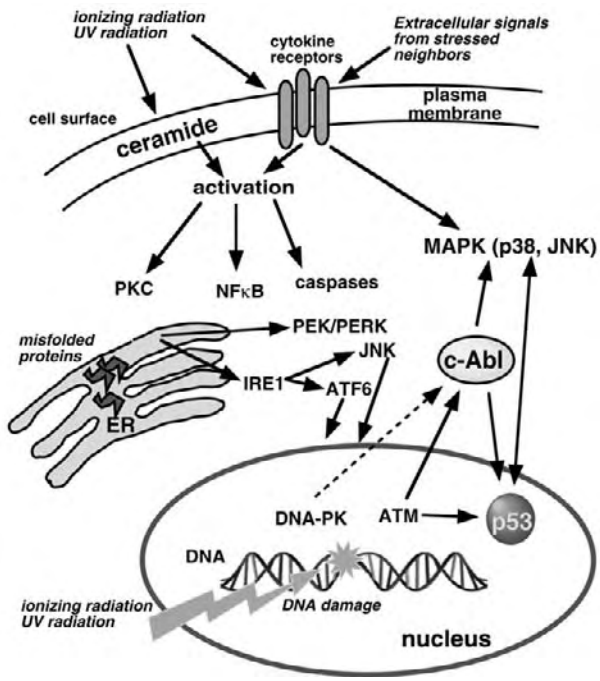


Figure 1 Major sites in the eukaryotic cell where stress signaling can originate, and some of the key signaling pathways activated in response. Many of these pathways will be discussed in detail in the following chapters in this section.

The Cytoplasm

Signaling can also be initiated by non-DNA-damage cellular stress. For instance, conditions including nutrient deprivation and exposure to certain toxins can cause accumulation of unfolded or misfolded proteins in the endoplasmic reticulum, triggering a complex unfolded protein response (UPR) (reviewed in [12]). This response has two major components, the first of which shuts down new protein synthesis, and appears to be mediated by the PEK/PERK protein kinase [13,14]. The second component of the UPR up-regulates expression of genes whose products are involved in protein folding and in the degradation of misfolded proteins. This pathway is initiated by activation of the IRE1 kinase, and the signal is transduced through both ATF6 and the JNK pathway [15]. These signal transduction pathways are discussed in the chapter by David Ron. Stresses that disrupt the normal function of other organelles, including the cytoskeleton and mitochondria, can also initiate signals leading to apoptosis [16].

The Plasma Membrane

Exposure to toxins or infection can give rise to a stress signal originating in the plasma membrane. Engagement of cell surface receptors, such as the tumor necrosis factor (TNF) receptor superfamily or the interleukin 1 (IL-1) receptor may lead to activation of NFκB or initiation of the caspase cascade (reviewed in [17]). Activation of TNF receptors initiates hydrolysis of sphingomyelin to produce ceramide, which acts as a second messenger. Irradiation of enucleated cells can also initiate ceramide signaling in the plasma membrane, clearly indicating signaling of radiation damage in the absence of DNA-damage-specific signals [18].

Extracellular Signals

Cells can also respond to stress or injury at a distance through communication with other cells. Cytokines, growth factors, and hormones are released in response to internal cues or external stressors, and their internalization or binding to receptors of other cells can set in motion signal cascades and alter transcriptional programs within the target cell. Such extracellular communication appears to be a basis of the so-called "bystander effect," where DNA damage in one cell can result in altered protein expression in undamaged cells in the same culture [19,20].

Signal Transduction

Once a stress situation has been recognized and signaling is initiated, numerous pathways exist for the amplification and direction of the message. Redox signaling, to be discussed in the chapter by Bruce Demple, is a common mechanism for signal amplification in cells. Reactive nitrogen (RNS) or oxygen species (ROS) can be generated by the p450 system or the electron transport machinery in the mitochondria in response to stress. Alteration of the cellular redox environment may directly affect protein function, such as ROS-mediated oxidation of Tyr phosphatase 1B, which inhibits its function and leads to activation of target proteins including ErbB1 [21]. Nitric oxide similarly has been shown to modulate the function of p21ras [22] through nitrosylation.

Protein Modification and Signaling

Protein modification is a major mechanism for stress signal propagation within the cell, and perhaps the most common modification is alteration of phosphorylation state. Many kinase cascades are activated by various stresses; one of the best studied kinase pathways associated with DNA damaging stress is that involving the ATM kinase. Activation of ATM by DNA damage leads to phosphorylation of both p53 and the c-Abl protein tyrosine kinase. In the case of c-Abl activation, this leads to activation of p73 and promotes apoptosis [23]. Stress signaling through the c-Abl kinase is discussed in a later chapter by Jean Wang, while the chapter by Lavin *et al*. focuses on ATM signaling.

The complexities of p53 activation by DNA damage and other stresses have been studied extensively, as reviewed in detail in the chapter by Anderson and Appella. Briefly, at least 18 sites of regulatory posttranslational modification of p53 have been described, including sites of phosphorylations, acetylations, and sumoylation. Modifications of these sites vary in response to different stresses and the activation of different upstream regulatory pathways. For instance, distinct patterns of p53 modification have been reported in ionizing radiation-treated, UV-irradiated, or senescent human primary fibroblasts [24,25]. Agents inducing double-strand breaks, such as ionizing radiation, activate the ATM kinase pathway, resulting in phosphorylation of p53 at Ser15, Ser20, Ser9, and Ser46 [26], as well as activating a number of additional kinases phosphorylating further sites in p53. In contrast, agents such as UVC that induce bulky lesions in DNA activate the ATR [27], p38 MAPK [28], and JNK [29] kinase pathways, resulting in phosphorylations at Ser15 and Ser37, Ser33 and Ser46, and Thr81, respectively. Such modifications of p53 subsequently affect its interactions with other proteins and its sequence-specific DNA-binding and transactivation activities, resulting in differential gene transcription profiles (Fig. 2). Thus p53 activation can promote such diverse outcomes as apoptosis [30,31], reversible cell cycle arrest [32], or a permanent



Cell Cycle	Apoptosis	DNA Repair	Senescence	Anti-angiogenesis	Other
CIP1/WAF1	*BAX*	*XPC*	*CIP1/WAF1*	*VEGF*	*MDM2*
ClnG	*BCL-X*	*DDB2*	type I PACAP	*IL-8*	*FRA1*
ClnD1	*PAG608*	*GADD45A*		*HIF1α*	*ATF3*
WIP1	*FAS/APO1*	*PCNA*		*MMP2*	*14-3-3σ*
EGF-R	*KILLER/DR5*	*p53R2*		*BAI-1*	*Rb*
Rb	*TRUNDD*			*TSP1 & 2*	*c-MYC*
BTG2	*TRID*				*ninjurin*
PCNA	*SIAH*				*MAP4*
GADD45A	*IGF-BP3*				*amyloid*
14-3-3σ	*PIG1 to PIG14*				*PIR121*
TGF–α	*14-3-3σ*				*WIG1*
SIAH	*MAP4*				
IGF-β	*TSP1 & 2*				
PIG1 to PIG14	*bFGF*				
inhibin-β	*GML*				
GML					

Figure 2 Diverse stress signals can be transduced by a variety of pathways through p53. This, in turn, results in the execution of different cellular programs, mediated in part by the activation or repression of transcription of a variety of genes, including the examples listed here.

cell cycle arrest indistinguishable from senescence [33,34]. The many different possible modifications and binding interactions of p53 could serve as a means to tailor the specificity of p53 responses to suit the originating stress or the specific cellular environment.

MAP Kinase Pathways

Protein activation by phosphorylation is a common mechanism for intracellular signal transduction, and the mitogen-activated protein kinase (MAPK) cascades provide a major example of this mechanism. The MAPK signaling cascades are three distinct but analogous pathways comprising the ERK-1/ERK-2 mitogen-activated pathway and the stress-activated protein kinases (SAPK), p38 and c-Jun N-terminal kinase (JNK). The MAPK/ERK pathway is activated primarily by mitogenic stimuli such as extracellular growth factors, as well as during physiological processes such as T-cell activation in immune response [35]. Conversely, the SAPK/JNK and p38 pathways are activated predominantly by stresses, such as UV or ionizing radiation, heat shock, or inflammatory cytokines. There is also a regulatory phosphatase system for the down-regulation and inactivation of each of these signaling cascades. The degree to which the different signaling cascades are activated and the duration of the activation appear to shape the cellular response and can result in altered gene transcription, cell cycle arrest, or apoptosis. The interplay of the various MAPK pathways is discussed in greater detail in the chapter by Blattner and Herrlich.

Functional Genomics and Proteomics Approaches

In addition to, and often consequent to, the modification of proteins, the cellular response to stress also alters the abundance of specific mRNA transcripts. This can occur both through alteration of message stability (discussed in the chapter by Miriam Gorospe) and via transcription factor modification leading to alteration of transcription programs. By changing the transcripts and subsequently the proteins manufactured in response to specific stress situations, the cell makes components available to promote or protect against cell death, to repair damaged DNA, or to halt cell cycle progression and maintain damage checkpoints.

The advent of functional genomics has vitalized interest in the transcriptional basis of stress response. Techniques such as serial analysis of gene expression (SAGE) and microarray analysis allow assessment of expression of thousands of genes in a single experiment [36,37]. For instance, SAGE has been used to identify around 30 transcripts apparently induced by p53 activation [38], while microarray analysis has been widely applied to the study of responses to stresses including ionizing [39,40] and UV [41] irradiation, hypoxia [42], alkylating agents [43], and toxic metals [44–46]. The data being collected in such experiments are extremely complex. Although some common factors emerge, such as increased expression of genes such as *CDKN1A* indicating activation of the p53 pathway, there is a profound heterogeneity in stress-responsive genes. Different "profiles" are beginning to emerge, however, based both on the initiating stressor (Fig. 3) and the responding cell type (Fig. 4). Mathematical approaches are being developed for analysis of this sort of data, including hierarchical clustering, principal component analysis, and multidimensional scaling analysis. Meanwhile, techniques for similar high-throughput analysis of protein changes occurring after stress are also being developed. Through the combination of these sophisticated approaches, we may soon be able to unravel much more of the complex interplay of signals involved in cellular stress response.

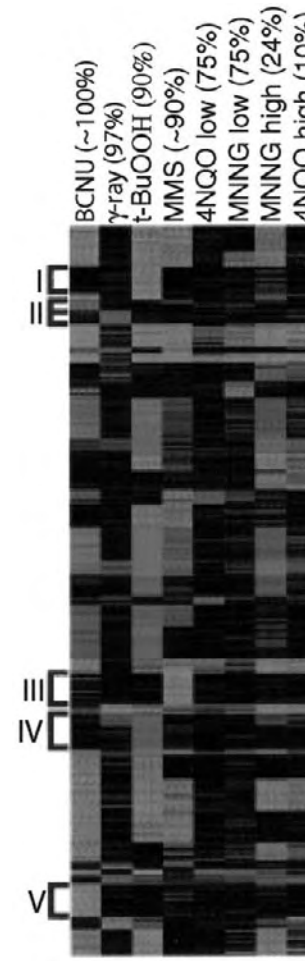


Figure 3 Global transcription profiles in *S. cerevisiae* exposed to different damaging agents. Log-phase cells were exposed to the indicated agents for 1 hr. For each treatment, the percent survival (in parentheses) was determined by colony formation. Expression of 2324 genes scored as responsive on Affymetrix arrays has been grouped into 30 clusters by K-means clustering. The six indicated clusters contain members appearing to respond only to a particular agent (Clusters I through IV). Treatments were as follows: 200 μM BCNU, 300 Gy γ-ray, 5 mM *t*-BuOOH, 0.1% MMS, 4NQO at 2 (low) or 8 (high) μg/ml, and MNNG at 8 (low) or 27 (high) μg/ml. (Reproduced with permission from [43].)

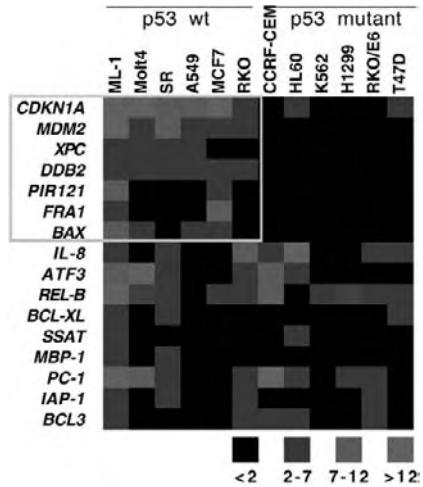


Figure 4 Variability of induction of a small number of genes in cell lines differing in functional p53 status. Cells were exposed to 20 Gy γ-rays, RNA was harvested 4 hr later, and relative gene induction was determined by quantitative single-probe hybridization [47]. The relative fold induction is indicated as a shade of gray according to the scale at the bottom of the figure. A black square represents no significant induction of a particular gene in that cell line. The box highlights genes dependent on wild-type p53 for robust induction by ionizing radiation.

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CHAPTER 299

Signal Transduction in the *Escherichia coli* SOS Response

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SOS Response

Inducing Signals and Transcriptional Control

An organism's DNA is often exposed to potential mutagens such as chemicals and UV light. *Escherichia coli* has a regulated system, the SOS response, that is induced in response to damage to its genome [1–4]. When the cell's DNA suffers damage or the normal replication process is stalled, a region of single-stranded DNA is formed. The RecA protein coats this single-stranded DNA, forming a nucleoprotein filament and in turn activating RecA for its coprotease function. The RecA/ssDNA filament serves as the signal for the SOS response, which governs the cellular response to the damage. The RecA/ssDNA filament stimulates the self-cleavage of the LexA repressor, thus inactivating it as a repressor (Fig. 1). This leads to expression of the SOS-regulated genes, many of which are involved in DNA repair. Many of the genes induced in the SOS response are involved in DNA damage repair and tolerance and cell cycle control, such as *polB*, *sulA*, and *uvrA* [1,5]. Among the genes induced are those that code for two members of the newly termed *Y-family* of low-fidelity DNA polymerases: *dinB*, also known as DNA Pol IV, and *umuDC*, also called DNA Pol V [6].

A recent genomic analysis demonstrated more than 40 LexA-regulated loci in *E. coli* (Table I) [7]. By including an Ind^- allele of *lexA* in this analysis, several genes were identified that are induced following UV irradiation in a LexA-independent manner, including genes coding for proteins involved in replication, the heat-shock response, and purine, pyrimidine, and RNA metabolism [7]. Decreased levels of some messages were also found following UV irradiation, including some genes involved in carbon and lipid metabolism, and in septum formation prior to cell division. These results suggest yet another level of regulation of this system.

Posttranscriptional Control in the SOS Response

After induction of the SOS response, UmuD and UmuC are translated and UmuD persists as the homodimer of the full-length protein for about 20 min, at which point it undergoes a RecA/ssDNA nucleoprotein filament-facilitated self-cleavage reaction similar to LexA cleavage in which the Cys24–Gly25 peptide bond is severed to form UmuD'. The $UmuD'_2C$ complex (DNA Pol V) is a low-fidelity lesion-bypass polymerase; the RecA/ssDNA filament and single-stranded DNA-binding protein (SSB) are also required *in vitro* for the polymerase activity of $UmuD'_2C$ [8,9]. Uncleaved UmuD also seems to have an important role in protecting the cell against excessive mutations as a result of DNA damage by serving as a DNA damage checkpoint effector [10,11]. Also, $UmuD'_2C$ is able to inhibit homologous recombination, whereas $UmuD_2C$ is not, an interaction that may serve to prevent recombination in favor of translesion synthesis [12].

The SOS-induced *dinI* gene is able to inhibit the RecA/ssDNA-mediated cleavage of UmuD [13]. Recent evidence suggests that the role of DinI is to modulate the rate of UmuD cleavage [13]. Earlier *in vivo* work showed that overexpression of DinI inhibited LexA cleavage [14]. DinI binds to the RecA/ssDNA filament more strongly than UmuD does but less strongly than LexA *in vitro*, a finding that supports the idea that competition for binding sites on RecA/ssDNA filaments allows DinI to attenuate the rate of UmuD cleavage while LexA cleavage is apparently less affected [13].

Ending the SOS Response and Return to the Uninduced State

As the cell recovers from DNA damage, the amount of ssDNA decreases and so the inducing signal abates, thereby resulting in the accumulation of intact LexA repressor and

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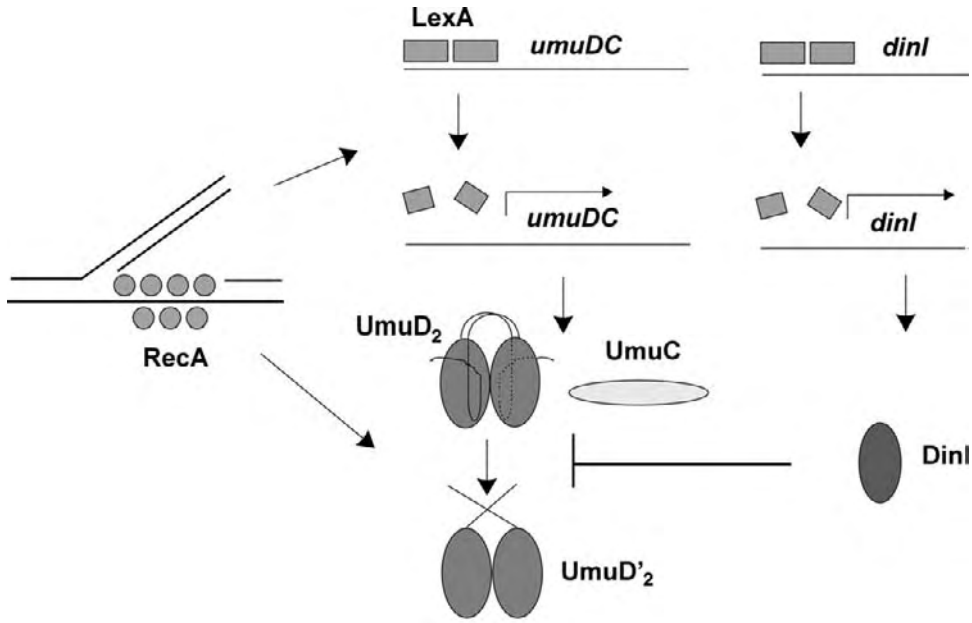


Figure 1 Induction of the SOS response showing two of the operons under LexA control. After DNA damage, RecA forms a filament on the single-stranded DNA that presumably results from the cell's failed attempts at replication (left), initiating a cascade of events including cleavage of the LexA repressor.

thus halting the general SOS response [1]. Because Pol V is a low-fidelity DNA polymerase, its activity is tightly regulated to prevent general use as a DNA polymerase. An additional level of control seems to be provided by the degradation of $UmuD_2$ and UmuC by the Lon protease and the selective digestion of UmuD' in the UmuD·D' heterodimer by the ClpXP protease [15,16]. While both UmuD and UmuD' form stable homodimers, the UmuD·D' heterodimer is more stable in solution than either homodimer [17]. The sequestration of UmuD' in the heterodimer may provide a way to target UmuD' for proteolysis [18].

LexA Cleavage and Other Self-cleavage Reactions Regulating the SOS Response

Researchers have known for some time that similarities exist between the self-cleavage reactions of LexA repressor, phage λ cI repressor, and UmuD [1]. All three self-cleave in a RecA/ssDNA nucleoprotein filament-dependent fashion, yet all three have autocatalytic capabilities in the absence of the RecA/ssDNA nucleoprotein filament at high pH [1]. These proteins utilize a Ser/Lys dyad active site for cleavage, and cleave either between Ala-Gly or Cys-Gly sequences. Additionally, cleavage of each of these three proteins results in distinct physiological events [1]. Thus, when the LexA repressor is cleaved, it loses its repressor activity and the genes it regulates become induced. Similarly, in the case of λ cI repressor protein, cleavage results in induction of phage genes. When UmuD undergoes cleavage, it converts itself, together with UmuC, from a DNA damage checkpoint effector to the lesion bypass polymerase, DNA Pol V.

A striking similarity between the structures of *E. coli* signal peptidase and UmuD was identified by Paetzel and Strynadka [19]. They found that the root mean square deviation (RMSD) for 69 common Cα carbon atoms between UmuD and signal peptidase was only 1.6 Å, despite the fact that there is only about 17% sequence identity between the aligned residues [19]. Signal peptidases contain a Ser/Lys dyad active site and their structures consist mainly of β-strands [19]. Several other recent structural studies involving this class of proteins have given significant insights into the control and mechanism of their cleavage reactions.

Crystal Structure of the LexA Repressor

Recently, the crystal structures of four different mutant forms of LexA were solved: two that are noncleavable due to mutations in the cleavage site, one that is noncleavable due to an active site mutation, and one that contains mutations to create a "hypercleavable" LexA coupled with an active site mutation to eliminate cleavage and thus facilitate crystallization [20]. The solved structures demonstrate that the C-terminal catalytic domain, like signal peptidase, consists mainly of β-strand structural elements [20]. Additionally, LexA also exhibits a high degree of structural similarity to UmuD', giving an RMSD of 0.96 Å for 81 C-terminal catalytic domain Cα atoms [20]. The several different solved structures of LexA exhibit two different but structured conformations in the cleavage site region: one in which the cleavage site lies nestled in the active site and the other in which the cleavage site is about 20 Å away from the active site [20].

It is proposed that, consistent with an earlier hypothesis based on a mutational analysis, the interconversion between the two structured forms is the basis for regulating the

Table I SOS-Induced Genes in *E. coli*[1]

Gene	Function/Possible function
lexA	Transcriptional repressor; regulator of SOS regulon
recA	Recombination, regulation of SOS response, role in SOS mutagenesis
umuC	DNA pol V, SOS mutagenesis
umuD	Forms complex with UmuC, role in DNA damage checkpoint
dinB/dinP	DNA pol IV
dinA/polB	DNA pol II
uvrA	Nucleotide excision repair, involved in damage recognition
uvrB	Nucleotide excision repair, involved in damage excision
uvrD	Nucleotide excision repair, helicase
ruvA	Daughter strand gap repair; Holliday junction helicase
recN	Recombinational double-strand break repair
ssb	Single-stranded binding protein
ydjQ/dinM/sosD	Putative excinuclease; Homolgous to *uvrC*
dinI	Modulates the rate of RecA/ssDNA-dependent UmuD cleavage
fis	Transcription factor
suhB	Transcriptional regulator
ttk	Putative transcriptional regulator
ydeO	Putative ARAC-type regulatory protein
ftsK	ATP-dependent DNA translocase, involved in chromosome segregation during cell division [36,37]
grxA	Glutaredoxin coenzyme for ribonucleotide reductase
ycgH	Putative ATP-binding transport system component
glvB	PTS system, arbutin-like IIB component
ybeW	Putative DnaK homolog
ibpB	Heat-shock protein
sulA	Suppressor of Lon, inhibits cell division
dinD/pcsA	DNA-damage inducible protein, cold-sensitive mutant
oraA	Regulator
dinF	Putative membrane protein [38], immediately downstream of *lexA*
sbmC	Resistance to Microcin B17
molR	Molybdate regulator
dinS	Transposase
dinG	Putative helicase
hokE/ydbY	Host killing protein, ensures stable maintenance of plasmids harboring it

[1]Largely from reference 7, with a cutoff of two-fold (LexA-dependent) induction to be included in this table. See also references 1, 2, and 5. Also noted as induced are the following proteins of unknown function: *yebG, yigF, arpB, yafO, yafN, yebF, yafP, ydiY, ydjM, yebE, ybiN, yceP, yjiW, yifL, ydeT, ysdAB, dinQ, ybfE*.

cleavage reaction [20,21]. In the two forms that are observed in the crystal structures, the catalytic core is mostly the same but there is a shift in the position of the cleavage site region. However, both conformations are stabilized by distinct sets of hydrophobic and hydrophilic interactions [20]. It is suggested that the RecA/ssDNA filament facilitates cleavage by binding preferentially to the cleavable conformation of LexA, thereby altering the equilibrium between the two forms. Extrapolation from cryoelectron microscopy experiments indicates that LexA may nestle in a groove in the RecA/ssDNA filament, thereby stabilizing the less stable cleavage-competent conformation [20,22]. Recent experiments with UmuD suggest that it may be stabilized in a cleavable conformation in a similar manner by repositioning the active site via interactions with the RecA/ssDNA filament [23,24]. The LexA side-chain ε-amino group of Lys156 is responsible for deprotonating the catalytic residue Ser119, but the ε-amino group itself is protonated in the noncleavable form of LexA [20]. The energetic cost of deprotonating and burying the ε-amino group of Lys156 in order to lower the pK_a sufficiently for catalysis likely provides an additional barrier, and thus level of control, to cleavage [20].

Structural Insights into the Cleavage of $UmuD_2$

NMR and crystal structures of $UmuD'_2$ show that the protein consists of a globular C-terminal domain that contains the dimerization domain, the catalytic residues Ser60 and Lys97, and flexible N-terminal arms [23,25]. A recent NMR study determined the structure of the $UmuD'_2$ homodimer, but the $UmuD_2$ homodimer was not amenable to NMR structure determination [23]. However, biochemical data indicate that the UmuD·D' heterodimer is a good model for the structure of the $UmuD_2$ homodimer, and NMR analyses yielded some insights into its structure [26,27]. Whereas secondary structural elements in the $UmuD'_2$ and UmuD·D' dimers are largely similar to each other and to those in the X-ray structure, the RMSD between $UmuD'_2$ homodimers in the NMR and X-ray structures is quite different at 4.59 Å [23,28]. Much of this difference can be accounted for in the more mobile exterior regions of the protein in solution [23]. In this solution structure of $UmuD'_2$, the active site Ser60 and Lys97 residues are not near enough to affect catalysis, whereas in the crystal structure, the ε-amino group of Lys97 points directly at the side-chain of Ser60 [23,25]. This suggests that the conformation of the C-terminal domain of UmuD' in the crystal may approximate its conformation when it is in contact with the RecA/ssDNA filament, which is required for cleavage [23]. The conformation of the N-terminal arm of UmuD as determined by cross-linking is similar to that of the linker region of LexA in the cleavable conformation [20,27]. Cleavage of $UmuD_2$ occurs intermolecularly, while the crystal structure of LexA indicates that cleavage in this case is intramolecular [20,23]. This may indicate an idiosyncratic feature of this family, but it is possible that subtle changes in the conformation of the linker region on LexA could produce intermolecular cleavage in this case as well [20].

Structures of Y-Family Polymerases

There are now crystal structures of the Y-family polymerases *Sulfolobus solfataricus* DinB and *Saccharomyces cerevisiae* Polη [29–32]. There are four reported structures of two different *S. solfataricus* DinBs (Dbh and Dpo4), including Dpo4 with DNA substrates bound [29,30,32]. In general, the Y-family polymerases possess the canonical shape of a right hand that is characteristic of other polymerases, but with shorter finger and thumb domains [33,34]. All of the structures determined thus far are observed in the "closed" conformation, whether they have substrate bound or not [33,34]. Thus, it appears that the PolY family polymerases do not use the same type of induced fit mechanism as the replicative polymerases, which alternate from open to closed conformations upon DNA binding.

In the structure of the complex, Dpo4 makes limited and nonspecific contacts with the newly forming base pair [29]. Moreover, the bound dsDNA is B-form, which does not allow the protein to access the minor groove to check the fidelity of the new base pair [29]. The minor groove of the DNA substrate bound to Dpo4 is solvent accessible, whereas in classical DNA polymerases it is bound by the protein. The DNA substrate bound to classical DNA polymerases is typically A-form, allowing the protein to bind the minor groove to check the fidelity of replication [35]. Some of these structures also show the presence of appended domains that are likely to play a role in providing additional DNA binding energy [29–31]. One of these, the "little finger" domain in the Dpo4 structure, is observed binding the double-stranded DNA in the major groove [29]. Finally, Dpo4 accommodates a bulged template base in its active site, which also provides enough space to bind a cyclobutane thymine dimer and may in part explain its substrate specificity [29].

Conclusions

The ultimate result of DNA damage, whether accurate repair such as nucleotide excision repair, homologous recombination, or translesion synthesis, is governed by several levels of control including transcriptional activation and posttranslational modification. Translesion synthesis itself is accomplished by a cascade of signaling events achieved through noncovalent interactions as well as covalent modification (self-cleavage) of several of the proteins involved. Translesion synthesis is executed by a specialized class of polymerases that appear to be uniquely suited to their role as low-fidelity DNA polymerases. It seems certain that there are many more insights to be gained into when and with which tools cells respond to an assault on their genetic material.

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CHAPTER 300

Oxidative Stress and Free Radical Signal Transduction

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Introduction: Redox Biology

During the past two decades, considerable attention has been focused on issues related to oxidative stress and the biology of free radicals. *Oxidative stress* refers to excess production of various reactive species (H_2O_2, superoxide, singlet oxygen, etc.) or to insufficient antioxidant defenses in the cell [glutathione (GSH), superoxide dismutase, catalase, etc.] [1]. Environmental exposure produces oxidative stress through airway injury, irradiation, and exposure to compounds that disrupt cellular electron transfer pathways. Actively generated free radicals can play physiological roles, including in cellular signaling pathways [2–5]. High-level production of superoxide and another free radical, nitric oxide, contributes to immunological attack on pathogens and tumor cells [6,7]. The main cellular components are all subject to damage during oxidative stress, so it is not surprising to find that cells have evolved pathways to respond dynamically to oxidative stress by altering gene expression [2,8].

Oxidative Stress Responses in Bacteria: Well-Defined Models of Redox Signal Transduction

Study of oxidative stress responses has focused especially on *Escherichia coli* and *Salmonella enterica* (*typhimurium*) as model systems amenable to combined genetic and biochemical analysis [5,9,10]. Studies in the Gram-positive bacterium *Bacillus subtilis* [11], or in medically or agriculturally important organisms such as *Pseudomonas aeruginosa* [12] and *Xanthomonas oryzae* [13], are now beginning to yield similar fundamental insights in those systems.

Response to Superoxide Stress and Nitric Oxide: SoxR Protein

The SoxR regulatory system of *E. coli* (Fig. 1) was discovered by genetic analysis of strains with elevated defenses against superoxide-generating agents such as paraquat and quinones [14,15]. The system works in two stages of transcriptional control; only the first stage involves direct redox signal transduction [16,17]. As described later, SoxR protein is activated in cells exposed to oxidative stress or nitric oxide; activated SoxR then stimulates expression of just one gene, *soxS*. The resulting increase in the level of SoxS protein, itself a transcription activator in the AraC/XylS family [18], leads to the up-regulation of 42 genes and the repression of 58 genes [19]. Many of the induced genes have obvious functions in oxidative defense (e.g., Mn-containing superoxide dismutase, or endonuclease IV for oxidative DNA damage), but many others (e.g., the *acrAB*-encoded efflux pumps) serve broader functions [20].

The redox-sensing SoxR protein is a homodimer of 17-kDa subunits, each of which contains a single [2Fe-2S] iron-sulfur cluster anchored to the protein's only four cysteine residues near the C terminus [21]. In resting SoxR, these clusters are in the reduced (+2) state, which can be detected *in vivo* using electron paramagnetic resonance spectroscopy [22,23]. Upon cellular exposure to agents such as paraquat, which sets up a heavy flux of superoxide in the cell and consumes NADPH by cyclic reduction and autoxidation [24], the [2Fe-2S] centers are oxidized to the +3 state. This one-electron oxidation converts SoxR to a powerful activator of *soxS* transcription, which elevates the soxS mRNA level up to 100-fold [4].

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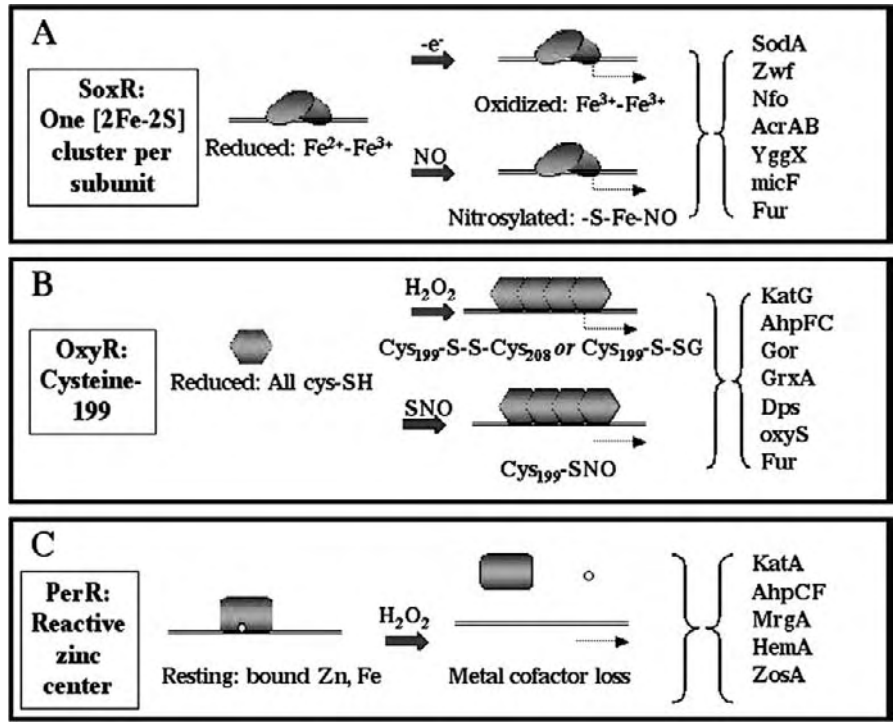


Figure 1 Representative systems of redox regulation. Each box depicts the main aspects of one of the regulatory systems described in the text. In all three cases, transcriptional activation is indicated by a dotted, right-angle arrow at the target DNA (thin parallel lines). (A) The SoxR system of *E. coli*. Each 17-kDa subunit of the homodimer contains a [2Fe-2S] cluster, which is in the reduced state in the absence of free-radical stress. Nonactivated SoxR binds its DNA site in the *soxS* promoter with high affinity. Oxidative stress exerted by superoxide-generating agents (such as paraquat) one-electron oxidizes the centers, which converts SoxR to a potent transcriptional activator without changing its DNA-binding affinity. Alternatively, nitric oxide activates SoxR by reacting with the iron-sulfur centers to form dinitrosyl-iron complexes anchored via cysteine residues in the protein. Activation of SoxR triggers expression of the *soxS* gene, whose protein product (not shown) then activates the many genes of the regulon. Among the many functions stimulated by activated SoxR are manganese-containing superoxide dismutase (SodA), glucose-6-phosphate dehydrogenase (Zwf), the DNA repair enzyme endonuclease IV (Nfo), the AcrAB efflux pump, and the YggX protein involved in maintenance of iron-sulfur clusters (P. Pomposiello, A. Koutsolioutsou, and B. Demple, submitted). In addition, secondary regulators are under SoxR control: the micF antisense RNA that down-regulates OmpF expression; and Fur protein, which controls many genes involved in iron uptake. (B) The OxyR system of *E. coli*. Resting OxyR is in the reduced state and does not bind most of its DNA targets. Oxidation by H_2O_2 causes structural changes that convert the protein to a DNA-binding tetramer. Cysteine-199 is the critical residue for this response and forms a disulfide, although there is controversy (see text) as to whether this involves cysteine-208 or a molecule of glutathione. A form of OxyR with a sulfenic acid based on cysteine-199 (not shown) has also been reported. In addition, nitrosothiols produce a different activated form of OxyR containing S-nitrosylated cysteine-199. Activation of OxyR stimulates the expression of many genes not activated by SoxR, although there is a limited overlap (e.g., Fur protein). The functions include catalase (KatG), alkyl hydroperoxide reductase (AhpFC), GSH reductase (Gor), glutaredoxin-1 (GrxA), and the protective DNA-binding protein Dps. As seen with SoxR, secondary regulators control still more genes; in addition to the Fur repressor, the oxyS RNA acts posttranscriptionally on the expression of several other genes. (C) The PerR system of *B. subtilis*. The Fur homolog PerR is a repressor of genes that respond to H_2O_2. The active (repressing) form of PerR contains zinc and iron (small circle in the cartoon); oxidation causes these metals to be lost from the protein and DNA-binding affinity is lowered dramatically. Functions regulated by PerR include catalase (KatA), AhpFC, the protective DNA-binding protein MrgA, HemA and other heme biosynthetic enzymes, and the ZosA protein involved in zinc uptake.

These events can be reproduced *in vitro* using purified SoxR protein [25,26]. SoxR isolated under air has iron-sulfur clusters in the oxidized state, and the protein has full transcriptional activity specific for *soxS*. SoxR stimulates transcription by increasing the rate of open complex formation by RNA polymerase [27]. Chemical reduction using sodium dithionite yields the +2 state of the [2Fe-2S] clusters, which switches off the transcriptional activity. The activity is restored by reoxidation [25,26].

SoxR binds tightly to its *soxS* target site independently of the iron-sulfur centers [28] or their oxidation state [26]. The SoxR binding site is centered between the –35 and –10

elements of the *soxS* promoter [27]. This promoter has an unusually long spacer of 19 bp (versus the usual 17 ± 1 bp) between the RNA polymerase recognition elements, which are otherwise good matches to consensus sequences. The *soxS* promoter has very weak basal activity, but mutant promoters with spacers of 18, 17, or 16 bp are highly expressed *in vivo* independent of SoxR [29]. The model for SoxR transcription activation is analogous to that for the Hg^{2+}-sensing MerR protein [30]: DNA structural remodeling that compensates for the excessively long spacer. Recent crystallographic analysis of the SoxR-related BmrR protein [31] is consistent with this view.

SoxR is also activated by nitric oxide, and the chemistry of this process is fundamentally different from the one-electron oxidation just described. Nitric oxide directly modifies the [2Fe-2S] clusters to form dinitrosyl-iron-dicysteine complexes anchored to the protein [32]. These nitrosylated complexes are quite stable *in vitro*, which allowed NO-modified SoxR to be repurified for analysis. SoxR activation by NO may play a role in bacterial resistance to the cytotoxic attack of NO-generating macrophages [33,34].

Both the oxidation and the nitrosylation of SoxR are very rapidly reversed in intact bacteria when the oxidative stress or NO exposure is stopped [32]. The former observation, together with maintenance of SoxR in the reduced form in the absence of stress, implies that the [2Fe-2S] centers are actively reduced, probably enzymatically. Although a report of a SoxR reductase activity [35] has not been followed up, a recent paper presents genetic evidence of a SoxR-reducing system [54].

The reversal of the dinitrosyl complexes in NO-treated SoxR to regenerate unmodified [2Fe-2S] clusters may be of even more general significance. Nitrosylation of various types of iron-sulfur clusters is a general feature of NO toxicity in all cell types [36]. Delineating the mechanism by which such protein damage is corrected (possibly by removal of the nitrosylated clusters and the resynthesis of new ones) should provide general insights into how cells handle this basic problem.

Response to H_2O_2 and Nitrosothiols: OxyR Protein

The *oxyR* regulatory system (Fig. 1) was identified by genetic analysis of *S. enterica* and *E. coli* stains in which inducible resistance to H_2O_2 [37] was activated constitutively [38]. OxyR protein both senses oxidative stress and activates the >60 genes of the *oxyR* regulon [39]. OxyR protein is a member of the LysR family of transcriptional regulators [40], and close homologs of the *oxyR* gene are widely represented in bacterial genomes [5].

Like SoxR, OxyR is a positive regulator that exists in a resting state in nonstressed cells [4,5]. Upon cellular exposure to H_2O_2 or nitrosothiols [41,42], the protein is activated to stimulate transcription of the regulon genes. Unlike SoxR, the activity of OxyR is regulated largely by increased DNA binding [43,44], as is the case for many prokaryotic signal transduction systems. The positioning of the protein upstream of the RNA polymerase binding site stimulates transcription through protein–protein contacts with the C-terminal domain of the polymerase α subunit [45]. The *oxyR* regulon includes genes such as *katG* (HP-I catalase-hydroperoxidase), *gor* (GSH reductase), and the *ahpFC* operon (alkyl hydroperoxide reductase) [39].

The molecular basis for OxyR activation has been explored to yield a detailed model of both the redox activation and the stimulation of transcription [46]. However, the mechanism of redox sensing is still subject to some debate [41]. At the DNA-binding level, redox activation converts OxyR from a predominantly dimeric form to a tetramer, which binds consecutive repeats of a recognition element and bends the DNA [43].

The proposed mechanism of redox activation of OxyR by H_2O_2 is the formation of an intramolecular disulfide in the protein [46], and genetic and biochemical evidence supports this view. For example, of the six cysteine residues in the OxyR polypeptide, one (cysteine-199) is absolutely required for full activity in response to oxidative stress, and the other (cysteine-208) has a significant effect. Peptides have been isolated from oxidized OxyR that contain a disulfide bond linking cysteines 199 and 208 (although the other four cysteine residues of the protein had been replaced by alanines for this experiment). These observations led to a model in which oxidative activation of OxyR occurs through the formation of an intramolecular disulfide bond linking cysteine-199 and cysteine-208. The structural basis for this specificity is proposed to be both the proximity of the two cysteine residues, and a basic residue near cysteine-199 that increases its reactivity with oxidants [44].

A recent report challenges this view [41]. That work indicates that there are various activated forms of OxyR, none of which contains the cysteine-199/cysteine-208 disulfide. Instead, Kim *et al.* [41] identified a form with cysteine-199 as a sulfenic acid (-SOH) in air-oxidized OxyR, or with a mixed disulfide linking cysteine-199 and GSH [41]. Such mixed disulfides were detected biochemically as a prominent product in OxyR activated *in vivo* [41]. However, note that GSH is not necessary to activate OxyR *in vivo* [46].

GSH and glutaredoxin-1 (a small, thioredoxin-like protein with a redox-active cysteine pair) evidently play physiological roles in restoring reduced OxyR following oxidative stress [3,47]. The overall view is that these proteins participate in thiol/disulfide exchange reactions that yield reduced OxyR, ultimately at the expense of NADPH through the activity of GSH reductase. This control would establish a negative feedback loop in the system, since the *gor* and *grx1* (glutaredoxin-1) genes are activated by OxyR [3,47]. However, *gor* and *grx1* are not required to maintain OxyR in the reduced state in unstressed cells [46].

Nitrosothiols have also been shown to activate OxyR, which was proposed to occur by generating a cysteine nitrosothiol (-SNO) in the protein [42]. New data support this view [41], but also show that different activated forms (-SNO, -SOH, and –S-SG) have variable affinity and transcription-activating capacity for different promoters. The discrepancy with earlier

studies [46] was rationalized by pointing out that they had employed mutant forms of OxyR with four of the six cysteines substituted by other amino acids [41]. These observations point to a general phenomenon in which different stress signals give rise to different patterns of gene activation even while operating on the same sensor/transducer protein. For the SoxR/SoxS system, current data indicate that different activated forms of SoxR would merely influence the *level* of SoxS protein. Differences in the pattern of gene expression would then reflect the degree of SoxR activation, rather than changes in its specificity.

Parallels in Redox and Free-Radical Sensing

Systems that seem to share the thiol/disulfide mechanism of redox sensing used by OxyR have now been identified in several contexts. A particularly interesting example is the *E. coli* Hsp33 chaperone, which is activated by H_2O_2 through the formation of intramolecular disulfide bonds [48]. This activation might provide for increased cellular capacity to handle oxidatively damaged proteins. The four cysteines involved are ligands for a zinc atom in the resting protein; oxidation eliminates the metal and activates the chaperone function [49]. A similar mechanism may govern the redox activation of the *B. subtilis* Fur homolog PerR, which governs an oxidative stress regulon (Fig. 1) [50]. Reversible disulfide bond formation (whether intramolecular or mixed) makes physiological sense as a regulatory mechanism because it links redox sensing to overall metabolism through NADPH and the oxidation state of intermediaries such as thioredoxin and glutaredoxin [5,47].

Disulfide bond formation in eukaryotic regulatory proteins has also been proposed. A comprehensive review of this and related questions for many mammalian systems has appeared recently [2], and the topic will not be further covered here. In plants, mRNA translation in *Chlamydomonas reinhardtii* chloroplasts is governed by disulfide bond formation in a regulatory protein [51,52]. Other examples will surely emerge, though we await a eukaryotic example of *direct activation* occurring through disulfide bond formation, and the role of mixed disulfides needs further exploration.

Examples analogous to the SoxR mechanism remain to be described, although the facile nature of one-electron oxidation and reduction of an iron-sulfur cluster should lend itself to redox signaling in various contexts. Another prokaryotic example of redox sensing using an iron-sulfur cluster is the *E. coli* Fnr protein, which acts as a repressor of genes involved in aerobic metabolism. Fnr harbors a [4Fe-4S] cluster that is sensitive to oxygen; exposure to O_2 converts the single cluster to a pair of [2Fe-2S] clusters and releases the protein from the DNA [53].

Themes in Redox Sensing

The ability of cellular proteins to detect changes in redox status and the presence of potentially toxic free radicals is likely a fundamental aspect of biology. Known examples of redox-regulated responses already include transcriptional, translational, and enzymatic control. Other key features might include feedback loops that return the system to a "ground state" (e.g., the role of reduced glutathione and glutaredoxin-1 in OxyR regulation) and the use of molecular "canaries in the coal mine"; that is, reactions that damage and inactivate most proteins actually switch on sensors such as OxyR and SoxR.

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CHAPTER 301

Budding Yeast DNA Damage Checkpoint: A Signal Transduction-Mediated Surveillance System

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Introduction

The control of cell cycle progression is of fundamental importance for all cells. Alterations in the mechanisms that oversee the coordination between the various DNA metabolic processes and the alternation of the different cell cycle phases frequently lead to uncontrolled proliferation or cell death [1]. *Checkpoints* are genetically controlled systems that are deeply involved in these coordination efforts and are activated upon sensing the presence of altered structures in the cell. The molecular effect of activating these pathways is not yet fully understood. Upon detection of DNA damage and triggering of the checkpoint, a transcriptional activation response can be observed, which involves several genes acting in various DNA repair pathways, and cell cycle progression is delayed (Fig. 1A). This is supposed to provide the cell with enough time to repair the lesions before replicating or segregating the chromosomes, although no formal proof of this hypothesis has been produced yet [2]. Detection of damaged DNA during the replication phase of the cell cycle leads to an expansion of the S phase by controlling origin firing and by intervening in the stability of the replication forks [3–7]. During the S phase, tight control over the integration of the different repair pathways with the progression of the replication fork is critical for preventing potentially lethal events, such as replication fork collapse and unscheduled recombination processes. Recent work has shown that the checkpoint response indeed acts by protecting the integrity of stalled replication forks and possibly by favoring alternative replication strategies, thus allowing the DNA polymerases to overcome the lesions [5,7–10]. We can predict that mutations in these surveillance pathways would have profound effects and, in fact, are responsible for several predispositions to genome instability and tumor development.

Checkpoints are thought to be similar to signal transduction cascades with proteins, devolved to sensing the abnormalities that elicit a signal that, through a kinase cascade, is relayed to effectors that directly or indirectly control cell cycle progression and DNA metabolic processes (Fig. 1B) [11]. The basics of cell cycle checkpoints are well conserved by evolution, while most of the information obtained on the molecular details of these mechanisms derives from the study of genetically amenable simple organisms such as budding and fission yeasts. In this chapter, we provide a short summary of the state of the art with regard to the checkpoint-mediated signal transduction pathways, with special emphasis on budding yeast.

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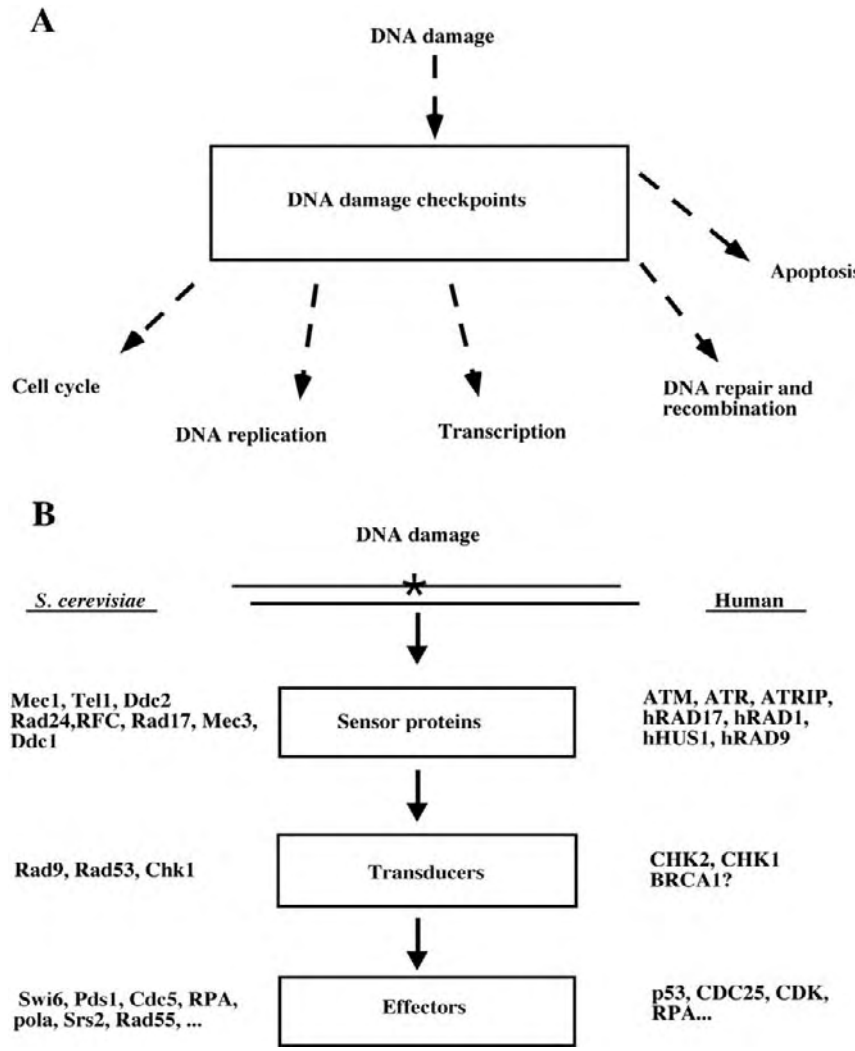


Figure 1

Sensing

Cells can recognize chromosome alterations and stalled replication forks, and this ability triggers a particular signal that results in a cascade of phosphorylation events. Very little is known about how this happens. How does the cell realize that its DNA has been damaged and how do different types of damage trigger the same response? Much work has been devoted to solve these questions, in the last few years, and there is evidence to suggest that single-stranded DNA might represent the checkpoint activating signal [7,12–14]. Genetic analysis has allowed the identification of some factors that are likely upstream in the signaling pathway. For the DNA damage checkpoint, these are represented by the Rad24, Rad17, Mec3, Ddc1, Mec1, and Ddc2/Lcd1 proteins of *Saccharomyces cerevisiae* [15–17]. All of the corresponding genes are present in all other eukaryotic cells, even though the names are different and confusing (Fig. 2).

Rad24 is a protein that bears some degree of homology to the five replication factor C (RFC) subunits and can be purified to RFC2-5 [18,19]. Rad17, Mec3, and Ddc1 are associated in a heterotrimeric complex, which *in silico* studies was predicted to have a toroidal structure very similar to that of proliferating cell nuclear antigen (PCNA) [20,21]. RFC1-5 is required to load PCNA at the primer-template interface during DNA replication; it is thus possible that a Rad24-modified RFC complex could be involved in loading the Rad17 complex onto damaged DNA. Moreover, conflicting evidence has suggested that at least one of the three subunits composing the heterotrimeric toroid might have exonucleolytic activity and thus be involved in DNA damage processing [22–24].

Mec1 is a protein kinase, of the PI3 family that is absolutely essential for checkpoint activity and is associated with the product of the *DDC2/LCD1* gene, which has been reported to mediate Mec1 binding to DNA [25–27]. The molecular details of its function and regulation have not been completely elucidated yet. All available data show that Mec1 targets are phosphorylated in response to genotoxic treatment or replication fork arrest, but this could happen in one of two ways. The kinase could be normally inactive and its catalytic activity could be triggered after the detection of some chromosomal structure (e.g., ssDNA). Because Mec1 is fairly similar to a

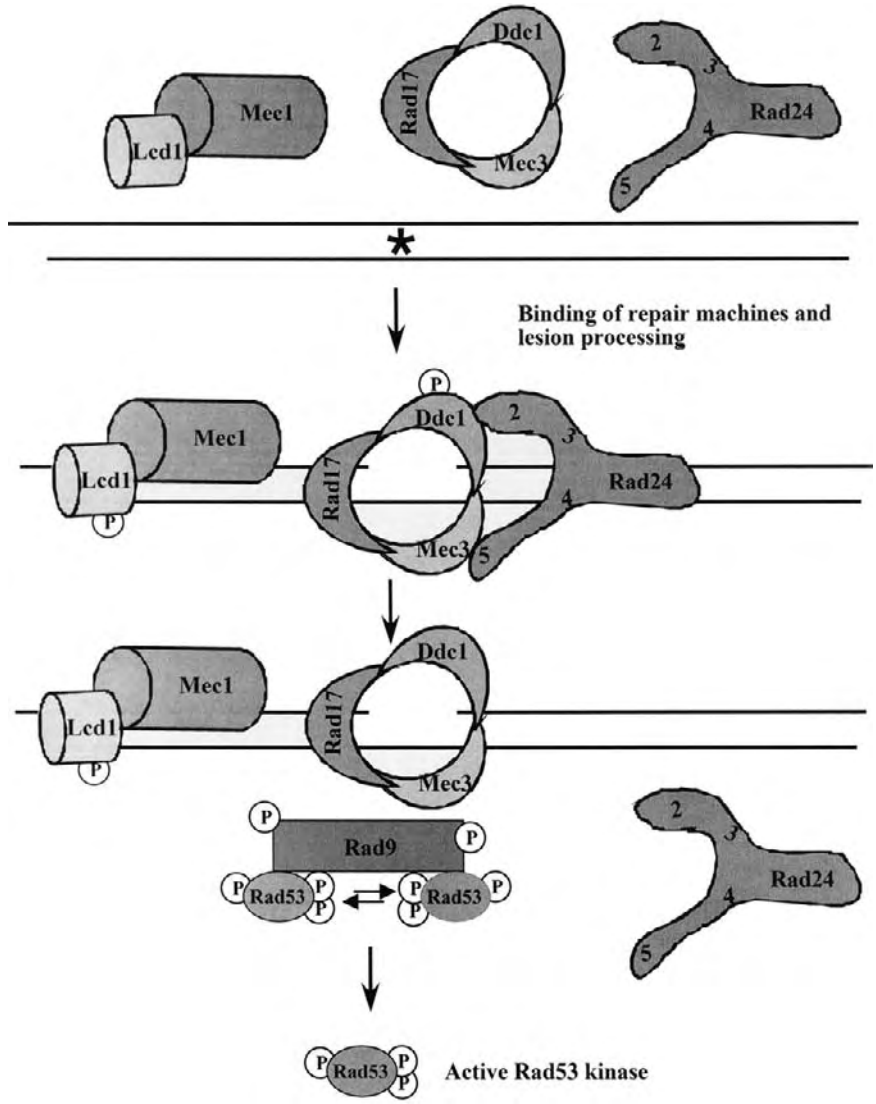


Figure 2

well-studied mammalian protein kinase (DNA PK), which binds to and is activated by DNA, one could imagine that binding of Mec1 to damaged DNA could lead to the activation of its catalytic function. On the other hand, it is also possible that the kinase could be constitutively active and only after DNA damage recognition does it encounter its substrates. In this second option, it is not the kinase catalytic activity that is regulated but rather the availability of the substrate.

Several groups, working on different organisms, have tried to obtain a clear demonstration explaining how all this could happen, although the results are somewhat contrasting [27–30]. Mec1 has several known targets and it has been demonstrated that the genetic requirements for their Mec1-dependent phosphorylation may be different. In fact, Rad53, a downstream kinase, is activated by Mec1-induced phosphorylation in a pathway that requires *RAD24* and the heterotrimeric complex [11]. On the other hand, Ddc2/Lcd1, which is presumably constitutively associated with Mec1, is phosphorylated in response to DNA damage, also in a strain lacking the *RAD24*, *RAD17*, *MEC3*, *DDC1* genes [15,26]. Thus, either we imagine a different regulation of Mec1 catalytic activity toward different targets, or we have to believe that the kinase is always active (or it could be activated upon DNA binding), and the substrates become available only following DNA damage.

Several groups have suggested that recruitment of the substrates could be, directly or indirectly, mediated by the Rad17 complex (Fig. 3) [31–34]. It is still not known how Mec1 and its substrates are brought in proximity to DNA after genotoxic treatment. Intriguingly, recently it has been shown that Mec1-Lcd1 and the Rad17 complex can be independently recruited onto damaged DNA [31–33]. It is hard to imagine that Mec1 or Lcd1 could themselves detect any kind of DNA alteration at any site of the genome. One possibility would be that DNA damage might alter the topological state of a chromosomal domain, thus unmasking the checkpoint signal and restricting the search for the lesion to limited portions of the genome. Another option, which is not mutually

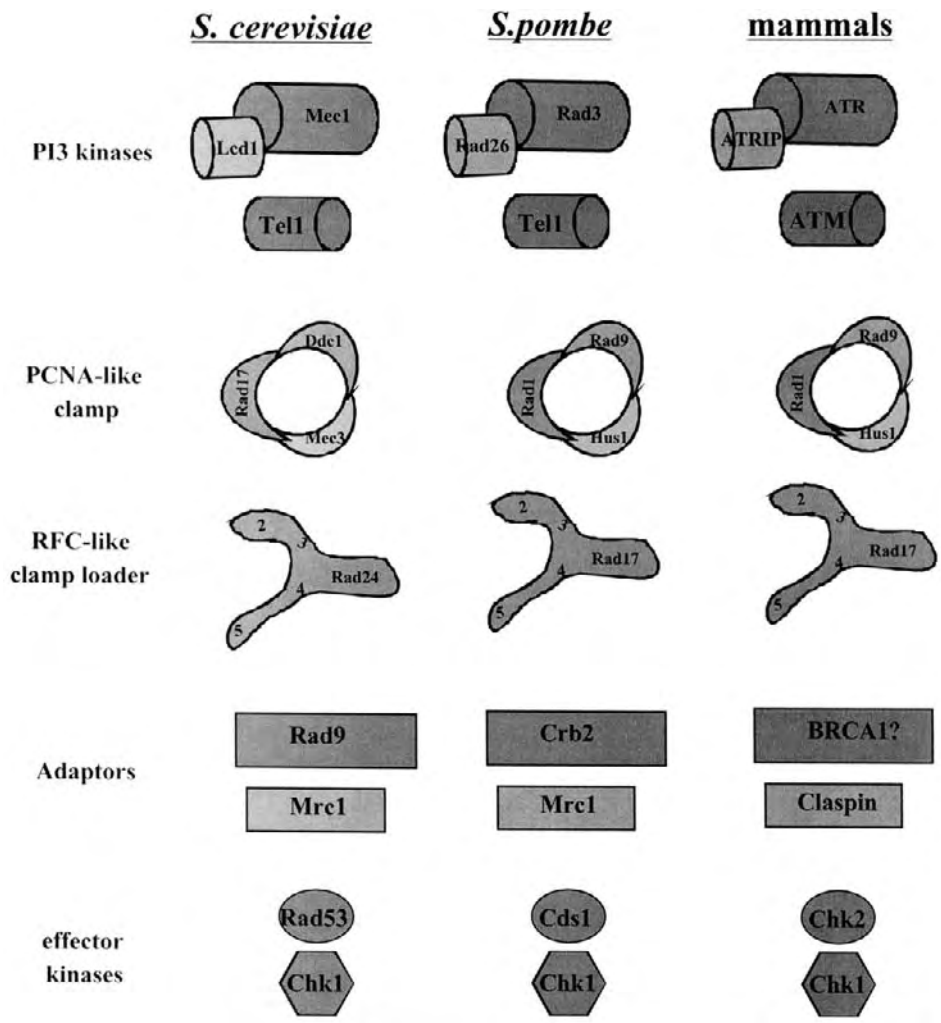


Figure 3

exclusive with the former one, predicts that it is the DNA repair proteins themselves that recognize DNA damages and by doing so trigger the checkpoint response, either by directly recruiting checkpoint proteins or by generating some intermediate DNA structure that can be recognized by the checkpoint. In this latter view the checkpoint response would be tightly connected to DNA repair functions. In this view it is intriguing that the Rad50/Xrs2/Mre11 complex, involved in double-strand break processing, has been reported to be required for DNA damage checkpoints triggered by double-strand breaks [35–37].

Downstream Events

Another important substrate of Mec1 kinase is the product of the *RAD9* gene, whose most likely human homolog is BRCA1, an oncosuppressor involved in breast cancer. The role of these modifications and how they are interconnected is not clear yet. Mec1-dependent hyperphosphorylation of Rad9 seems to be required for checkpoint activity: Hyperphosphorylated Rad9 captures Rad53 kinase molecules, promotes their transphosphorylation by Mec1 and, by increasing their local concentration, allows them to undergo autophosphorylation events that result in activation of Rad53 kinase activity. Rad9, thus functioning as a solid-state catalyst for Rad53 activation, works as an adaptor between Mec1 and Rad53 leading to signal amplification [38–42].

What happens after Rad53 has been activated is still unclear; in fact, very little is known about Rad53 targets. In G2, one possible substrate is Cdc5, a protein required for mitotic exit and for adaptation following double-strand break formation. Cdc5 is phosphorylated following genotoxic treatment in a Rad53-dependent manner; moreover genetic data suggest that Cdc5 could be a critical Rad53 target [43–45]. Swi6, a factor involved in cell-cycle-dependent transcription of several genes, among which are cyclins, has been shown to be a direct substrate of Rad53 *in vitro*, and this phosphorylation inactivates this protein [46]. Furthermore Rad53 activity causes modifications of the phosphorylative state of several enzymes involved in DNA replication, recombination, and repair [47–50].

Another kinase, Chk1, is activated by Mec1, even though the molecular details of this event are not completely clear.

It has also been reported that Chk1 phosphorylation is dependent on Rad9 and that the two proteins physically interact; this suggests the existence of a mechanism similar to the one described for Rad53 activation. Chk1 is important for the function of the G_2/M checkpoint and regulates the anaphase inhibitor Pds1. Upon phosphorylation by Chk1, Pds1 is stabilized and prevents loss of cohesion between sister chromatids, a fundamental step for the metaphase/anaphase transition, causing cells to arrest in mitosis. In G_2 cells two branches departing from Mec1 seem to amplify and transduce a signal in order to make sure that the cell cycle is arrested on detection of DNA damage [43,51].

In response to genotoxic treatments, cells also activate the so-called "DNA damage regulon" (DDR), which includes several genes from different repair pathways. Transcriptional induction of the DDR is genetically controlled through activation of the checkpoint pathway. Hence, in this way, checkpoint activation increases the intracellular concentrations of repair enzymes. Among the DDR-controlled genes is *RNR2*, which encodes a subunit of ribonucleotide reductase and whose transcription is cell cycle regulated, showing a peak at the beginning of the S phase. This is an enzyme that carries out the rate-limiting step in deoxyribonucleotides (dNTPs) synthesis and, thus, controls the pool of dNTPs within the cell. In recent years it has been shown that a deletion of *MEC1* or *RAD53* is lethal in budding yeast, but this lethality can be rescued by overproducing *RNR2* or by mutating Sml1, an inhibitor of ribonucleotide reductase.

These results indicate that the level of the intracellular pool of dNTP is limiting in yeast cells and that in order to cope with problems in DNA replication during the S phase or repair synthesis, cells have to increase dNTPs levels by upregulating *RNR2*. Intriguingly, the checkpoint feeds into the regulation of this critical activity in atleast two ways. In fact, as mentioned earlier, activation of the DNA damage response signal transduction cascade triggers transcription of the DDR genes. This is achieved through the activation, mediated by Rad53, of yet another kinase, namely, Dun1 [52]. Dun1 also phosphorylates the above-mentioned Sml1 protein and by doing this it causes Sml1 degradation by the ubiquitin pathway [53–55]. Thus, by activating Dun1, the checkpoint not only brings about more Rnr2 protein, through an increase in transcription, but also activates ribonucleotide reductase by extinguishing the Sml1 inhibitor.

The DNA damage checkpoint response is thus a very complicated signal transduction pathway, with several branches that likely interconnect and coordinate all DNA metabolic processes in the cell. In the last 5 years much has been learned about the molecular mechanisms underlying its function, but many important details are still unknown. The study of simple eukaryotes, like budding and fission yeast, thanks to their amenability to genetics studies, will be critical for the complete understanding of such complex phenomenon.

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CHAPTER 302

Finding Genes That Affect Signaling and Toleration of DNA Damage, Especially DNA Double-Strand Breaks

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Introduction

The total response of an organism to DNA perturbations is determined by many factors, including the nature of the lesions, ability to detect and tolerate the lesions, and status of the cell cycle at the time the damage occurs. The responses require transduction of damage information into cellular changes as well as processes for directly modifying the damaging effects of the lesions. The information can be transduced into a series of effectors that could direct a host of processes including apoptosis, replication inhibition, cell cycle arrest, lesion bypass, and repair. Some examples of elaborate DNA damage transducing systems are the ATM-p53 network of mammalian cells [1] or the RecA-LexA system from bacteria ([2] and elsewhere in this volume). Lesions are sensed through the ATM or RECA pathway, leading to posttranslation modifications of the p53 or the LEXA *transducer* proteins. Modifications of the transducer result in an orchestrated change in expression of more than 20 effector genes involved in many aspects of cell growth, repair, and even cell death in the case of p53.

The signaling elements could be the DNA lesions or their processed intermediates. DNA double-strand breaks (DSBs) are among the most biologically relevant lesions [3]. As indicated in Fig. 1, they are induced directly by a variety of agents or as a result of processing of single-strand lesions. DSBs can arise spontaneously during replication and they are an intrinsic part of meiosis leading to recombination [4]. Genetic and biochemical studies in yeast were the first to reveal that they can be repaired via recombination, a process with features common to nearly all organisms. End joining is another type of DSB repair common to most eukaryotes, although the nature of breaks may determine its efficiency [5].

Studies of DSBs have provided insights into signaling networks that deal with DNA lesions. Many kinds of lesions can produce signals that are transduced into biological effects and the responses are often lesion dependent (Fig. 1). Signaling targets could be genes that are relevant to repair and DNA metabolism. Repair may or may not require signal transduction depending on whether the full set of proteins is always available and active (constitutive), as appears to be the case for excision repair [6] and photoreactivation [7] of UV-induced lesions. DNA damage responses may also be *d*amage-*in*ducible (*DIN*) requiring the *de novo* production of proteins involved in repair or replication [7,8]. Alternatively, posttranslational modification such as phosphorylation may be required for activation of existing proteins, as is the case for many damage-induced cell cycle arrest phenomena.

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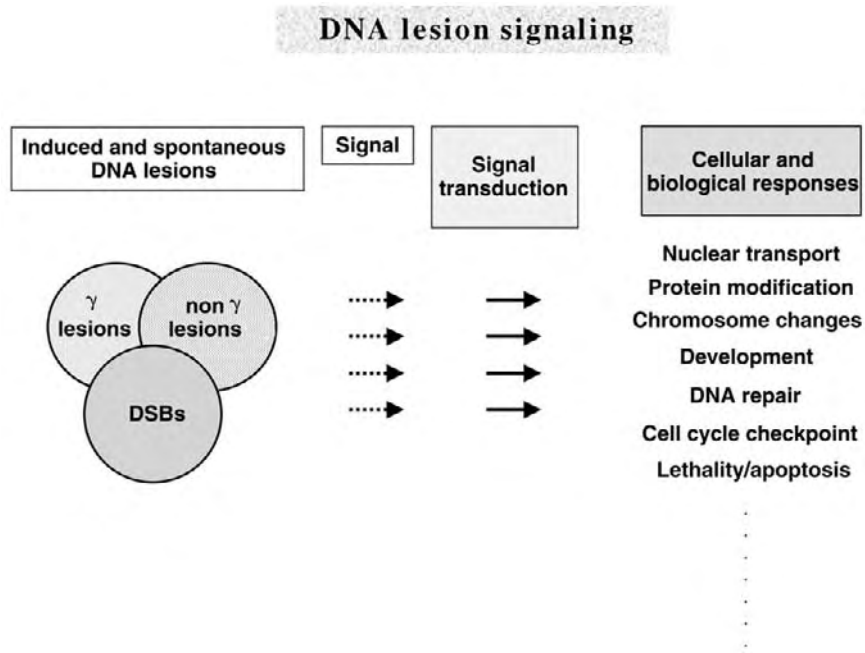


Figure 1 DNA lesion signaling in the yeast *S. cerevisiae*. DNA damage from agents such as ionizing radiation (gamma rays) are capable of inflicting DNA, protein, and lipid damage; however, DSBs appear to be the most biologically relevant lesions and they are responsible for most gamma-ray-induced lethality. Other DSB lesions such as those induced by MMS or HU (also see text) may result from unresolved replication intermediates that occur during the S phase of the cell cycle. Following the induction of DNA damage, yeast cells utilize a sensitive signaling system that can detect even a single DSB, transduce information to the cell cycle apparatus and in turn effect efficient repair of the lesion. This process requires gene products that act as (1) "sensors" of the type and magnitude of damage and (2) signal "transducers" that activate a wide range of cellular and biological responses, which are in turn "effectors" of cell cycle arrest and lesion repair or lesion toleration. Failure in any of these signaling processes can result in genome instability such as aneuploidy, deletions, and gross chromosomal rearrangements. In higher eukaryotes this instability can initiate the multistep process leading to cancer. Persistence of unrepaired DSB damage in nonessential (dispensable) DNA can also lead to cell lethality even in the presence of an intact DNA damage signaling system.

Studies using microarray expression approaches should reveal more of the network involved in *DIN* gene expression.

The budding yeast *Saccharomyces cerevisiae* has been an excellent model system for addressing the relationship between DNA lesions, signaling, and biological consequences. One of the great strengths of this organism is the opportunity to address genetically the components that connect lesion production to cellular changes. Moreover, there is the unique opportunity with budding yeast to follow morphologically cell cycle events in individual cells (Fig. 2). As cells transition from the G_1 to S phase, they develop buds and prior to mitosis (G_2/M) the resulting cells have a doublet appearance [9].

DNA damage could signal a variety of modifications including chromosome condensation, altered nuclear morphology, changes in cell cycle, and apoptosis. Because cell morphology reflects stages in the cell cycle in *S. cerevisiae*, yeast has been ideal for characterizing damage-induced cell cycle changes and for the isolation of mutants that affect the underlying processes. Cell cycle arrest is a hallmark signaling response to DNA damage. Cells monitor the status of chromosome integrity and possess elaborate, genetically controlled feedback networks, or checkpoints, to prevent cell cycle progression if there are chromosomal alterations [10] (Fig. 2). Checkpoints provide additional time for repair prior to cellular division. As discussed later, cell cycle arrest checkpoints have two facets: STOP (arrest) and GO (resumption of cell cycle progression). In yeast, genes regulating the STOP (solid lines, Fig. 2) and GO (dashed arrows, Fig. 2) functions have been identified at both the G_1/S or the G_2/M transitions, whereas only genes regulating STOP functions have been identified during replication (S-phase) arrest.

The strengths of yeast in addressing damage and cell signaling responses have been enhanced through global opportunities to examine genetic relationships. These include total analysis of gene expression [11] combined with functional evaluation of all genes that contribute to resistance of DNA damage [12]. Complete genome mRNA expression reveals the circuitry of responses to perturbations and may suggest participants in signaling pathways. Knowledge of which genes contribute to sensitivity to an agent may lead to identification of genes that play a critical role in damage signaling responses. In this chapter, we focus on approaches to the isolation of genes that play a role in signaling responses to DNA damage, particularly DSBs.

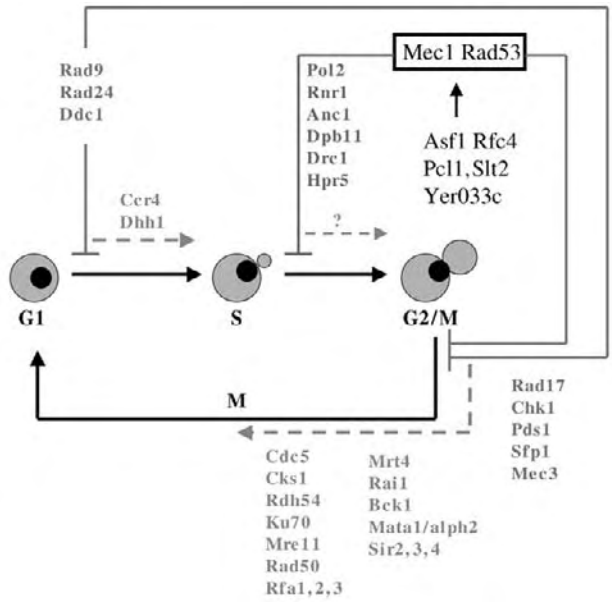


Figure 2 Genetic controls responsible for cell cycle arrest following DNA damage in the yeast *S. cerevisiae*. The G_1, S and G_2 stages of the cell cycle can be identified morphologically. Single unbudded G_1 cells progress through the cell cycle (solid arrows) and give rise to cells with small buds corresponding to the initiation of S phase. Cells progress into G_2 where the size of the daughter cell increases to approximately half the size of the mother cell. In the presence of unrepaired DSB damage, logarithmically growing cells can signal cell cycle arrest (⊥) at any of the three stages in the cell cycle: G_1, intra-S, and G_2/M transition. These damage-induced checkpoints are controlled by many genes that check for genetic integrity. Checkpoint genes such as *RAD9* cause cell cycle arrest, allowing greater time for repair. Mutations in the checkpoint genes can result in a lack of cell cycle arrest and reduced viability following DNA damage. There are also adaptation genes that enable cells to resume cell cycle progression, even in the presence of unrepaired damage (---»). Mutations in these genes cause prolonged cell cycle arrest and loss of viability following DNA damage. *CCR4* and *DHH1* are newly identified adaptation genes required for the resumption of cell cycling following DSB-induced G_1 arrest (T. J. Westmoreland *et al*. *J. Surg. Kes*., in press). Genes such as *RAD9, RAD24,* and *DDC1* are required for both the G_1 and G_2/M checkpoints. *MEC1* and *RAD53* provide intra-S and G_2/M checkpoints. The roles of some genes (such as *ASF1, PCL1, SLT2,* and *YER033C*) that are high-copy suppressors of the temperature-sensitive growth phenotype of *mrc1rad53* double mutants are less clear (see [53] for details).

Nature of DSB and Repair and Genetic Consequences

DSBs can appear spontaneously or result from a variety of physical and chemical agents. They can vary markedly, both in structure and in biological responses. They are observed during DNA replication or meiosis and are intrinsic to mating-type switching in yeast. The ends of DSBs come in many forms. Random, radiation-induced DSBs have *dirty* ends often associated with base or sugar damage [13]. The *in vivo* expression of endonucleases such as the mating-type switching enzyme HO or restriction enzymes produces clean, site-specific breaks with blunt or recessed cohesive ends [5]. Many anticancer agents such as bleomycin are described as radiomimetic although the actual structure of the resulting DSBs differs from those produced by radiation [14]. Also, agents that interfere with DNA replication such as hydroxyurea (HU), the single-strand alkylating agent methylmethane sulfonate (MMS), or topoisomerase inhibitors such as camptothecin produce DSBs and/or unresolved replication intermediates that are specific to the S phase of the cell cycle.

DSBs can be repaired by recombination with homologous chromosomes or sister chromatids or by direct end joining [5,15,16]. In the absence of recombination, DSBs may lead to loss of essential chromosomal material via large deletions, rearrangements, and aneuploidy. However, in yeast, lethality can also be indirect as shown for a persistent unrepaired DSB in dispensable DNA [17–19]. The biological responses to specific types of DSBs vary in yeast, suggesting that the nature of the break influences recognition, signaling, and repair. In our recent study, many gamma-ray-sensitive mutants were sensitive to one or more of the following DSB-inducing agents: bleomycin, camptothecin, MMS, or HU, indicating that a variety of repair/checkpoint pathways are required to tolerate a wide spectrum of DSB lesions [12]. Furthermore, DSBs induced by ionizing radiation are almost exclusively repaired by recombination, which explains why radiation resistance in Rad^+ haploid cells is G_2 dependent [20]. An undamaged homolog is available for recombinational repair via the *RAD52* group of repair genes. Surprisingly, *in vivo* expression of *Eco*RI results in little or no lethality in recombination-deficient *rad52* strains in spite of the large number of DSBs. Unlike radiation-induced DSBs, these breaks are repaired almost exclusively by end joining [21].

Checkpoint Activation and Adaptation as Signaling Responses to DSBs

Because bud status identifies cell cycle stage, *S. cerevisiae* is ideal for examining the signaling impact of DNA damage, particularly DSBs, on cell cycle progression using time-lapse photomicroscopy. Following induction of radiation or HO-induced DSBs, cells arrest (STOP) predominantly in the G_2/M phase of the cell cycle [9,17]. This ensures both greater opportunities to repair lesions prior to division and accurate chromosome segregation. Inactivation of many of the genes within these checkpoint pathways, such as *RAD9*, renders cells sensitive to ionizing radiation [9,22]. Damaging agents also induce arrest at G/S preventing entry into S phase [23–25], and there is also an S-phase checkpoint that monitors successful completion of replication [26]. Cells arrested at G_2/M can, after a period of time, resume cell cycling even if the damage remains unrepaired. This GO process has been termed *adaptation* [27]. The duration of the checkpoint arrest at G_2/M may in part depend on the strength or persistence of the original inducing signal. Yeast have a robust recognition system for detecting DSBs. A single, site-specific HO-induced DSB is sufficient to signal prolonged G_2/M arrest, suggesting an exquisitely sensitive system for sensing a break and amplifying and transducing the signal to effectors that arrest the cell cycle machinery [9,17].

The DSB signaling response can occur even if it is induced in a completely dispensable plasmid [17] or yeast artificial chromosome (YAC) vector [18,19], under conditions where the break may or may not be repairable. In the absence of repair the break leads to loss of the vector. If there is rapid repair, there is no checkpoint response suggesting that induction of a DSB is not sufficient *per se* to signal a checkpoint response [18]. Surprisingly, in the absence of repair, the DSB can signal not only cell cycle arrest but also cell death even though there is no loss of genetically relevant material. It is interesting that some nonrepairable DSBs do not cause arrest or lethality [19].

DNA Damage Signaling Networks

Extensive DNA damage signaling networks have been revealed through genetic screens with yeast. DNA damage-induced checkpoint arrest and adaptation is a complex multicomponent signaling process involving more than 40 genes that can affect the G_1/S, S, and G_2/M phases of the cell cycle (Table I, Fig. 2). As described later, many genetic and biochemical strategies have been used to screen for checkpoint signaling mutants. They extend from signaling responses elicited by random radiation-induced DSBs or site-specific HO-induced breaks to DNA replication stress induced by agents such as MMS or HU. Knowledge of genes involved in damage-induced checkpoints has enabled the development of secondary screens that do not rely directly on DNA damage responses.

Identifying Checkpoint Defects by Screening Radiation-Sensitive Mutants

The first checkpoint mutant, *rad9*, was identified in a screen of radiation-sensitive *S. cerevisiae* mutants sensitive to ionizing radiation [9]. This *d*amage *s*ensitive *mu*tant screening approach (*DSMU*; see Table I), assumes that checkpoint defects will sensitize cells to damage. Therefore, simple visual screening of DNA damage-sensitive mutants for cell cycle progression has been successful in identifying many checkpoint and adaptation mutants (Table I). Recently, a broad

Table I Screening for Genes Involved with DNA Damage Checkpoint and Adaptation Responses in the Yeast *Saccharomyces Cerevisiae*

Gene	Essential	Phosphorylated	Checkpoint function	Screening method	First report of checkpoint mutant
RAD9	No	Yes	G2/M checkpoint arrest following DNA damage or replicative arrest G1/S checkpoint after damage	*DSMU*	[9] [23]
RAD17	No	No	G2/M arrest following DNA damage or replicative arrest	*DSMU & DRAC*	[28]
MRT4	No	?	G2/M checkpoint arrest following DNA damage as diploid and adaptation to the G2 checkpoint as haploids	*DSMU*	[12]
RAI1	No	?			
BCK1	No	Yes?	Adaptation to G2/M checkpoint as haploid		
MRE11	No	Yes	Complex is required for phosphorylation of Rad53 and Chk1 to activate the G2/M checkpoint following damage	*DSMU*	[29]
XRS2	No	Yes			
RAD50	No	No			
KU70	No	No	Adaptation to G2/M checkpoint	*DSMU & DECP*	[30]
MRE11	No	Yes			
RAD50	No	No			
RPA(RFA1,2,3)	Yes	Yes			
TID1(RDH54)	No	?	Adaptation to G2/M checkpoint	*DSMU*	[31]
MRC1	No	Yes	S phase checkpoint	*DSMU*	[32]
DUN1	No	Yes	G2/M checkpoint following damage; mutant defective in DNA damage-induced transcription of *RNR2* and *RNR3*	*DITR*	[33]
HRR25	No	Yes?	Defective in DNA damage-induced transcription of *RNR2* and *RNR3*	*DITR*	[34]
SWI4	No	Yes?			
SWI6	No	Yes			
SFP1	No	?	G2/M checkpoint arrest following DNA damage	*DITR*	[35]
CRT1	No	Yes	Effector of DNA damage and replication arrest checkpoint pathway; required for transcriptional activation after damage	*DITR*	[36]

Table I *continued*

Gene	Essential	Phosphorylated	Checkpoint function	Screening method	First report of checkpoint mutant
RAD24	No	Yes	G2/M damage checkpoint S + G2/M	*DRAC*	[37]
MEC1,	Yes	No	incomplete replication and damage		
RAD53	Yes	Yes	checkpoint		
MEC3	No	No	G2/M damage checkpoint		
PDS1	No	Yes	G2/M checkpoint arrest following DNA damage	*DECM*	[38]
CDC5	Yes	Yes	Adaptation to G2/M checkpoint	*DECP*	[27]
CKB2	No	?			
POL2	Yes	No	Defective in S phase checkpoint and *DUN1* dependent transcription	*SRA*	[39]
CKS1	Yes	?	Suppressor of *RAD53*-dependent replication (S-phase) checkpoint	*SRA*	[26]
RFC5	Yes	?	Suppressed by overexpression of *RAD53*	*SRA*	[40]
DDC1	No	Yes	G1/S and G2/M checkpoints following DNA damage	*SRA*	[41]
RNR1	Yes	?	Suppression of the *mec1* and *rad53*	*SRA*	[42]
ANC1	No	?	replication defect		
DPB11	Yes	?	Suppression of *pol2* and *dph2* replication defect	*SRA*	[43]
DRC1	Yes	?	Suppression of *dpb11* replication defect	*SRA*	[44]
SML1	No	Yes	Suppression of *mec1* and *rad53* replication defect	SRA	[45]
CHK1	No	Yes	G2/M checkpoint arrest following DNA damage	*HOMS*	[46]
CHL12(CTF18)	No	?	Defective in S phase checkpoint when combined with *rad24*	*HOMS*	[47]
RAD55	No	Yes	*RAD53*-dependent activation by phosphorylation	*CHDP*	[48]
HPR5(SRS2)	No	Yes	S-phase checkpoint	*CHDP*	[49]
SIR2,	No	?	Adaptation to G2/M checkpoint	*ENCHR*	[50]
SIR3,	No	Yes?			
SIR4,	No	Yes?			
MAT	No	?			
HUG1	No	?	*MEC1*-dependent arrest following DNA damage or replication	*SAGE*	[51]
RFC4	Yes	?	S + G2/M incomplete replication and damage checkpoint	*SYNL*	[52]
YER033C	No	?	S + G2/M incomplete replication	*SSYNL*	[53]
PCL1	No	Yes?	and damage checkpoints		
SLT2	No	Yes?			
ASF1	No	?			

Total = 50

Phosphorylation status: Yes? = phosphorylated but not yet examined following DNA damage; ? = not examined; No = not detected

Approaches for identifying checkpoint and adaptation defective mutants:

*DSMU-d*amage *s*ensitive *mu*tants that have a posttreatment checkpoint defect

DITR-mutants with altered *d*amage *i*nduced *t*ranscription

DRAC-mutants that are *d*efective in *r*eplication *a*ssociated *c*heckpoint

DECM-checkpoint mutants among mutants that are *de*fective in *c*hromosome *m*echanics

DECP-direct screen for mutants *de*fective in *c*ell *p*rogression (adaptation) following induction of DNA damage

CHOM-mutants with *c*heckpoint *hom*ology, *i.e.,* homology to known checkpoint genes in the same and/or other species

ENCHR-genes that *en*hance *ch*eckpoint *r*esponse to DNA damage when expressed in high copy

*SRA-S*uppression of *r*eplicative *a*rrest and/or lethality by deletion or overexpression.

*SAGE-S*erial *a*nalysis of *g*ene *e*xpression in the presence of HU

*SYN-Syn*thetic *l*ethality with the *rfa1-Y29H* ts allele

*SSYNL-S*uppression of *syn*thetic *l*ethality of a *mcl1rad53* double mutant

CHDP-Direct screen for *ch*eckpoint *d*ependent *p*hosporylation of known recombination gene products

functional genomics approach was used in *diploid* yeast that identified 107 new genes that enhanced sensitivity to gamma radiation when deleted. Using *DSMU* screening, the mutants *bck1*, *mrt4,* and *rai1* were found to be defective in postirradiation G_2 arrest, similar to *rad9* [12]. Surprisingly, these mutations resulted in an adaptation defect in haploids where they experienced prolonged G_2 arrest after irradiation. This is unlike *RAD9* mutants, which have a defective checkpoint response in both haploid and diploid cells. These effects suggest modifiers of checkpoint signaling that may result from differences in transcriptional controls associated with mating type (*MAT*) and/or ploidy.

Checkpoint Mutants Revealed through Screening DNA Replication Mutants

Signaling defective mutants have been identified through secondary screens with replication mutants. Some temperature-sensitive (*ts*) mutants of replication-essential genes arrest in late S phase at high temperature. The arrest is due to activation of a checkpoint that detects replication-induced lesions (possibly DSBs). Therefore, mutations in damage-sensing checkpoint genes, when combined with *ts* mutants, should also be *d*efective *i*n *r*eplication *a*ssociated *c*heckpoints (*DIRAC*; Table I). Four *ts* cell division cycle (*cdc*) mutants including *cdc13* were not viable in combination with *rad9* or *rad17* mutations if held at the restrictive temperature for a short time [28]. Both RAD mutants were required for checkpoint arrest following UV or gamma-ray damage, suggesting they failed to sense DSBs, caused by replication arrest of the *CDC* mutants. The *cdc13* mutant was used in another *DIRAC* screen to identify the *m*itosis *e*ntry *c*heckpoint mutants *mec1, mec2 (rad53), mec3,* and *rad24* [37]. Further analysis with various *CDC* mutants and other damaging agents including hydroxyurea established that the *RAD17, RAD24,* and *MEC3* were specific to the G_2/M checkpoint, whereas *RAD9* was active at both G_1/S and G_2/M checkpoints. The essential *MEC1* and *MEC2* genes were required for both the S phase and the ionizing radiation-induced G_2/M arrests, suggesting two overlapping damage signaling checkpoint pathways (Fig. 2).

Using direct *s*uppression of *r*eplication *a*rrest (*SRA*; Table I), *S*-phase *a*rrest *d*efective (*sad*) mutants including *sad1/rad53* and *sad2/pol2* were initially isolated based on HU sensitivity [26]. In a second-site suppressor screen, the HU sensitivity of *sad1/rad53* was suppressed by mutations in *CKS1*, a subunit of the Cdc28p kinase required for normal G_1-to-S and G_2-to-M cell cycle transitions. The Cdc28p kinase appears to be a downstream target of Cdc5p kinase implicated in checkpoint adaptation; therefore, it appears to be involved in both S-phase and G_2/M checkpoints [26].

Because *RAD53* is central to damage-induced signaling responses and it is essential, high-copy suppressors of lethality were sought. Interestingly, overexpression of the ribonucleotide reductase gene *RNR1* completely suppressed the lethal effects of *rad53*Δ, and *mec1*Δ mutants but did not suppress the HU or UV-induced lethality or checkpoint defects [42]. Furthermore, deletion of the *SML1* gene was found to suppress the replication defects of *mec1* or *rad53* deletions without affecting their checkpoint functions [45]. These results indicated that two separate checkpoint functions exist in S phase, either dealing with damage or replication stress. Surprisingly, overexpression of the transcriptional regulator *ANC1* was found to be a weak suppressor of the *mec1*Δ replication defect, possibly through the transcriptional regulation of other genes [42]. Using *SRA* screens, both Dpb11p [43] and Drc1p [44] have also been identified as participants in the S-phase replication checkpoint. These proteins appear to interact with the C-terminal domain of DNA Pol2p. This domain participates in the sensing of DNA damage to prevent entry into mitosis when DNA is not replicated [43].

Recently synthetic lethality genetic screens (*SYNL*; Table I) have further expanded the role of replication factors in checkpoint controls. For example, the *RFC* complex (*RFC1-5*), which is a processivity factor for DNA polymerases 2 and 3, plays a role in checkpoint signaling. The *rfc4-2* mutant was lethal in combination with a mutant *(rfa-t11)* of *RFA1* [52] that encodes a subunit of the DNA-binding protein RPA required for G_2/M adaptation [30]. Both the *rfc4-2* and *rfa1-t11* mutants were defective in the G_2/M checkpoint, as determined by decreased arrest of *cdc13* or *cdc15* mutants at the nonpermissive temperature. The *rfc4-2* cells were found to have a defective S-phase checkpoint in response to MMS damage [52].

Screening for Checkpoint Defects

Screening for checkpoint defects associated with damage-induced changes in transcription also identifies kinase mutants. *D*amage-*i*nduced *t*ranscription (*DITR*; Table I) is an important signaling response to radiation. Genes associated with checkpoint function frequently influence gene expression by altering transcription. Among the *d*amage-*i*nducible (*DIN*) genes are those required for synthesis of ribonucleotide reductase (i.e., *RNR1*, *RNR2,* and *RNR3*). By fusing *RNR3* to a β-galactosidase color reporter gene, 5 complementation groups of DNA *d*amage-*un*inducible mutants were isolated [8,33]. One of these transcriptionally defective signaling mutants (*dun1)* is partially defective in the G_2/M checkpoint response [54]. It is a nuclear protein kinase that autophosphorylates following DNA damage in a *RAD53*-dependent manner and subsequently is required for inactivating the Crt1p transcriptional repressor (by hyperphosphorylation), which is required to tolerate replicative stress and maintain genome stability [36]. Thus, *DUN1* (through inactivation of *CRT1*) derepresses *RNR2* and *RNR3* following damage. Other candidate G_1/S and S-phase checkpoint genes include *HRR25*, *SWI6,* and *SWI4.* Mutations in these genes result in sensitivity to HU and MMS treatment as well as defective damage-induced (*DIN*) transcriptional signaling responses [34].

Central components of the damage-induced signaling pathway are the Mec1p and Mec2p/Rad53p kinases, which are essential for replication and are involved in both S and

G_2/M responses. Phosphorylation is clearly important in checkpoint responses because more than half of the associated gene products are phosphorylated, many in a damage-specific manner (Table I). The G_2/M checkpoint arrest involves two parallel *MEC1*-dependent pathways that contribute equally to arrest [55]. These pathways utilize upstream damage "sensor" functions attributed to the combined actions of the *RAD9, RAD17, RAD24, MEC3,* and *DDC1*gene products [41,56]. Both Rad9p and Ddc1p appear to be phosphorylated following DNA damage. Furthermore, Rad53p is also phosphorylated following damage in a Mec1p-dependent process. Rad53p in turn phosphorylates downstream effectors [46] such as the *CDC5* gene product required for checkpoint adaptation [27] and the *RAD55* gene product required for recombinational repair [48]. In a second parallel pathway, the checkpoint protein Chk1p functions in a Mec1-dependent manner to maintain abundant levels of Pds1, an anaphase inhibitor when DNA damage is present [46,55]. The Cdc5p, Rad55p, Chk1p, and Pds1p effectors are all activated by a *MEC1*-dependent phosphorylation cascade in response to damage.

Screen for Altered Checkpoint and Adaptation Responses to a single DSB

A single, persistent HO-induced DSB in a nonessential plasmid or YAC can induce a prolonged G_2 arrest followed by lethality. However, some strains do not exhibit this prolonged arrest and quickly adapt to the damage and resume cell cycle progression. Using such a strain, a high-copy library was screened for genes that would confer *en*hanced G_2 *ch*eckpoint *r*esponses (*ENCHR*; Table I) and lethality following induction of a HO break in dispensable DNA. Multiple copies of the silent mating type locus *HMRa* resulted in the *ENCHR* phenotype and this was attributed to the locus being able to titrate proteins involved in chromatin silencing [50]. This led to the finding that deletion of the multifunctional chromatin modification genes *SIR2, SIR3,* or *SIR4* has the same effect. Both the mating type status and the *SIR* genes determined the checkpoint and lethal responses to the single DSB. These results suggest that the transcriptional status of the cell (in this case subject to mating-type controls) can greatly influence checkpoint responses or lethality arising from a persistent DSB. Furthermore, since a *rad9*Δ *sir4*Δ mutant did not show prolonged G_2 arrest, but did exhibit DSB-induced lethality, the two responses to a DSB appear to involve separate pathways [50].

A single unrepairable DSB system has also been used to address the ability of cells to reenter the cell cycle, that is, to adapt to a break, and to screen for mutants that are *de*fective in *c*ell *p*rogression (*DECP* screen; Table I). A site-specific break in *MAT* was created in a dispensable copy of chromosome III in cells that could not repair the DSB because they were deficient in recombination (i.e., *rad52*). Cells mutated in *cdc5* and *cdk2* were permanently arrested as budded cells in a *RAD9*-dependent manner [27]. Furthermore, in diploid cells lacking recombinational repair, adaptation is a prerequisite of genomic rearrangement or break-induced replication [57]. Therefore, adaptation promotes the survival of diploid cells carrying one or a few persistent DSBs. Cells lacking Yku70p also permanently arrested as budded cells following induction of a single DSB. This was suppressed by mutations in *RAD50*, *MRE11*, or the ssDNA-binding protein complex *RPA* [30]. Using a derivation of the *DSMU* screening approach, the *RAD54* homolog *RDH54/TID1* was identified as an adaptation gene [31]. Following induction of a single HO-induced DSB, *tid1*Δ cells showed prolonged arrest in G_2/M that was *RAD9* dependent. This prolonged arrest was similar to that seen for *yku70*Δ cells; however, extensive resection of the DSB, characteristic of the *yku70*Δ mutant, was not seen in the *tid1*Δ strain. Recently, the Rad50/Mre11/Xrs2 complex has been shown to be required for both the S-phase and G_2/M checkpoints [29]. Specifically this complex is required for damage-specific phosphorylation of Rad9p, Rad53p, and Chk1p. Because Rad9p is also required for G_1/S arrest following damage, deletions of *RAD50, MRE11*, or *XRS2* may affect this checkpoint as well.

Other Screens for DNA Damage Checkpoint Pathway Genes

Several other screens have also identified genes associated with checkpoint control. For example, *CHK1* was identified in *S. cerevisiae* based on a *hom*ology *s*earch (*HOMS*; Table I) against the mammalian and *Schizosaccharomyces pombe* Chk1p [46]. Similarly, the yeast Chl12p is related to the yeast Rad24p; it provides a redundant replication checkpoint function [47].

The *PDS1* (*p*recocious *d*isassociation of *s*ister chromatids) checkpoint mutant was first identified by its *de*fect in *c*hromosome *m*echanics (*DECM*; Table I) [58]. Subsequently, it was shown to have a damage-associated checkpoint function [38]. Another approach, serial analysis of gene expression (*SAGE*; Table I), revealed a nonannotated transcribed gene *HUG1* that was highly expressed following HU treatment. Subsequently it was shown to affect checkpoint responses [51]. Using a variation of the *DSMU* screen and examining all *RAD52* group genes for damage-inducible *ch*eckpoint-*d*ependent *p*hosphorylation (*CHDP*; Table I), Rad55p was found to be a *terminal effector* of the *MEC1*-dependent checkpoint pathway since it was phosphorylated following DNA damage in a *RAD53*-dependent fashion [48]. In a similar manner, the *CHDP* approach identified Hpr5p (Srs2p) as being phosphorylated in response to damage in a *MEC1, RAD53*, and *DUN1*-dependent manner [49].

High-copy *s*uppressors of *syn*thetic *l*ethality (*SSYNL*; Table I) were identified that rescued growth of a *mrc1 rad53 mec1* triple mutant at a nonpermissive temperature. These mutants also suppressed the HU sensitivity of *rad53* mutants. One of them, *ASF1*, which is involved in chromatin remodeling, was later shown to have additional interesting checkpoint-related functions [46]. The mutant is gamma-ray sensitive, spontaneously arrests in G_2, and exhibits high levels of spontaneous transcription of a damage-responsive reporter,

suggesting a role in replication or S-phase checkpoint response ([12]; unpublished).

Implications of DNA Damage Checkpoint Signaling

Genomic instability has been implicated as an early event in cancer progression. Defects in genes that regulate DNA damage checkpoints have been suggested to be predisposing factors for the onset of cancer because of their influence on genome stability. Mutations in many of the yeast genes described earlier, particularly those associated with the S-phase checkpoint, result in destabilization of the genome. The conservation of gene function among most eukaryotes predicts that many of these yeast genes that are critical for damage signaling and checkpoint control will have orthologs in human cells with similar roles in checkpoint signaling pathways. For example, Mec1 is the yeast ortholog of the human checkpoint gene ATR [11]. Mutations within ATR are responsible for sensitivity to DNA damaging agents and a predisposition for cancer. Also, mutations in the C-terminal domain of the breast cancer gene BRCA1 abrogate mammalian checkpoint response at G_2/M following DNA damage [59]. A large superfamily with repeats of the conserved BRCT domain first identified in the C terminus of BRCA1 have also been found in many damage-responsive checkpoint proteins including yeast *RAD9*, *DPB11*, and the large subunit of RFC [60]. Thus, characterization of new checkpoint genes in yeast will undoubtedly lead to the identification of new cancer gene targets and the elucidation of their underlying molecular role(s) in neoplastic progression as well as other diseases.

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CHAPTER 303

Radiation Responses in *Drosophila*

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Introduction

Four decades after the discovery of X rays, H. J. Muller established *Drosophila* as a preeminent model for an emerging field of *radiation biology*, receiving a Nobel Prize for showing a cause-and-effect relationship between radiation and heritable mutations [1]. We now understand that radiation exposure promotes genotoxic damage, causing wholesale chromosomal alterations that jeopardize viability and reproductive capacity. Consequently, all organisms have evolved sophisticated mechanisms that respond to and counteract these effects. In eukaryotes, radiation responses can be divided into three phases: damage sensors, signal transmitters, and effectors that promote (1) cell cycle arrest, (2) DNA repair, and (3) apoptosis. Except where specified we use the term *radiation* in the broad sense, not necessarily distinguishing UV rays versus X rays.

Sensors and Transmitters

Initial Detection of DNA Damage

Sensors must have the ability to discriminate damaged from normal DNA, and they must show a defect in the DNA damage checkpoint when mutated. A current model for the sensors in *Schizosaccharomyces pombe*, describes a multicomponent complex composed of Hus1, Rad1, and Rad9 that is recruited to damaged DNA by R17 associated replication factor C (RFC) [2–6]. Though, we do not yet know which gene products act as direct sensors of DNA damage in flies, the best candidates are *Drosophila* orthologs of *S. pombe*'s gene products recruited to damaged DNA via the RFC.

Signal Transmission by Kinases

Once DNA damage is recognized, signal transmitters including the ATM and ATR kinases are activated. In mammals, ATM responds exclusively to ionizing radiation (IR), whereas ATR responds to IR, UV radiation, and stalled replication forks [7]. The *Drosophila* ATM/ATR homolog, *mei-41*, was isolated as a meiotic mutant four decades ago [8] and has been well characterized as a pleiotropic locus affecting DNA repair, chromosome instability, and defective replication control [9]. A second ATM/ATR like gene is found in the fly genome (CG6535) and, from sequence alignments, this is a likely ortholog of ATM, whereas Mei-41 more closely resembles ATR [10]. In mammals, ATM and ATR activate the downstream serine/threonine kinases Chk1 and Chk2, depending on the signals they receive [11] and, similarly, in flies, genetic studies suggest *mei-41* (ATR) functions upstream of *grapes*, the fly homolog of *chk1* [12]. Though *mei-41* and *chk2* mutant flies exhibit similar damage checkpoint defects [13], tests for genetic interactions between these have not been reported. Like their mammalian counterparts, *Drosophila* Chk1 (Grapes) and Chk2 kinases probably function to modulate the activity of damage response effectors.

Effectors

Cell Cycle Arrest

Cell division cycles in early *Drosophila* embryos are divided into two stages, defined by the midblastula transition (MBT), which occurs 120 min after fertilization. Cell cycle divisions prior to this (referred to as *pre-MBT*) are controlled by maternally derived transcripts, whereas the *post-MBT* phase is controlled by zygotically produced transcripts.

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The first 13 nuclear cycles do not contain G_1 and G_2 stages, and proceed as a syncytium (without cytokinesis). A G_2 phase is added to the cell cycle during the 14th embryonic division, which is accompanied by cytokinesis. Syncytial embryos (1–13 cycles, pre-MBT) and cellular embryos (14–16 cycles, post-MBT) show very different responses to X-ray irradiation (Table I). In addition, syncytial embryos can eliminate damaged nuclei by incorporation into the yolk, and these are quickly replaced through rapid nuclear divisions of syncytial embryos. This elimination process cannot be accomplished in cellular embryos (owing to an emergence of cell membranes); therefore, alternative methods, such as programmed cell death, are involved. Su and Jaklevic [14] speculate that these different radiation responses reflect an emphasis on velocity of replication in syncytial embryo, and replication fidelity in cellular post-MBT embryos.

When post-MBT embryos are exposed to X-ray irradiation, delays are observed at both G_2/M and metaphase–anaphase transitions. These responses are evidently important for reducing the incidence of broken chromosomes [14]. In the wing discs, flies mutated for *mei-41*, *grapes*, *chk2*, and *14-3-3ε* exhibit G_2/M checkpoint defects of varying degrees, indicating these genes act in a common pathway whose elements are outlined in Fig. 1 [9,15,16]. The onset of a G_2/M transition checkpoint during normal development involves a Cdc25 homolog, String, and Cdks. String may activate Cdks by removing inhibitory phosphates, and activated Cdks drive the G_2/M transition together with mitotic cyclins. The inhibitory phosphorylation of Cdks may be necessary during damage-induced G_2/M arrest, as previously demonstrated from mammalian studies [19,20]. Induction of p21 is a major regulator of damage-induced G_1 cell cycle arrest in mammals [21–23], but interestingly, Dacapo, the fly ortholog of p21, is not responsive to radiation [17,18], though it can induce G_1 cell cycle arrest when ectopically expressed in eye discs [18,24]. Therefore, in flies, current evidence suggests that one or more p21-independent pathways specify radiation-induced cell cycle arrest.

DNA Repair

Some of the first mutants in DNA repair genes were identified in the 1970s in flies as mutagen-sensitive (mus) or meiotic recombination mutants (mei) [25–27]. Currently, approximately 30 mus genes and 2 mei genes are known or inferred to comprise the DNA repair pathway [28]. (For a comprehensive review, see [10,29].)

Apoptosis

The propensity toward radiation-induced apoptosis varies dramatically during development and appears to correlate with the proliferative potential. Tissues in early embryos and larval imaginal discs are particularly sensitive to radiation-induced apoptosis, whereas later stage embryos and adults are not. This pattern is also reflected in an organism's viability [30]. Figure 2 outlines an emerging model for radiation-induced apoptosis in *Drosophila*. In early embryos, *reaper* (*rpr*) is transcriptionally activated in response to radiation via an enhancer region that contains a binding site for Dp53 [31].

In functional studies, both Dp53 and the *rpr* region are required for radiation-induced apoptosis [24,31]. Another gene mapping near the *reaper* region, *sickle* (*skl*), was recently identified as a radiation-responsive gene capable of inducing apoptosis [32–34]. Rpr and Skl antagonize the function of inhibitor of apoptosis proteins (IAPs) by releasing activated caspases from IAP-mediated inhibition [35]. Another regulator of caspases, Dark, is required for radiation-induced apoptosis like its human counterpart, Apaf-1[36]. This gene is induced by X-ray and UV exposure [37–39], but whether it is a target of Dp53 has not been determined. In mammals, Chk2 phosphorylates and activates p53 in response to irradiation [40]. Because mutations in the *Drosophila chk2* gene also exhibit defects in both cell cycle arrest and apoptosis, it is reasonable to hypothesize that at least the apoptotic defects reflect p53-dependent functions. Requirements for the *Drosophila* homologs of ATM and ATR in apoptosis have not been tested.

Conclusions: What Can We Learn from the *Drosophila* Model?

It has been nearly a full century since T. H. Morgan used the fruit fly as a model to launch the science of genetics.

Table I Damage Induced Checkpoints Associated with Onset of MBT

			Radiation induced arrest		
	Cleavage cycles	Phases	G2	Metaphase/ Anaphase	Other responses
Precellularized embryos	1–13		No	No	Metaphase chromosome align improperly Abortion of mitosis
Cellularized embryos	14–16		Yes	Yes	—

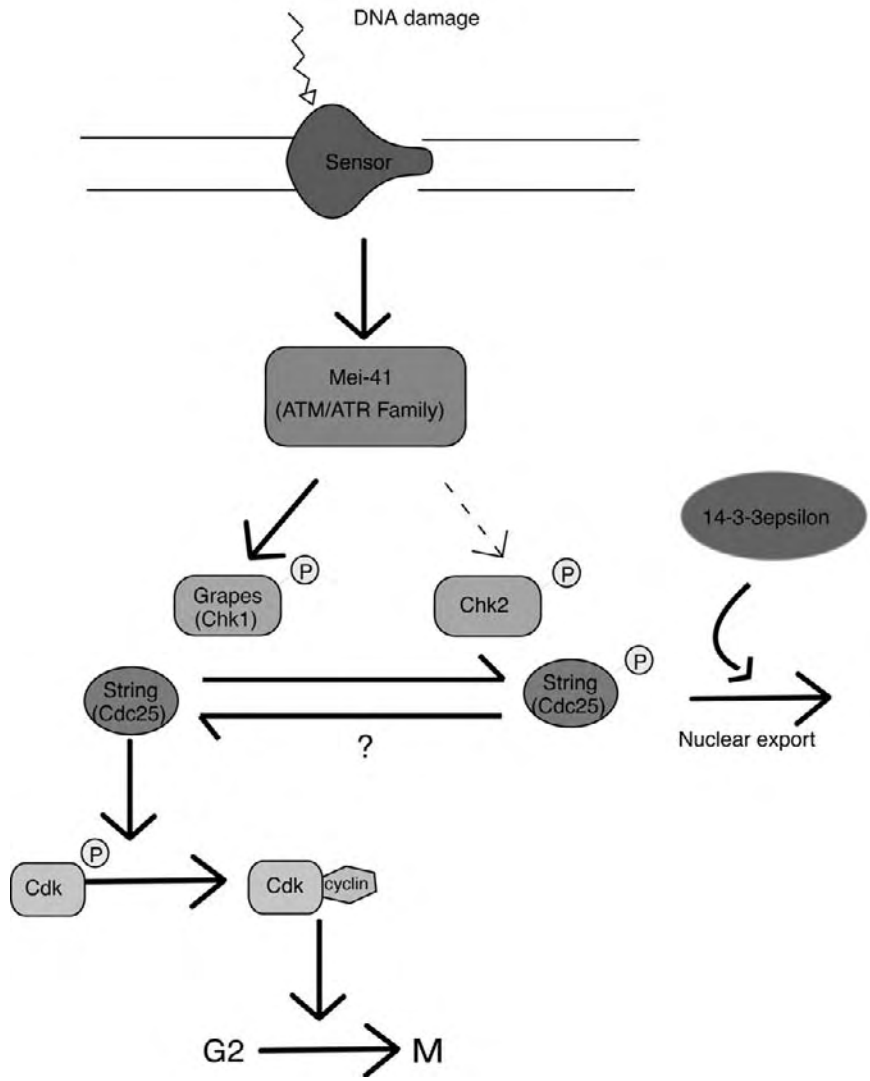


Figure 1 Organization of damaged-induced cell cycle arrest in *Drosophila*. The dashed arrow indicates the inferred orthologous relationship from mammalian studies. Phosphorylation of target proteins is also inferred. Upon radiation-induced damage, MEI-41 activates Grapes or Chk2, leading to the nuclear export of the phosphatase, String. The phosphorylated form of Cdks prevails due to nuclear export of String and, thus, a G2/M transition does not take place.

As a metazoan, *Drosophila* offers fundamental insights regarding the ways in which developmental programs exert constraints on adaptive responses to radiation damage. Although preferential attention is often focused on pathways that are shared by common descent, equally important insights can be gained by an awareness of what has not been conserved. For instance, p53 earned status as a "guardian of the genome," specifying a cell cycle arrest and a DNA repair program that can switch to an apoptotic option if the repair program fails. Current evidence from studies in *Drosophila* contradicts this notion since a robust cell cycle arrest program is triggered in irradiated flies, but *Drosophila* p53 evidently plays no part in this response [24,31]. Similarly, the requirement for induction of p21 as the predominant p53 effector of G_1/S arrest is widely documented in mammals, yet the *Drosophila* homolog of p21, Dacapo, is dispensable for radiation-induced G_1/S arrest (though it is competent to do so [18,24]) and is unlikely to be a radiation-responsive Dp53 target gene [17]. Together, these observations suggest that members of the p53 family can specify the apoptotic fate without first engaging a cell cycle arrest/DNA repair program. An evolutionary corollary related to these observations is that, among the damage responses governed by p53, cell cycle arrest could be a more recently acquired pathway. Continued elucidation of shared and distinct features of radiation-induced damage responses in this model should uncover important insights that help us understand, and perhaps treat, human disease.

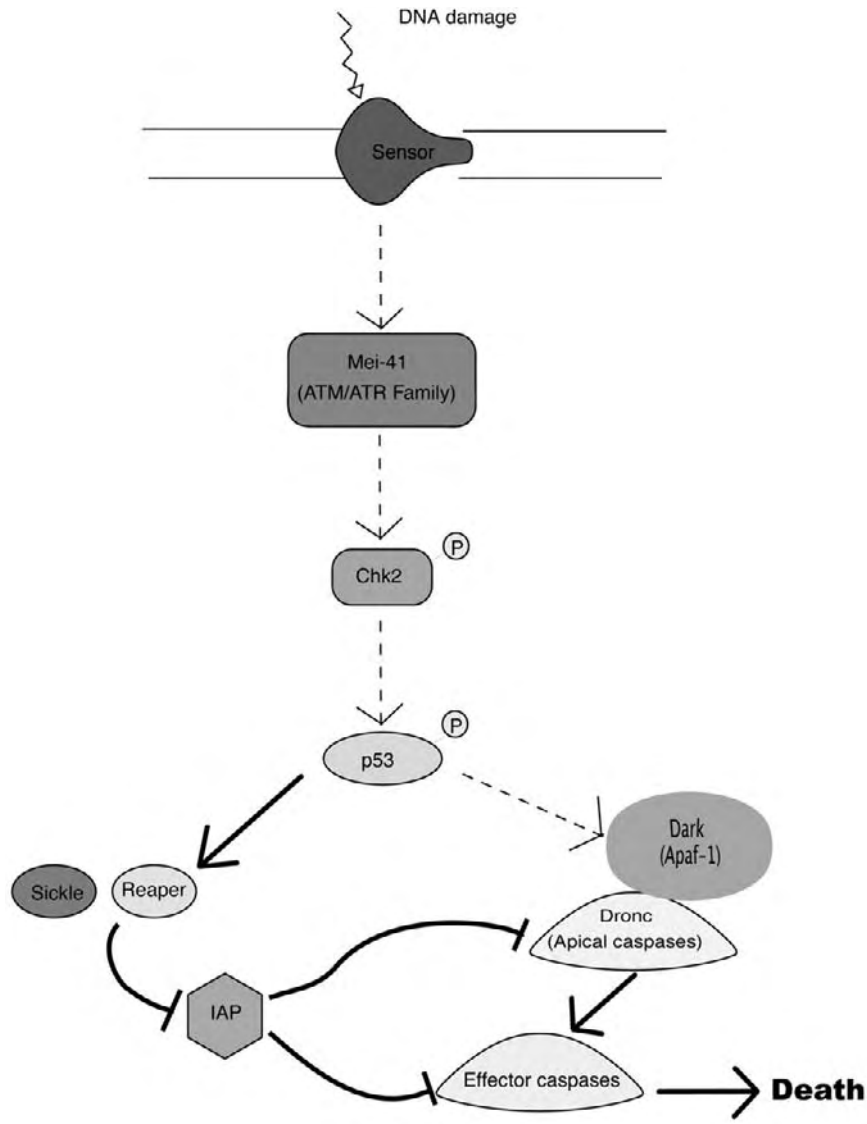


Figure 2 Organization of damage-induced apoptosis in *Drosophila.* The dashed arrows and phosphorylation of target proteins are inferred from orthologous relationships. Upon radiation-induced damage, Dp53 activates IAP antagonists Reaper and possibly Sickle, releasing IAP inhibition from caspases and leading to cell death.

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CHAPTER 304

Double-Strand Break Recognition and Its Repair by Nonhomologous End Joining

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Introduction

Many different agents can damage DNA, often with disastrous consequences. The most dangerous form of DNA damage is the double-strand break (DSB)—a single unrepaired DSB can induce a cell to undergo apoptosis [1]. DSBs can also directly inactivate key genes, lead to chromosomal translocations, or generate unstable chromosomal abnormalities [2], which may ultimately lead to cancer development. DSBs are induced by ionizing radiation and radiomimetic chemicals but can also be generated during site-specific recombination events (for example, during V(D)J recombination in the immune system and meiotic recombination) and during DNA replication.

Because the effects of DNA DSBs are potentially so serious, eukaryotes have elaborate systems to repair the damage rapidly or to stop DNA replication and cell division until the damage has been repaired (Fig. 1; [3]). Here, we consider nonhomologous end joining (NHEJ), a major DSB repair system, drawing on data obtained from the mammalian system and the highly conserved *Saccharomyces cerevisiae* system.

Repair of DSBs: Homologous Recombination and NHEJ

Eukaryotic cells have two major DSB repair systems: homologous recombination (HR) and NHEJ [4,5]. HR involves 5′ resection of the damaged DNA; invasion of the 3′ end of the broken DNA into a homologous DNA duplex molecule—a sister chromatid or a homologous chromosome; and replication of the damaged DNA using the homologous DNA as a template. Because no genetic material is normally lost during HR, this is the DNA repair pathway of choice throughout most of the life cycle of *S. cerevisiae* and other lower eukaryotes.

NHEJ, by contrast, does not require homologous DNA. Instead, the broken DNA ends are brought together and religated, often after limited nuclease digestion to remove damaged bases. Thus, repair of DSBs via NHEJ generally results in loss of some genetic material. Nevertheless, NHEJ is a major DSB repair pathway in mammals, possibly because it is an efficient way to remove potentially lethal DSBs.

Several of the principal players in NHEJ (Fig. 2) were originally identified by studying mutant mammalian cell lines that are hypersensitive to ionizing radiation and defective in DSB rejoining and V(D)J recombination (reviewed in [4,6]). Cells of complementation groups IR5 and IR7 lack Ku80 and DNA-PKcs, respectively. These proteins, together with Ku70, form a DNA-activated nuclear serine/threonine kinase called DNA-dependent protein kinase (DNA-PK; [7]). DNA-PKcs itself is a large protein that is a member of the phosphoinositol 3-kinase-like family of kinases (PIKK; see Smith and Jackson elsewhere in this series). There are yeast homologs of the two small Ku subunits but no obvious DNA-PKcs homolog. Cells of complementation group IR4

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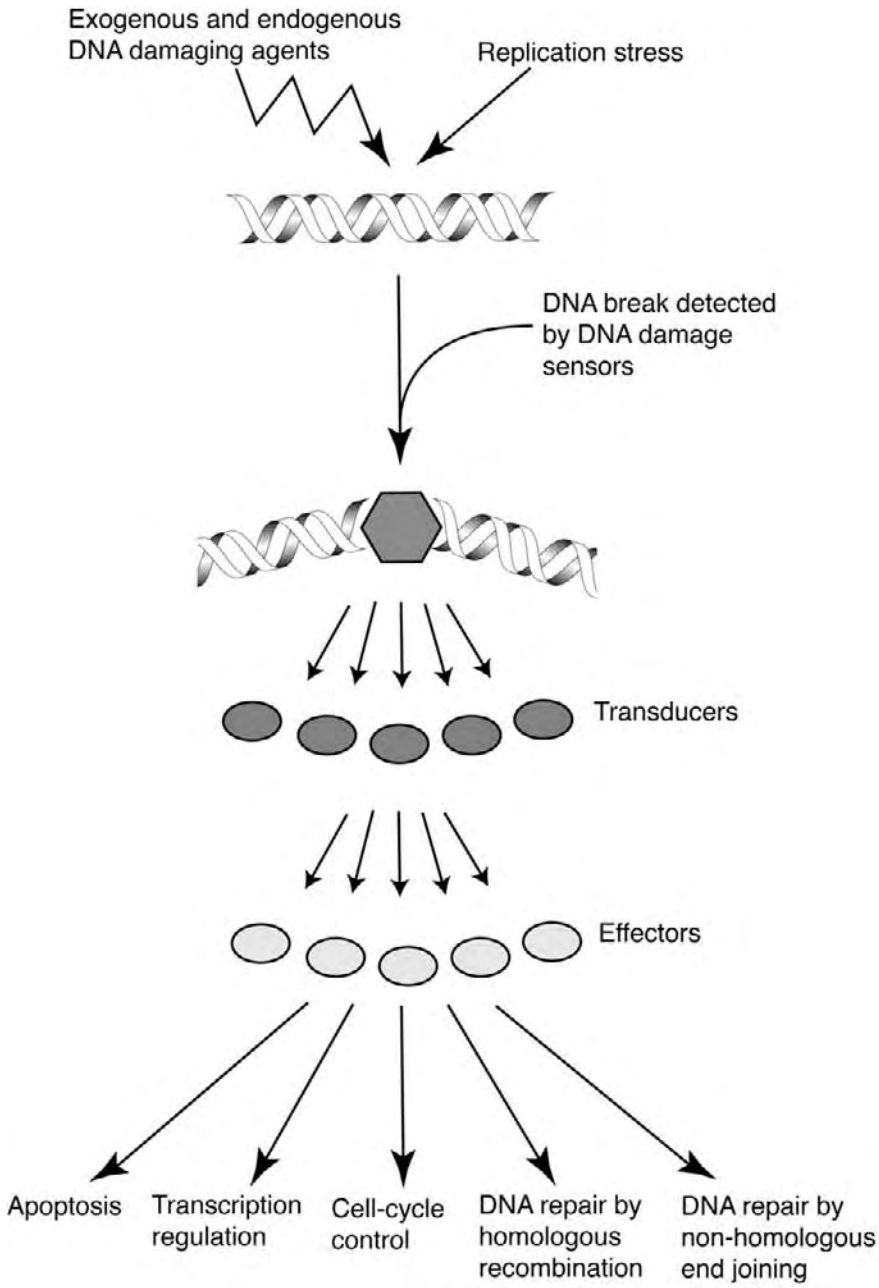


Figure 1 General outline of the cellular response to DNA double-strand breaks. For simplicity, responses are shown as an arbitrary number of separate arrows. In reality, the number of pathways and their interactions with each other are much more complicated, allowing the cell to deal with each and every DNA double-strand break that occurs rapidly and efficiently.

lack the small nuclear phosphoprotein XRCC4, a protein that forms a tight, specific complex with DNA ligase IV [8,9]. This is the ligase responsible for rejoining DNA at DSBs and thus the effector of the NHEJ pathway. Again, close homologs of XRCC4 and DNA ligase IV exist in yeast (Lif1p and Dnl4p, respectively; [10–12]).

Other evidence linking DNA-PK and ligase IV to NHEJ comes from studies on spontaneous mouse mutants and knock-out mice. The severe-combined immunodeficient (Scid) mouse strain has no mature circulating T or B lymphocytes and is hypersensitive to ionizing radiation and radiomimetic drugs. Scid mice lack active DNA-PKcs [13,14], thus indicating the importance of DNA-PKcs in both DNA repair and V(D)J recombination. More recently, knock-out mice have been derived in which DNA-PK components are missing (reviewed in [7]). Most of these are immunodeficient and hypersensitive to ionizing radiation, but, interestingly, mice lacking DNA ligase IV or XRCC4 [15–17] are embryonic lethal (see later discussion).

In addition to these players, the human Mre11/Rad50/Nbs1 complex (Mre11p/Rad50p/Xrs2p in *S. cerevisiae*) has been implicated in NHEJ. This complex may be involved in

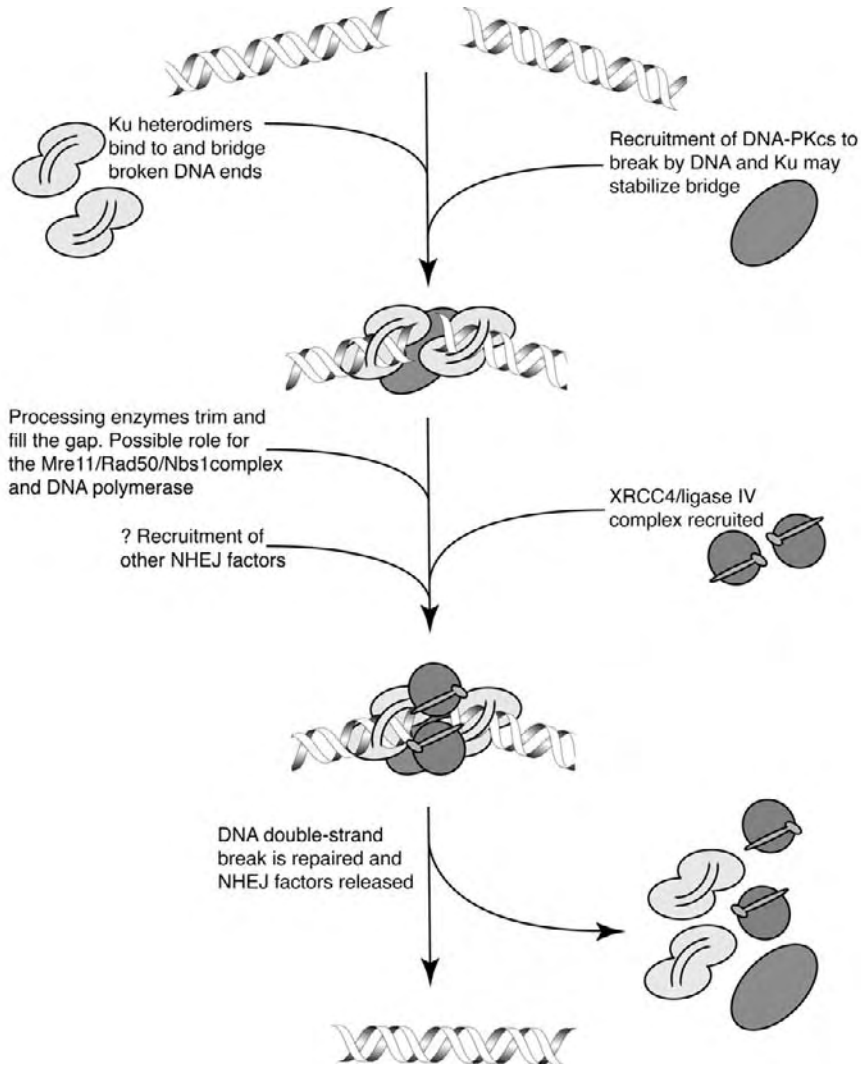


Figure 2 Schematic showing the major known players in NHEJ in mammalian cells. Some of the potential additional NHEJ components are described in the text where attention is also drawn to aspects of this model for which we have recently gained new insights.

preparing the DNA for ligation via the nuclease activity of Mre11p [18]. However, it also has a role in checkpoint signaling in mammalian cells (reviewed in [5]) and in yeast [19–21]. Finally, two new NHEJ factors have been identified recently (see later discussion).

Recognition of DNA DSBs

Substantial *in vitro* evidence indicates that the ends of a DNA DSB are initially recognized by two Ku heterodimers [6,7,22], which may interact to bring together the DNA ends [23,24]. Subsequent recruitment of DNA-PKcs may help to stabilize this bridging complex and to recruit or activate other factors needed for NHEJ (Fig. 2). The recent publication of the crystal structure of Ku bound to DNA provides support for this model [25]. In this structure, the Ku heterodimer forms an open ring that can be threaded onto a DNA end. One side of this ring forms a cradle that protects one surface of the DNA double helix. The other side of the ring is considerably more open, a finding that suggests how other NHEJ factors can access the DSB, including the processing enzymes needed to prepare the DNA for ligation. The structure also helps to explain many of the *in vitro* properties of Ku. For example, the observation that Ku makes contacts almost exclusively with the sugar-phosphate backbone of the DNA explains why Ku binds with high affinity to DNA ends irrespective of sequence or structure [22].

The structure also explains why Ku can only be loaded onto free DNA ends (reviewed in [7]) and becomes trapped if linear DNA is subsequently circularized [26]. However, the data beg the question: How is Ku released from DNA once the DSB has been repaired? One possibility is that Ku is released before or concomitant with ligation. Alternatively, Ku's removal from ligated DNA might require its structural rearrangement or proteolytic digestion.

Signal Transduction

DNA-PKcs can bind to DNA by itself [27–29] and is activated by binding to single-stranded DNA [30,31], possibly through a conformational change [32,33]. Nevertheless, mammalian DNA-PKcs can be regarded as a signaling kinase that links DNA damage sensing by Ku to DNA repair—the kinase activity of DNA-PKcs is certainly necessary for NHEJ [34]. *In vivo*, DNA-PKcs probably never binds alone to DNA. Instead, the weak intrinsic DNA-end binding activity of DNA-PKcs is greatly enhanced and stabilized by the Ku70–Ku80 heterodimer [27,29,33,35].

The DNA-PKcs–Ku complex preferentially phosphorylates proteins attached to the same DNA molecule [33] although targeting of non-DNA-bound substrates can also occur. Numerous *in vitro* substrates for DNA-PKcs have been defined, including several transcription factors, chromatin components, and replication protein A (reviewed in [7]). Although it remains unclear which, if any, of these are true physiological substrates, it seems reasonable to assume that other NHEJ components will be phosphorylated by DNA-PKcs to increase their intrinsic activity and to encourage the formation of multiprotein complexes. Indeed, XRCC4 is a phosphoprotein *in vivo* and is an effective substrate for DNA-PK *in vitro* [8,36]. The Ku subunits and DNA-PKcs itself are also phosphorylated *in vitro* by DNA-PKcs, modifications that may help in the disassembly of the DNA-PK complex at the time of or after DNA ligation [37].

DNA Repair

Once the DNA ends on either side of a DSB have been brought together and tidied up, the break is repaired by DNA ligase IV in mammalian cells and by Dnl4p in yeast. These enzymes form tight complexes with XRCC4 and Lif1p, respectively, interactions that probably regulate their activity [38]. Certainly, XRCC4–DNA ligase IV promotes DNA end joining *in vitro* [39], a reaction that is stimulated by the addition of Ku [23,40] and by DNA-PK [41].

The recent determination of the crystal structure of XRCC4, both alone [42] and in a complex with a peptide from DNA ligase IV [43], yields some mechanistic insights into NHEJ. XRCC4 alone forms an elongated dumb-bell-like tetramer, but in the XRCC4–DNA ligase IV complex, the helical tail of XRCC4 is partly unwound to produce a flat interaction site. The sites of contact within this complex are highly conserved from yeast to man, suggesting that the mechanism of interaction between XRCC4 and DNA ligase IV has been maintained throughout evolution.

Other Sensors and Transducers of DNA Damage

Although Ku is an important DNA sensor in the NHEJ system, it is not the only sensor for DNA DSBs. Indeed, signaling to the cell cycle apparatus seems to occur mainly through related PIKKs: ATM and ATR in mammalian cells [44,45] and Tel1p and Mec1p in *S. cerevisiae* [3]. For these signaling kinases, we know less about how the damage is initially sensed because no Ku homolog has been found for these enzymes, although a number of proteins have been proposed as possible damage sensors [3]. Recent work in yeast in our laboratory and elsewhere has yielded some information about how Mec1p, the yeast ATR homolog, might be recruited to DNA damage by the protein Lcd1p (also called Ddc2p and Pie1p; [46–49]). A human homolog of Lcd1p has also been identified ([50]; P. Reaper and S. P. Jackson, 2001, unpublished data) and its properties are likely to match those of yeast Lcd1p.

Interestingly, as mentioned earlier, transgenic animals lacking functional XRCC4 or DNA ligase IV are embryonic lethal, whereas mice lacking Ku are viable. One explanation for this apparent inconsistency is that in Ku-defective mice, the broken DNA ends can be detected by other DNA damage sensors, including those that signal to the checkpoint apparatus and to other DNA repair mechanisms. In mice that have defective XRCC4 or DNA ligase IV, however, Ku can still bind to the DSBs and this may result in a nonproductive complex that prevents other cellular responses to DNA damage being activated. Thus the cell, and indeed the whole organism, is unable to avoid lethal genomic instability. Further evidence for this proposition comes from recent work by Adachi *et al.* [51] in DT40 cells, which shows that $LIG4^{-/-}$ cells are more X-ray sensitive than $Ku70^{-/-}$ cells, but that $Ku70^{-/-}/LIG4^{-/-}$ double-mutant cells have a similar sensitivity to $Ku70^{-/-}$ cells.

New Factors in NHEJ

We now know a considerable amount about NHEJ but there may still be unknown factors in both the yeast and the mammalian system. For example, Ooi *et al.* [52] recently developed a microarray-based screen to look for additional NHEJ factors in *S. cerevisiae*. Their screen reidentified several known NHEJ components, but also identified a new factor required for NHEJ, Nej1p, which has been identified independently by three other groups using different approaches [53–55]. Nej1p (also called Lif2p) interacts with Lif1p but it does not stimulate NHEJ simply by regulating Lif1p levels because it is required for efficient NHEJ even when Lif1p is overexpressed in the nucleus. More work is needed to discover Nej1p's exact mode of action and the identity of any mammalian homologs of this yeast protein. Interestingly, Nej1p is expressed only in haploid yeast cells, an observation that provides an elegant explanation for why NHEJ is very inefficient in yeast diploid cells where HR is the preferred means of DSB repair.

Another new NHEJ component was recently identified by looking for the gene defect in a family of human SCID individuals who were also radiosensitive. Unlike Scid mice, human beings who lack T and B cells do not have defective DNA-PKcs. Instead, Artemis, a novel DNA double-strand

break repair/V(D)J recombination protein, is mutated in this subset of SCID patients [56]. Artemis belongs to the metallo-β-lactamase superfamily so it may be involved in DNA processing. However, the precise function of Artemis in NHEJ remains to be elucidated.

Future Prospects

Although we have learned much about NHEJ, many questions remain. For example, is the conformation of DNA-PKcs altered on binding DNA and Ku? What are the *in vivo* physiological substrates for DNA-PKcs? How is the DNA-PK complex removed from the DNA repair site? In addition, mammalian and yeast cells possess multiple DNA damage sensing systems that must be tightly integrated, but we are a long way from understanding how this is brought about. Recent work also indicates that intimate links exist between NHEJ and the control of chromatin structure [57], telomere maintenance [6,58], retroviral integration [59,60], and innate immunity [61]. By understanding all of these aspects of the NHEJ machinery, we should gain insights into how genomic stability is maintained in the face of constant endogenous and exogenous insults, knowledge that should lead to better therapeutic and preventive measures for cancer and other human diseases.

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CHAPTER 305

Role of ATM in Radiation Signal Transduction

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Introduction

To maintain the integrity of the genome, cells have evolved several mechanisms that recognize DNA damage and signal this to the DNA repair machinery, to cell cycle checkpoints, and to transcriptional control. Although there have been exhaustive reports on the nature of the lesions arising in DNA in response to a variety of damaging agents and on the mechanisms of repair, the ability of the cell to recognize these lesions and signal to the appropriate cellular machinery has only recently begun to be unraveled. The description of a number of human genetic disorders characterized by chromosomal instability and cancer predisposition has accelerated our understanding of the process of DNA damage recognition. One such syndrome, ataxia-telangiectasia (A-T), has been a focal point because of the universal sensitivity to ionizing radiation and because of the central role the gene product involved plays in radiation signal transduction. This syndrome is characterized by immunodeficiency, neurodegeneration, radiosensitivity, meiotic defects, and cancer predisposition [1,2]. Radiosensitivity is observed in patients exposed to therapeutic radiation [3,4] and in cells in culture [5,6]. The basis of the radiosensitivity remains unknown but can be explained by a failure of A-T cells to respond appropriately to double-strand breaks in DNA and the presence of residual breaks at longer times postirradiation [7]. It has been suggested that residual chromosomal breaks in A-T cells lead to oxidative stress for which there is evidence in both human cells and in *Atm*-gene disrupted mice [8–10]. Failure of mutant ATM to respond to double-strand breaks in DNA appears to account for the multiple cell cycle checkpoint defects in A-T cells [11,12]. Of these, radioresistant DNA synthesis (RDS) was the first to be described [13,14]. RDS may be explained by a defect in radiation-induced degradation of Cdc25A phosphatase that normally prevents dephosphorylation of Cdc2 kinase and a transient block in DNA replication [15]. Alternatively, it could be due to failure to inhibit cyclin-dependent kinase activity [16] or by defective signaling through BRCA1 [17].

The defect in regulating the passage of cells from the G_1 to S phase can be explained primarily by a defective p53 response in irradiated A-T cells [18,19] but it is also evident that ATM controls the G_1/S checkpoint through several intermediate phosphorylation reactions [20]. The exact basis for the G_2/M checkpoint defect in A-T cells remains unresolved but there is evidence that defective inhibition of Cdc2 cyclin B kinase may at least be partially responsible [16].

ATM is a member of a family of proteins that share a phosphatidylinositol 3-kinase (PI3K) domain [21]. This group includes the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), A-T and rad3-related protein (ATR), and proteins in other organisms responsible for DNA damage recognition or cell cycle control [22]. ATM kinase is rapidly activated by ionizing radiation to phosphorylate a series of substrates involved in radiation signaling [23]. However, evidence also exists to show that ATM can be

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regulated at both the transcriptional and translational levels [24–26]. A more widespread role for ATM in events other than DNA damage recognition exists including receptor signaling, cellular proliferation, K^+ channel activity, and insulin signaling pathways [27–30]. It is possible that ATM plays a direct role in these processes, or that in its absence cellular homeostasis is altered by oxidative stress or some other form of perturbation, leading to the myriad of defects described in A-T cells. Here we initially focus on the function of ATM; its role in DNA damage recognition and its relationship to other DNA damage recognition systems; intermediates phosphorylated; and pathways activated to help coordinate the cellular response to radiation. A possible role in more general signaling is discussed later.

Sensing Radiation Damage in DNA

Exposure of cells to ionizing radiation causes direct damage to DNA as well as ionized and excited species through interaction with water, also capable of damaging DNA [31]. A multitude of damage results, including base modifications and single- and double-strand breaks (DSBs) in the backbone of DNA [32]. Of these the DSB is the most lethal lesion. DNA DSBs also arise normally during the process of V(D)J recombination in B- and T-cell ontogeny and during meiosis and mitosis [33,34].

In both yeast and mammalian cells these breaks are repaired by three mechanisms: single-strand annealing, homologous recombination (HR), and nonhomologous end joining (NHEJ) [35]. The relative importance of these mechanisms was thought to vary between yeast and mammals but recent data suggest this may not be the case [36]. NHEJ is primarily responsible for repair of DSBs in G_1 and early S phase, whereas HR predominates in G_2/M [37]. The appearance of breaks in DNA is rapidly detected by several enzyme systems as outlined in Fig. 1. Poly(ADP-ribose) polymerase (PARP) responds rapidly to single-strand breaks in DNA by transferring ADP-ribosyl from NAD^+ to proteins involved in chromatin structure (histone proteins) and in DNA metabolism (topoisomerases, DNA replication factors, and PARP itself) [38]. Addition of chains of negatively charged poly(ADP-ribose) to these proteins alters their capacity to bind DNA and is responsible for their inactivation.

These protein alterations would be expected to increase the access of proteins involved in DNA repair. Concurrently with the recognition of single-strand breaks by PARP, DSBs are detected by a group of sensor proteins. In *Schizosaccharomyces pombe*, Rad3, a homolog of ATM and ATR, recognizes damage in DNA in conjunction with Rad1, Rad9, Rad17, Rad26, and Hus1 [39]. The corresponding proteins in human cells form a complex, and reduced extractability from nuclei after DNA damage points to tighter binding to damaged DNA. Recent results suggest that after DNA damage

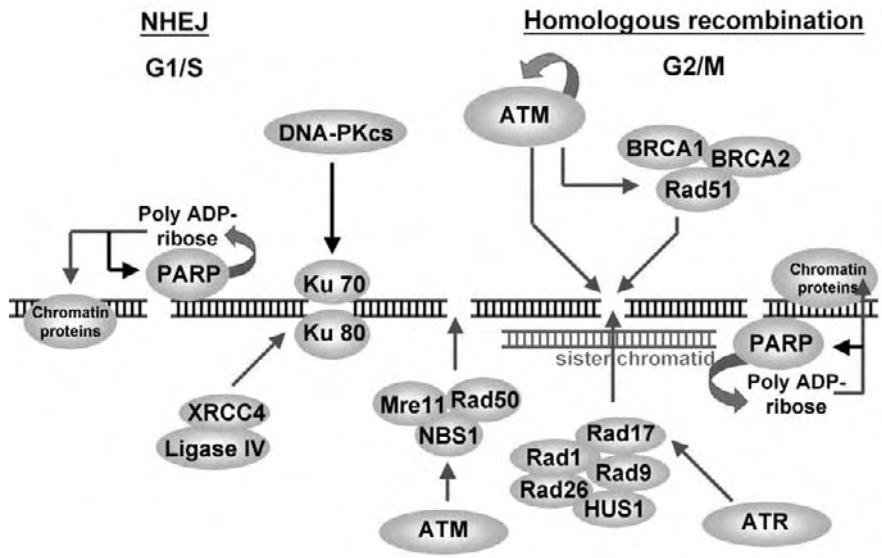


Figure 1 Detection of strand breaks in DNA. PARP is rapidly activated by single- and double-strand breaks in DNA to poly ADP ribosylate a number of proteins including itself. This modification alters their ability to bind to DNA and in turn assists in the processing of the break. DSBs are repaired by two major mechanisms: NHEJ and HR. Recognition of the DSBs involves the coordinated action of several proteins and complexes including DNA-PKcs and the Ku heterodimer that recognize free ends in DNA followed by recruitment of the DNA ligase IV/XRCC4 complex to seal the break. This occurs in concert with the Mre11/Rad50/Nbs1 complex, at least for some of these breaks. ATM also senses breaks in DNA through the HR pathway and phosphorylates and activates a number of downstream effector proteins including Nbs1 and BRCA1. Evidence for indirect involvement of ATM through Rad51 and Mre11 also exists.

Rad17 recruits the Rad1/Rad9/Hus1 complex, which is required for ATR phosphorylation of Rad17 and triggering of the checkpoint signaling cascade [40]. NHEJ is responsible for initiating the process of DSB repair primarily in G_1 phase cells. Characterization of radiation-sensitive mammalian mutants and the generation of gene-disrupted mutant mice have identified DNA-PKcs, a heterodimer of Ku70 and Ku80, ligase IV, and XRCC4 as central players in this process [41]. Mutants in any of these genes are hypersensitive to ionizing radiation, defective in DSB repair and V(D)J recombination, and have abnormalities in telomere maintenance [42–44]. The Ku heterodimer binds to the ends of a DSB and recruits and activates DNA-PKcs leading to the phosphorylation of a number of substrates implicated in DNA repair. There is also evidence that Ku recruits the XRCC4-ligase IV complex to DNA ends to complete the process of end joining [45].

Another complex, Mre11/Rad50/Xrs2, was also shown to participate in NHEJ of breaks in *Saccharomyces cerevisiae* [46]. Homologs of Mre11 and Rad50 have been identified in mammalian cells and Nbs1 (nibrin), the gene defective in Nijmegen breakage syndrome (NBS), appears to be the functional counterpart of Xrs2 [47]. In response to radiation, the Mre11/Rad50/Nbs1 complex is localized rapidly to sites of DNA DSBs and associates in discrete foci [48,49]. The Mre11 complex is also involved in homologous recombination, meiotic recombination, and telomere maintenance [50]. Cross-talk between the Mre11 complex and ATM, in recognizing DSB in DNA, is supported by overlap in phenotype between the three syndromes that arise when these genes are mutated: A-T (ATM), NBS (Nbs1), and A-T like syndrome (Mre11) [51]. Further support for this is derived from the observation that ATM phosphorylates Nbs1 to mediate cell cycle control and minimize sensitivity to radiation [52]. Thus it is evident that several mechanisms have evolved to recognize and repair DSBs in DNA. The relative importance of NHEJ and HR in this process is governed by cell cycle position but it is not clear how the various recognition systems coordinate their activities in the recognition and repair processes.

The Mre11 complex together with the Rad52 epistasis group of genes also participates in HR in organisms ranging from *S. cerevisiae* to mammals [53,54]. In response to DNA damage, Rad52 binds to free DNA ends, which may facilitate processing by an exonuclease activity of the Mre11 complex, which localizes rapidly to the break [55]. The resulting 3' overhangs represent a recognition structure for RPA binding and Rad51 multimerization that, in the presence of the Rad51 paralogues, BRCA1 and BRCA2, invades the homologous duplex DNA to achieve exchange between damaged and intact DNAs. DNA polymerase fills in the resulting gap followed by ligation and strand resolution. Colocalization of Rad51 and the BRCA proteins to nuclear foci at the sites of DNA damage suggests that those structures participate in the HR process [56]. This association, induced by ionizing radiation, is accompanied by increased phosphorylation of BRCA1 and, as discussed later, at least some of this is carried out by ATM kinase [52,57]. ATM may also participate at the level of Rad51 because radiation exposure causes tyrosine phosphorylation of Rad51, which is both ATM and c-Abl dependent and this facilitates complex formation between Rad51 and Rad52 [58]. In chicken DT40 cells, characterized by high homologous recombination frequencies, disruption of the ATM gene altered the kinetics of Rad51 and Rad54 focus formation [59]. $ATM^{-/-}$ $Ku^{-/-}$ (NHEJ-deficient) DT40 cells were more sensitive to radiation and displayed high levels of radiation-induced chromosome aberrations whereas the $ATM^{-/-}$ $Rad54^{-/-}$ (HR-deficient) cells showed only slightly increased aberrations suggesting that ATM is on the HR pathway.

ATM Signaling: Recognition of Breaks in DNA

ATM is primarily activated as a preexisting protein by ionizing radiation and radiomimetic agents [60,61]. The exact mechanism of activation is as yet unclear but it is clear that this molecule undergoes autophosphorylation, which is expected to be responsible for or contribute to activation [52]. Agents that break DNA activate ATM so that the initiating signal may be a relaxation of the superhelical density of chromatin, which will be rapidly transmitted to ATM in complexes associated with chromatin. The initiating event may be a conformational change or dissociation from other members of a protein complex such as BRCA1-associated genome surveillance complex (BASC) [62], which would allow access to ATP and protein substrates. In this context recent evidence points to a greater retention of a portion of ATM in nuclear aggregates, postirradiation [63].

This retained ATM colocalizes with the phosphorylated form of histone H2AX (γ-H2AX) and in foci with Nbs1, suggesting that it is being targeted to sites of DSBs. Phosphorylation of H2AX occurs with rapid kinetics after irradiation at sites of DSBs and there is evidence that this is dependent on both ATM and ATR [64,65]. A host of proteins are either phosphorylated directly by ATM or their phosphorylation is ATM-dependent during radiation signal transduction (Table I). To identify potential substrates Kim *et al.* [66] employed a rapid peptide screening assay and showed that the sequence S/TQ is a minimal essential requirement and that the presence of hydrophobic or negatively charged amino acids N terminal of S/T were positive determinants for activity. Candidate substrates identified included ATM itself, Nbs1, BRCA1, p53, Mre11, Rad17, and Werner's syndrome protein (WRN).

Oriented peptide library analyses were also used to define preferred amino acid motifs for ATM [67]. As will be discussed later, it was not surprising that key substrates predicted for ATM would be involved in DNA damage response and repair as well as cell cycle checkpoint activation. Because ATM phosphorylates proteins involved in both DNA damage recognition and cell cycle control, an important question is whether these substrates are simultaneously phosphorylated or are distinguished by their distribution in different subcompartments in the nucleus. Andegeko *et al.* [63] have

Table I Substrates for ATM Kinase and ATM-dependent Modification of Proteins

Protein	Site (amino acid)	Direct	Indirect	Function
p53	Ser15	+		G1/S checkpoint
	Ser20		+	G1/S and G2/M checkpoints
	Ser376 (dephosphorylation)		+	G1/S checkpoint
Mdm2	Ser395	+		G1/S checkpoint
Chk2	Thr68	+		G1/S, S and G2/M checkpoints
53BP1	Several (unidentified)	+		cell cycle (developmentally regulated)
BRCA1	ser 1387	+		G2/M checkpoint/radioresistance
	ser 1423	+		
	ser 1457	+		
	ser 1524	+		
CtIP	ser 664	+		DNA damage response/Induction of GADD45
Nbs1	ser 278	+		S phase checkpoint/
(nibrin)	ser 343	+		Mre11/Rad50/Nbsi foci/radioresistance
Rad51	ser 645		+	Assembly of rad51 complex
c-Jun	ser 63		+	Oxidative stress signaling
	ser 73		+	
Pin2/TRF1	ser 219	+		Telomere maintenance/G2/M checkpoint/radioresistance
BLM	Thr 99	+		chromosome integrity
	Thr 122	+		
I KappaB	?		+	gene expression via NF-kappa B
c-Abl	ser 465	+		cellular response to radiation
Histone H1	desphosphorylation			chromatin remodelling
SMC1	ser 957	+		sister chromatid cohesion
	ser 966	+		

addressed this issue to some extent by revealing that a slow migrating (phosphorylated) form of Nbs1 was found with the retained form of ATM, but in contrast neither the basal nor phosphorylated forms of the cell cycle checkpoint kinase, Chk2, were detected in the retained fraction. However, it is evident that only a small proportion of activated ATM is confined to the retained nuclear fraction [63].

While double-strand breaks arise in DNA in response to radiation and other agents, breaks occur normally in some cells in specialized processes such as V(D)J recombination in lymphoid cells and during meiosis in germ cells [68,69]. Evidence for an involvement of ATM in recognizing these breaks arises from hypogonadism in A-T patients [70] and severe meiotic disruption in prophase 1 in $Atm^{-/-}$ mice [71,72]. A high incidence of chromosomal rearrangements involving both the T-cell receptor and Ig heavy chain genes is characteristic of A-T [73]. This does not appear to be due to defective V(D)J recombination in A-T cells [74,75]. However, both A-T patients and *Atm* gene-disrupted mice develop lymphoid tumours characterized by translocations involving antigen receptor genes [76,77]. Blocking of V(D)J recombination by crossing $Atm^{-/-}$ mice with recombinant activating gene (RAG) deficient mice delays the onset of tumors in these mice [78,79]. Atm is localized to breaks associated with V(D)J, being recruited at approximately the same time as RAG [80].This report also provides evidence that Ser18 phosphorylated p53, a product of ATM kinase activity, is also localized to the breaks with Atm.

These data together with results demonstrating direct interaction between ATM and p53 [81,82], suggest that ATM is being activated by V(D)J recombination breaks and that it is phosphorylating a known substrate in the vicinity of the break. Although ATM may not be essential for V(D)J recombination, it appears to play a surveillance role for intermediates, suppressing aberrant rearrangements [80]. These data also are supportive of phosphorylation of substrates in the immediate vicinity of the DNA damage. Double-strand breaks in DNA also up-regulate the nonreceptor tyrosine kinase, c-Abl, in an ATM-dependent manner [83,84]. ATM interacts with c-Abl through its SH3 group, and in A-T cells radiation induction of c-Abl kinase is defective. c-Abl also interacts with and phosphorylates DNA-PKcs, causing it to dissociate from the Ku/DNA complex [85], and inhibition of c-Abl kinase prevented down-regulation of DNA-PK [86].

Thus, there is evidence that ATM regulates the activity of DNA-PK postirradiation providing support for a convergence of these pathways of DNA damage repair.

Checkpoint Activation

G_1/S-Phase Checkpoint

Perhaps the best described signaling pathway initiated by ATM postirradiation is that through p53 to arrest the passage of cells from G_1 to S phase [18,19,87]. In this pathway ATM phosphorylates p53 on Ser15 and is required for Ser20 phosphorylation and Ser376 dephosphorylation [88]. p53 Becomes transcriptionally activated to induce the cyclin kinase inhibitor p21/WAF1, which associates with cyclin E-Cdk2 to block its activity, to prevent phosphorylation of downstream substrates, and to delay the passage of cells from G_1 to S phase [89–91; Fig. 2]. ATM phosphorylates Chk2 kinase on Thr68, which depends on the integrity of the forkhead associated (FHA) domain in Chk2 [92]. This phosphorylation is required for the subsequent autophosphorylation and activation of Chk2 [93], which enables it to exert its control on the G_1/S, S, and G_2/M checkpoints. This activation of Chk2 by ATM also requires Nbs1 since activation is defective in NBS cells [94]. Whereas wild-type Nbs1 could complement this defect in NBS cells, a construct mutated in the ATM phosphorylation site on Nbs1 (ser 343) failed to do so as well as a mutant form that abrogated the formation of the Mre11/Rad50/Nbs1 complex. Phosphorylation of p53 on Ser20 by Chk2, in response to radiation, stabilizes the protein and increases its availability to induce downstream effector genes such as p21/WAF1 [95,96]. Dephosphorylation of p53 at Ser376 occurs in response to radiation treatment and this is also ATM dependent [97]. The significance of this change to p53 stabilization/activation is not known.

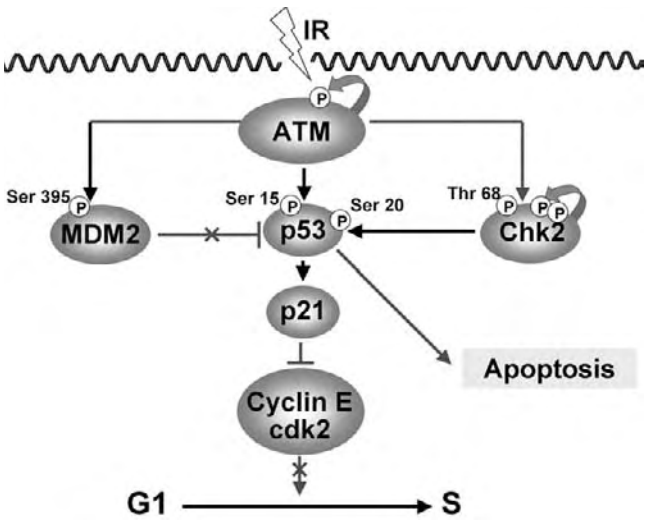


Figure 2 ATM activates the G_1/S checkpoint in response to ionizing radiation damage to DNA. ATM is rapidly activated by an unknown mechanism, in response to DNA breaks, to phosphorylate itself and three downstream effector proteins (p53, MDM2, and Chk2) to achieve arrest of cells at the G_1/S checkpoint. It directly phosphorylates p53 on ser15, which may alter its transcriptional capacity. Stabilization of p53 is achieved by phosphorylating Chk2 on thr 68, which leads to its subsequent activation to, in turn, phosphorylate p53 on ser20 leading to its stabilization. Negative regulation of MDM2 by ATM through phosphorylation on ser395 reduces its ability to bind to and mediate the ubiquitination and degradation of p53. Thus stabilization of p53 is ensured through two separate steps. When stabilized p53 is capable of inducing p21 and other downstream genes to inhibit cyclin E-Cdk2 kinase activity and bring about G_1 arrest.

The involvement of ATM signaling at multiple levels in a single pathway is further illustrated by the capacity of activated ATM to phosphorylate Mdm2, a negative regulator of p53, on Ser395 [98]. Expression of Mdm2 is controlled by p53, but Mdm2 in turn has a feedback effect, binding to p53, exporting it from the nucleus and promoting its degradation in the proteasome pathway [99]. ATM-dependent phosphorylation of Mdm2 is observed prior to p53 accumulation. Decreased reactivity of Mdm2 from irradiated cells to an anti-Mdm2 antibody, directed against an epitope containing Ser395, suggests that this is a site of phosphorylation *in vivo*, and phosphorylation at this site may reduce the capacity of Mdm2 to translocate p53 to the cytoplasm and ensure its stabilization [100]. Thus, it is evident that ATM acts directly on three different substrates and also has indirect effects on some of these, providing complex control of the G_1/S checkpoint.

S-Phase Checkpoint

Exposure of mammalian cells to radiation leads to a biphasic pattern of inhibition of DNA synthesis reflecting the greatest sensitivity in replicon initiation [101]. A much reduced inhibition of DNA synthesis in A-T cells in response to radiation, which was termed *radioresistant DNA synthesis* (RDS), represented the first report of a cell cycle anomaly [13,14]. This phenomenon is still not fully understood but may be explained by defects in more than one signaling pathway controlled by ATM. Beamish *et al.* [16] have shown that exposure of lymphoblastoid cells to radiation in S phase causes a rapid inhibition of cyclin A-Cdk2 accompanied by markedly increased binding of p21/WAF1 (Fig. 3). In contrast, radiation did not inhibit cyclin kinase activity in A-T cells in S phase nor was there any significant change in cdk-associated p21/WAF1 compared to unirradiated cells [16]. These observations are compatible with a reduced effect of radiation on DNA replication in A-T cells. A functional link has also been established between ATM, the checkpoint signaling kinase, Chk2, and Cdc25A phosphatase and its downstream target for activation, Cdk2, in S phase [15]. Radiation-induced degradation of Cdc25A requires both ATM and Chk2-mediated phosphorylation of Cdc25A on Ser123 and this prevents dephosphorylation of Cdk2, leading to a transient block in DNA replication (Fig. 3). Exposure of A-T cells to radiation failed to cause an increase in Cdk2 Tyr 15 dephosphorylation or inhibition of cyclin E-Cdk2 kinase activity, consistent with the radioresistant DNA synthesis phenotype. Furthermore, Chk2 alleles defective in catalytic activity or ability to interact with Cdc25A had a dominant interfering effect and abrogated the S-phase checkpoint [15].

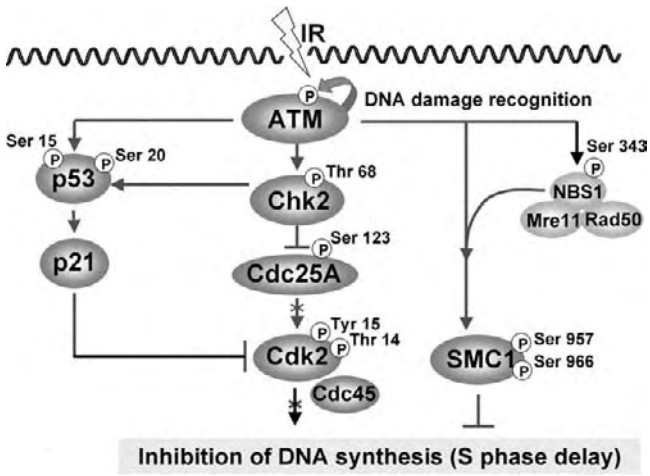


Figure 3 ATM activates the S-phase checkpoint. Radioresistant DNA synthesis was first described in cells from A-T patients lacking ATM. This phenomenon is also observed in NBS where the ATM substrate Nbs1 is defective and in the A-T like syndrome (ATLD) characterized by hypomorphic mutants in the Mre11 gene, a component of the Mre11/Rad50/Nbs1 complex. Radioresistant DNA synthesis is also observed when Chk2 is disrupted on its downstream pathway through Cdc25A/Cdk2. These observations point to an important role for ATM in inhibiting DNA synthesis through several separate but parallel pathways. In the first case ATM activates Chk2 as described for the G_1/S checkpoint, which in this case destabilizes Cdc25A by phosphorylation on Ser123, preventing its ability to remove the inhibitory Tyr 15/Thre 14 phosphorylations for activation of Cdk2, leading to inhibition of DNA synthesis. In a parallel but less well-described pathway, ATM activates the Mre11 complex and SMC1, a downstream target to inhibit DNA synthesis. Finally, the p53 activation pathway might also contribute through inhibition of Cdk2 kinase.

Similarities in phenotype between A-T and NBS including RDS provided another approach to understanding the basis of this phenomenon. After ionizing radiation exposure, Nbs1 is phosphorylated on Ser343 both *in vitro* and *in vivo* in an ATM-dependent manner [102–104].

In normal cells transiently transfected with Nbs1, radiation caused inhibition of DNA synthesis but when the Ser343 site was mutated prior to transfection, a significant reduction was seen in the extent of inhibition of DNA synthesis [104], indicating abrogation of the S-phase checkpoint by dominant interference. Furthermore, RDS was rescued by retroviral constructs expressing wild-type Nbs1 but not Ser343 mutants. Thus ATM phosphorylation of Nbs1 is an important part of the mechanism to ensure inhibition of DNA replication in response to radiation damage. Recent data suggest that the S-phase checkpoint is regulated by parallel pathways through Nbs1-Mre11 on the one hand and Chk2 on the other [105]. Concomitant interference with both of these pathways gave rise to RDS after exposure of cells to ionizing radiation. Thus it appears likely that ATM, by phosphorylating both Nbs1 and Chk2, triggers two pathways that inhibit distinct steps in DNA replication.

Evidence for parallel pathways is further supported by the demonstration that the structural maintenance of chromosomes protein, SMC1, is a downstream effector in the ATM/Nbs1 branch of the S-phase checkpoint [106,107]. It has been suggested that Nbs1 ceases to be a sensor of DSBs after phosphorylation by ATM and becomes an adaptor to facilitate the phosphorylation of SMC1 by ATM. Once phosphorylated SMC1 may participate in the S-phase checkpoint in a postreplicative role. A delay in radiation-induced phosphorylation of replication protein A (RPA), a component of the single-strand DNA binding protein complex, has also been implicated [108]. Furthermore UV-induced hyperphosphorylation of RPA (p34 subunit) is ATM dependent, and ATM kinase phosphorylates sites on p34 that are phosphorylated *in vivo* in response to UV irradiation [109]. The transitory delay in DNA synthesis postirradiation also appears to be mediated through a calmodulin-dependent regulatory cascade [110] and this pathway is defective in A-T.

G_2/M Checkpoint

Arrest of cells in G_2 phase in response to radiation damage leads to suppression of the mitotic index to protect cell viability [111]. Consequently, abrogation of G_2 arrest sensitizes cells to radiation [112]. This checkpoint is defective in A-T cells as evidenced by a lesser delay of cells, irradiated in G_2 phase, in progressing into mitosis [113]. Defective G_2-phase delay in A-T cells was confirmed by labeling cells in S phase with BrdU, blocking their passage into mitosis with nocodazole prior to irradiation (in G_2 phase), and scoring for ability to enter the next G_1 phase [11]. This was also achieved by distinguishing G_2 phase cells from mitotic cells using histone H3 phosphospecific antibody [114]. As observed with cells irradiated in S phase, when A-T cells were exposed to radiation in G_2 phase, there was no inhibition of Cdc2-cyclin B kinase activity and, unlike that obtained with irradiated control cells, no increase in p21/WAF1 associated with Cdc2 was observed [16]. Again this checkpoint defect is compatible with a lack of ATM signaling in A-T cells to the cyclin-kinase complex. As with S and G_1 phase checkpoints, it appears that ATM influences G_2 arrest by more than one pathway. It is well established that BRCA1 is involved in the cellular response to radiation and this involves phosphorylation on several sites mediated by both ATM and ATR [52,57,115]. ATM interacts with BRCA1 and this molecule is a substrate for ATM kinase both *in vitro* and *in vivo* [52]. Deletion of exon 11 of BRCA1 leads to a defective G_2 checkpoint and extensive chromosomal abnormalities [116].

A G_2/M checkpoint abnormality, similar to that seen in A-T cells, has been reported for the BRCA1-null cell line HCC1937 and a normal checkpoint was restored to these cells with BRCA1 transfection [17]. Transfection of HCC1937 cells with a mutant form of a major ATM phosphorylation site in BRCA1 (S1423A) failed to complement the defective G_2/M checkpoint. Thus, it appears likely that radiation-induced phosphorylation of BRCA1 on ser1423 by ATM is important in the regulation of the G_2/M checkpoint (Fig. 4). Because cells from NBS patients exhibited a normal radiation-induced G_2/M checkpoint, it appears that ATM phosphorylation of Nbs1 does not play a significant role for this checkpoint. BRCA1-mediated induction of GADD45 also leads to

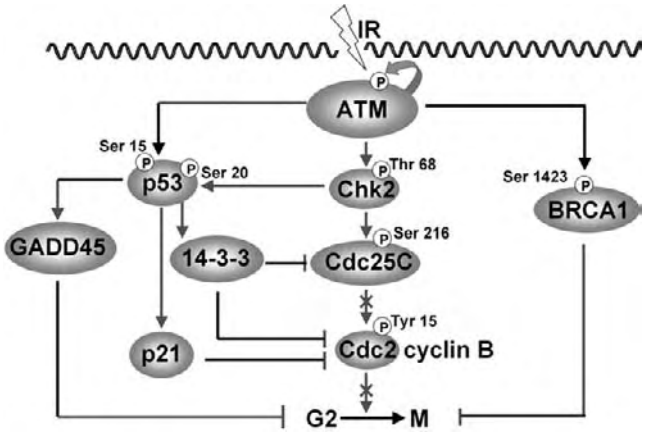


Figure 4 ATM involvement in G_2/M checkpoint control. The activation of the G_2/M checkpoint is no less complex than the S-phase checkpoint. Again ATM is a central player, via chk2 phosphorylation of Cdc25C on ser216 in S phase, and in response to DNA damage this site is maintained in a phosphorylated state. Inactivation appears to be mediated by binding to 14-3-3 protein, a p53 downstream effector. Again in this case activation of the p53 pathway is prominent to induce 14-3-3 protein and p21/WAF1, which can bind to and inhibit Cdc2-cyclin B kinase blocking cells at the G_2/M checkpoint. Induction of GADD45 in this pathway may also interfere with Cdc2-cyclin B kinase. To add to the complexity mutations in BRCA1, including an ATM phosphorylation site (ser 1423), leads to a defective G_2/M checkpoint indicating that ATM also controls the G_2/M checkpoint through BRCA1.

G_2/M phase delay but only in response to microtubule poisons, not DNA damaging agents, revealing that it is ATM independent [117].

Under normal proliferative conditions, the dual specificity Cdc25 phosphatase removes the phosphate group from tyrosine 15 on Cdc2 to facilitate mitotic entry [118]. As part of the G_2/M checkpoint activation, Cdc25C is phosphorylated on Ser216 and inactivated by binding to 14-3-3 protein and subsequently sequestered to the cytoplasm [119,120]. Both Chk1 and Chk2 phosphorylate Cdc25C on ser216 *in vitro* [121–124]. Since Cdc25C is already phosphorylated on ser216 in S phase it appears that DNA damage prolongs this state of phosphorylation [125]. As in the case of the G_1/S checkpoint it appears likely that ATM activates Chk2 to phosphorylate Cdc25C as part of the G_2/M checkpoint activation.

Role of ATM in More General Signaling

The presence of ATM in the nucleus is consistent with its role in sensing damage in DNA and signaling to cell cycle checkpoints. However, ATM is also extranuclear in proliferating cells with evidence for localization to endosomes [126] and peroxisomes [81]. In postmitotic human and mouse Purkinje cells and other neurons, ATM is predominantly a cytoplasmic protein [127,128]. A likely explanation for ATM activation by double-strand breaks in DNA is the binding either directly or indirectly to the lesion, which is supported by data revealing binding of ATM to DNA ends [129,130] and the recruitment of this protein to DNA double-strand breaks after ionizing radiation damage [131]. However, it is difficult to envisage the commonality that might occur when the same molecule is activated in the cytoplasm or in a subcellular organelle outside the nucleus. Support for a more general signaling role for ATM comes from a number of observations [132]. One of the earliest observations supporting a non-DNA damage role for ATM was the failure to record a cytoplasm to nucleus signaling after internalization of PHA in A-T lymphocytes [133]. Kondo *et al.* [134] demonstrated that Ca^{2+} mobilization, in response to both PHA and CD3 cross-linking, was defective in lymphocytes from A-T patients, and Khanna *et al.* [28] have provided evidence for defective signal transduction and Ca^{2+} mobilization after B-cell receptor ligation in A-T lymphoblastoid cells. Rhodes *et al.* [29] have shown that the ability of A-T fibroblasts to depolarize in response to increasing concentration of extracellular K^+ is significantly reduced compared to controls, and that the outward rectifier K^+ currents are largely absent in these cells.

Electrophysiological anomalies were also reported by Chiesa *et al*. [135] who showed that there was a significant decrease in the duration of calcium and sodium firing in Purkinje cells from $Atm^{-/-}$ mice. Other evidence for defective signaling in A-T includes greater demand for growth factors in A-T fibroblasts [136,137], poor growth capacity of fibroblasts from $Atm^{-/-}$ mice and from A-T patients [87], only partial protection against cell death in peripheral blood mononuclear cells from A-T patients by serum and added cytokines [138], and defective signaling through the EGF-receptor in A-T cells [25,26]. A more direct role for non-DNA damage activation of ATM kinase has been described in response to insulin [30]. Insulin activated ATM kinase to the same extent, but it did so somewhat slower than ionizing radiation, which led to the phosphorylation of 4E-BP1 (PHAS-1) on Ser111, while bound to eIF-4E. This appears to be the priming event for other phosphorylations on 4E-BPI, which lead to dissociation of the complex and availability of eIF-4E for the initiation of translation of mRNA. Furthermore, there is evidence for a reduction in the extent of dissociation of the complex in A-T cells in response to insulin. Thus while agents other than DNA damage can activate ATM kinase, it remains unclear whether the nuclear or extranuclear form is being activated. In addition unless ATM is present in soluble form in the cytoplasm, the additional problem of bringing substrates such as 4E-BPI-eIF-4E together with ATM in subcellular organelles (endosomes and peroxisomes) exists.

Putting aside the example of insulin-induced signaling through ATM, it might well be argued that all or most of the signaling defects present in A-T cells are indirect as a consequence of the loss of ATM. A possible unifying explanation for the myriad of defects at the level of the membrane in A-T cells might be oxidative stress for which there is accumulating evidence. It has been suggested that perturbation of the cellular balance of reactive oxygen species leads to oxidative stress in A-T, which may be responsible for several features of the phenotype including neurodegeneration [139]. Evidence for a state of oxidative stress in A-T is based

on abnormal response of these cells to agents that cause stress [140]; reduced antioxidant capacity [141]; increased oxidative damage in the cerebellum of $Atm^{-/-}$ mice [8,81]; and the capacity of antioxidants to reduce constitutively high levels of p53, p21/WAF1, and other proteins in A-T cells [9]. Increased oxidative stress in the absence of ATM could be due to a failure to sense and respond to reactive oxygen species (ROS) levels, deregulation of enzymes involved in preventing oxidative stress, or the presence of unrepaired breaks in DNA that activate components of the DNA damage response to deplete NAD [139]. Thus at least some of the series of non-DNA damage signaling defects in A-T cells might be due to indirect consequences of loss of ATM.

Perspective

The cellular response to radiation exposure is complex, involving nuclear-, cytoplasmic-, and membrane-mediated events. As outlined earlier, ATM plays a central role in the recognition and response to the major radiation-induced lesion in DNA, the double-strand break. In the nucleus a series of rapid protein phosphorylations occurs that is controlled by ATM, but it remains unclear how ATM is activated as an existing protein to achieve this. It is likely that alterations in the superhelicity of chromatin loops may rapidly signal the presence of breaks to ATM, dislodging it from an inhibition or inhibitory complex associated with chromatin to become autophosphorylated and, in turn, phosphorylate key substrates such as p53, BRCA1, and Chk2. Evidence exists for ATM autophosphorylation, postirradiation, but it has not been established how many such sites there are in ATM and how this is related to its capacity to phosphorylate specific substrates. Compartmentalization of different ATM substrates in different complexes such as BASC may determine accessibility and rapidity of phosphorylation. This may help to explain how it is that ATM can activate several pathways for DNA damage recognition and cell cycle control almost simultaneously.

To add to this complexity it is evident that ATM has a fine-tuning role in these pathways by phosphorylating more than one pathway intermediate directly or mediating phosphorylation through other protein kinases. A good example of this is the G_1/S checkpoint where the downstream targets include p53, mdm2, and Chk2. It is not difficult to envisage a central role for ATM in radiation signaling in the nucleus where the DNA damage is located and where the enzyme is present in abundance in proliferating cells. However, being able to establish a similar mechanism of activation in the absence of chromatin for extranuclear (nonmitochondrial) ATM is somewhat more difficult. It has been reported that the majority of ATM is extranuclear in postmitotic cells, and even in proliferating cells up to 30% of ATM is outside the nucleus. Insulin has been shown to activate ATM kinase, but it is not clear whether this involves nuclear or extranuclear ATM and what the mechanism is. It is possible that constitutive activation of ATM in the nucleus is prevented by association with other proteins in a complex and it becomes activated as referred to earlier by a reorganization of chromatin. Similarly, ATM may be kept inactive or residually active by interaction with a different protein outside the nucleus that dissociates in response to a different stimulus. We should point out that no one has demonstrated active ATM kinase in extranuclear ATM to date.

What happens when ATM protein is mutated or absent by the description of the A-T syndrome was established as long ago as 1926. In the absence of ATM, patients undergo a progressive cerebellar degeneration, they are prone to infection, and up to 30% die from mainly lymphoid tumors. Failure of appropriate DNA damage recognition leads to genomic instability, which is related to the propensity to develop tumors. What is of particular interest is the penetration of subtle but significant aspects of the phenotype in carriers of the defective gene. For some time it has been demonstrated that A-T heterozygotes are intermediate in their sensitivity to ionizing radiation; they have an increased risk for the development of cancer, especially breast cancer, and their relative risk for dying from other conditions is increased. The challenge ahead requires a greater understanding of how ATM is activated, what pathways it controls, and how it minimizes genomic instability and protects against cancer.

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CHAPTER 306

Signaling to the p53 Tumor Suppressor through Pathways Activated by Genotoxic and Nongenotoxic Stresses

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Introduction

The product of the human p53 tumor suppressor gene (*TP53*) is a 393-amino-acid polypeptide that functions as a homotetrameric transcription factor. p53 regulates the expression of genes that control cell cycle progression, the induction of apoptosis, DNA repair, and other functions involved in cellular responses to stress. Loss of p53 function, either directly through mutation or indirectly through several mechanisms, plays a central role in the development of cancer [1,2]. While the p53 protein normally is short lived and is present at low levels in unstressed mammalian cells, in response to both genotoxic and nongenotoxic stresses it accumulates in the nucleus where it binds to specific DNA sequences [2,3]. Genomic approaches have shown that p53 induces or inhibits the expression of more than 150 genes including *p21*, *GADD45*, *MDM2*, *IGFBP3,* and *BAX* [4], many of which mediate cell cycle arrest or apoptosis. p53 modulates DNA repair processes [5,6], and the arrest of cell cycle progression may provide time for the repair of DNA damage. In some circumstances, cell cycle arrest is permanent and indistinguishable from senescence [7]. Alternatively, stress signaling may initiate p53-dependent apoptosis [2]. The biochemical links between p53, G_1 arrest, senescence, and apoptosis are cell- and stress-type dependent. These observations suggest that specific posttranslational modifications to the p53 protein, at least in part, determine cellular fate. In turn, these modifications reflect the specific pathways that become activated in response to any particular stress condition. In this chapter, we highlight recent studies on the pathways that modulate p53 stability and activity in response to genotoxic and nongenotoxic stresses through covalent posttranslational modifications to p53 including the phosphorylation of serine and threonines and the acetylation of lysines.

p53 Protein Structure

The p53 polypeptide can be divided into three functionally distinct regions: an amino-terminal region (1–101, numbering for human p53) that interacts with regulatory proteins and the transcriptional machinery; a central, sequence-specific DNA-binding domain (102–292); and a carboxyl-terminal tetramerization and regulatory domain (293–393) (Fig. 1). In the N-terminal domain, residues 1–42 are required for transactivation activity and interact with the transcription factors TFIID, TFIIH, several TAFs, the histone acetyltransferases CBP/p300, and possibly PCAF, as well as the MDM2 ubiquitin ligase. Residues 17–28 form an amphipathic helix that

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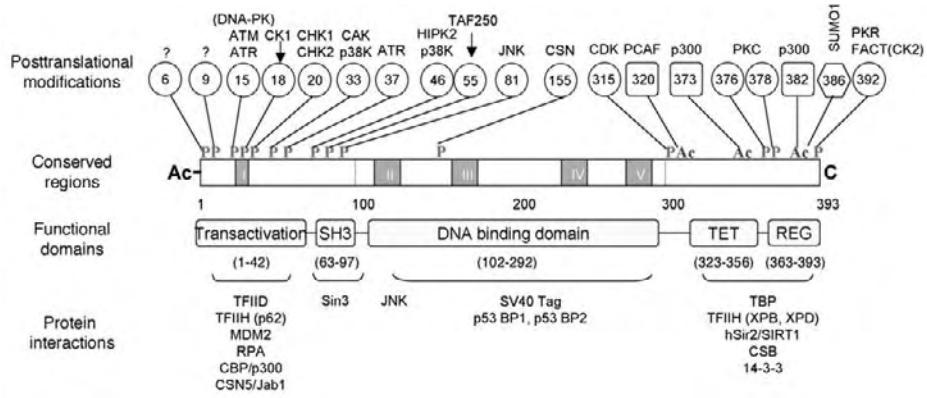


Figure 1 Protein domains, posttranslational modification sites, and proteins that interact with human p53. The 393-amino-acid human p53 polypeptide is represented schematically (box) with the five most highly conserved regions marked (I–V); postulated function regions and domains also are indicated. Residues ~1–42 comprise the transactivation domain; residues ~63–97 constitute a Src homology 3-like (SH3) domain that overlaps a poorly conserved proline- and alanine-rich segment (33–80); residues ~102–292 contain the central, sequence-specific, DNA-binding core region; residues ~300–323 contain the primary nuclear localization signal (NLS); residues 324–356 comprise the tetramerization domain (TET), which contains a nuclear export signal [86]; and residues 363–393 (REG) negatively regulate DNA binding by the central core to consensus recognition sites in oligonucleotides and interact in a sequence-independent manner with single- and double-stranded nucleic acids. Interaction regions for selected proteins are indicated below the polypeptide, and posttranslational modification sites (P, phosphorylation; Ac, acetylation) are indicated above the peptide together with enzymes that can accomplish the modifications *in vitro*. Lys386 may be modified by conjugation with SUMO1, an ubiquitin-like peptide. References are found in the text and recent reviews.

interacts directly with a hydrophobic cleft in the N-terminal domain of MDM2 [8], while residues 11–26 are reported to function as a secondary nuclear export signal [9]. Residues 63–97 comprise a proline-rich SH3 domain required for interaction with the Sin3 corepressor [10] and other proteins required for the induction of apoptosis. While residues 1–31 and 80–101 are highly conserved, especially among mammals, residues 32–79 are poorly conserved in sequence even among mammals.

The majority of tumor-derived p53 mutations affect the central domain and block or alter sequence-specific DNA binding either by destabilizing the domain or through changes to residues that directly contact DNA. The three-dimensional structure of the DNA-binding domain (DBD) bound to DNA was determined by X-ray crystallography [11]. The consensus DNA-binding site is composed of two 10-bp segments (RRRCWWGYYY) separated by 0–13 bp. The carboxy-terminal region contains the nuclear localization signal (312–324), a tetramerization domain (323–356), and a basic segment that binds certain DNA structures, including short single strands, four-way junctions, and insertions/deletions in a sequence-independent manner [12]. Only tetrameric p53 appears to be active as a transcription factor.

Posttranslational Modifications to p53

p53 activity is thought to be regulated largely through numerous posttranslational modifications that occur mainly in the N- and C-terminal regions (Fig. 1). Using monoclonal or affinity-purified polyclonal antibodies produced by several laboratories and companies that recognize specific, modified sites in human or mouse p53, increased phosphorylation at most of the 15 known phosphorylation sites has been demonstrated in response to the treatment of cells with DNA damage-inducing agents in Western immunoblot experiments (reviewed in [13]). Seven serines and two threonines in the N-terminal domain of human p53, specifically Ser6, 9, 15, 20, 33, 37, and 46 and Thr18 and 81, are phosphorylated in response to exposing cells to ionizing radiation or UV light. Recently, Thr55 was found to be phosphorylated in unstressed cells [14] and dephosphorylated after DNA damage (X. Liu, personal communication, 2001). Thus, all N-terminal serines and threonines in the first 89 residues of human p53 may be phosphory-lated or dephosphorylated in response to one or more stress conditions. In the C-terminal regulatory domain, Ser315 and Ser392 are phosphorylated; Lys320, 373, and 382 are acetylated; and Lys386 is sumoylated in response to DNA damage. Ser376 and 378 were reported to be constitutively phosphorylated in unstressed cells, whereas Ser376 was dephosphorylated in response to ionizing radiation [15]. Thr155 and Thr150 or Ser149 in the central site-specific DBD recently were reported to be phosphorylated by the COP9 signalosome (CSN)-associated kinase [16]; so far, these are the only sites in the central domain that have been reported to be posttranslationally modified.

Regulation of p53 Activity

The biochemical mechanisms that regulate p53 activity are complex and incompletely understood, but it is widely

believed that activation of p53 as a transcription factor involves two stages, as discussed next.

p53 Stabilization

First, in response to stress-activated signaling pathways, p53 is stabilized and accumulates in the nucleus. In unstressed cells, p53 protein is maintained at low steady-state levels and has a short half-life due to rapid, ubiquitin-dependent degradation through the 26S proteosome. Ubiquitin is a 76-amino-acid polypeptide that is transferred to lysine residues in proteins by ubiquitin ligases; multiple ubiquitination targets the protein to the 26S proteosome complex where it is degraded. At least three cellular systems that target p53 for ubiquitination have been described. In the G_0 phase of the cell cycle, the Jun N-terminal kinase (JNK) binds p53 and targets it for ubiquitination [17]. Activation of JNK in response to DNA damage allows JNK to phosphorylate p53 on Thr81, enhancing its transactivation potential and releasing JNK from p53. The COP9 signalosome (SSN) recently was shown to bind p53, phosphorylate it on Thr155 and nearby residues, and promote p53 degradation by targeting it for ubiquitination [16]. Inhibition of the CSN-associated kinase activity or mutation of Thr155 to valine resulted in increased p53 stability and its accumulation. The CSN kinase is thought to be constitutively active; thus, CSN is believed to contribute to the normal turnover of p53 in cells, but it is not known if modulation of the CSN kinase activity in response to stress contributes to p53 stability.

In dividing cells, the primary system that ubiquitinates p53 is the MDM2 E3 ubiquitin ligase. This activity of MDM2 is vital as shown by the rescue of Mdm2 knock-out mice from embryonic lethality by deletion of p53. Interestingly, loss of MdmX expression also results in embryonic lethality that is rescued by deletion of p53 [18]. MdmX is a structurally related protein that interacts with Mdm2 and blocks p53 degradation. A cleft in the N-terminal domain of MDM2 (amino acids 25–109) binds to an amphipathic helix (amino acids 17–29) in the transactivation domain at the N terminus of p53 [8], and binding is required for subsequent ubiquitination at multiple C-terminal p53 lysines. p53 is phosphorylated at several N-terminal sites that reside in or near the MDM2 binding site by kinases activated through several stress response pathways; this led to the hypothesis that phosphorylation might stabilize p53 by preventing its interaction with MDM2 [19]. Initially it was proposed that phosphorylation of Ser15 and 37 in response to DNA damage induced a conformational change in p53 that prevented its interaction with MDM2, thus inhibiting p53 ubiquitination [19]. Subsequently, phosphorylation of Thr18 and Ser20 were reported to negatively regulate the interaction of p53 with MDM2 [20–23]. Both Thr18 and Ser20 lie within the p53 N-terminal amphipathic helix that directly interacts with the N terminus of MDM2. Thr18 makes several hydrogen bonds with neighboring residues that stabilize the helix and that would be disrupted by phosphorylation. Consistent with the structural data, phosphorylation of Thr18, but not phosphorylation of Ser15, Ser20, or Ser37, was found to interfere directly with the interaction of an N-terminal p53 peptide with the N-terminal domain of MDM2 [22,24]. Nevertheless, changing Ser15 to alanine was shown to significantly decrease the ability of p53 to activate transcription and induce apoptosis in both human [19,25] and mouse [26] systems, and changing Ser20 to alanine in human p53 abrogated stabilization in response to DNA damage [20]. Taken together, these results suggest that phosphorylation of p53 Ser15 and Ser20 may indirectly affect complex formation with Mdm2.

One mechanism that would be consistent with the present results is increased competition for binding to the N terminus of phosphorylated p53 by other factors. For example, CBP/p300 interacts with the N terminus of p53, and binding is dramatically enhanced by phosphorylation of Ser15 [27]. Consistent with this finding, changing Ser6, Ser9, Ser15, or Thr18 (but not more distal serines) to alanine reduced acetylation of Lys382 [28]. Surprisingly, however, changing murine Ser23, the equivalent of human Ser20, to alanine had no measurable effect on transactivation, apoptosis, and Mdm2 binding in mouse ES cells, fibroblasts, or thymocytes [29]. Thus, in spite of the high degree of sequence conservation between human and mouse p53 in this region, the two species appear to regulate p53 stabilization and MDM2 binding somewhat differently. Several other proteins have been shown to interact with the N terminus of p53 (see Fig. 1), but the effect of p53 phosphorylation on their binding has not been examined.

Recent results suggest that other mechanisms also play an important role in regulating MDM2 activity. First, $p14^{ARF}$ can inhibit the activity of MDM2 by sequestering it in the nucleolus or through other mechanisms [30]. Second, MDM2 itself is a target for DNA damage-induced posttranslational modifications that may positively or negatively regulate its activity [31]. Third, several studies have shown that the C-terminal regulatory region of p53 is important for its stabilization. The circumstances under which each mechanism operates and their relative importance in regulating p53 stability have not been fully characterized, but the existence of several mechanisms may explain reported differences in results relating to different phosphorylation sites.

A fourth system that targets p53 for ubiquitin-mediated degradation is the E6 protein of human papillomaviruses. Papillomaviruses are small, DNA tumors viruses, and certain serotypes are strongly associated with cervical cancer. Like other DNA tumor viruses, papillomaviruses inactivate p53, both to allow cells to enter a state in which the DNA viral genome can replicate and to avoid virus-induced apoptosis. The papillomavirus E6 protein, in conjunction with a ~100-kDa cellular protein E6AP, functions as an ubiquitin ligase in a manner similar to MDM2 [32]. As a consequence, papillomavirus transformed human cells (e.g., HeLa) frequently have wild-type p53 genes but are functionally deficient for p53 activity.

p53 Activation

p53 accumulation alone is not sufficient to fully activate p53-dependent transcription [33]. Early studies by Hupp and

Lane [34] indicated that p53 is synthesized in a latent form that is incompetent for sequence-specific DNA binding; they proposed that subsequent to its synthesis p53 was activated to a DNA-binding competent state through posttranslational modifications that were postulated to induce a conformational change in the DBD of p53. Indeed, several modifications to the C terminus, including phosphorylation [34], acetylation [35,36], binding of antibodies, and truncation of the C-terminal 30 amino acids, were shown to result in increased p53 binding to oligonucleotides containing the p53 consensus recognition site. These results suggested that the C-terminal domain negatively regulated sequence-specific binding by the central domain. The C-terminal regulatory domain of p53 contains numerous basic residues and, subsequently, it was found to bind single-stranded DNA and RNA, as well as several DNA structures.

An alternative model to explain latency was proposed by Anderson *et al.* [37], who showed that the sequence-specific DNA binding of p53 was inhibited by long DNAs but not by short oligonucleotides (Fig. 2, top). These results suggested that strong non-sequence-specific binding by the C-terminal domain of tetrameric p53 prevented sequence-specific binding. Furthermore, relief of inhibition of sequence-specific DNA binding through removal of the C terminus only occurred in the presence of long DNA molecules. The interference model recently received indirect support from NMR-based structural studies on a dimeric derivative of p53 and its C-terminal truncated form [38]. No significant differences in the structure of the central DBD were observed between the two forms, suggesting that increased DNA binding did not result from an allosteric conformational change in p53 structure. However, the concept of a latent DNA binding form of p53 recently was challenged by the finding that unmodified p53 binds well to long DNAs containing consensus recognition sites [39]. Indeed, p53 was reported to be constitutively bound to chromatin at some recognitions sites *in vivo*, including sites in the *CDKN1A* (*p21, WAF1, CIP1*), and *MDM2* promoters [40]. Furthermore, genotoxic stress caused only a small increase in the amount of p53 bound to chromatin at these sites.

If DNA/chromatin binding is not the rate-limiting step for activation of p53, then what might this step be? Although direct attempts to demonstrate a role for p53 acetylation in

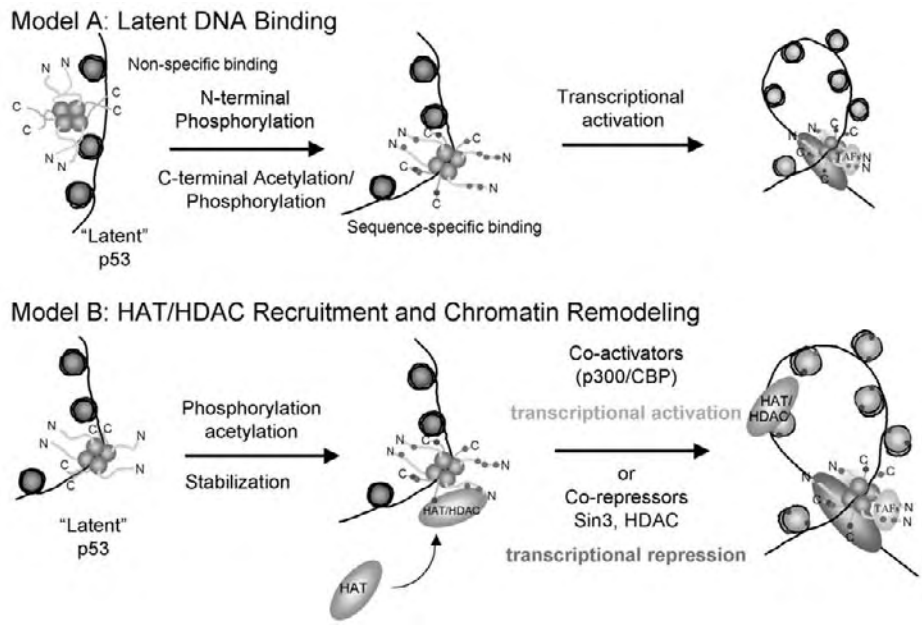


Figure 2 Models for the activation of p53 as a transcription factor. *Top:* Model A: Regulation of DNA binding by the C-terminal domain. Unmodified p53 exists as a latent tetramer incapable of sequence-specific DNA binding. Upon modification of C-terminal residues by phosphorylation or acetylation, latent p53 is converted into an active, sequence-specific, DNA-binding protein that then recruits elements of the transcription apparatus (e.g., TAFs). The conversion of latent p53 into active p53 might occur through an allosteric transition in the DBD [34] or as a consequence of the inhibition of nonspecific DNA binding to the C-terminal regulatory domain [37]. Recent NMR studies [38] argue against activation through an allosteric transition. *Bottom:* Model B: Recent studies suggest that p53 may be constitutively bound at some p53 consensus sites; thus, latency of DNA binding may be an artifact of the *in vitro* conditions employed [40]. Other studies have shown that both histone acetyltransferase (HAT) coactivators, such as CBP/p300, and histone deacetylase (HDAC) corepressors are recruited to p53 through stress-induced posttranslational modifications to the p53 N terminus. Although CBP/p300 and PCAF acetylate lysines at the C terminus of p53, mutation of these sites failed to demonstrate a strong effect on p53-mediated transcription [38]. Instead, recruitment of HATs and HDACs to promoter sites may alter chromatin structure in the vicinity of p53 promoters, suggesting that activation of p53-mediated transcription may result from the action of these coactivators and repressors on the modification state and structure of chromatin in the vicinity of p53 promoters [39].

transcriptional activation *in vivo* have been unsuccessful [41], the importance of histone acetyltransferases (HATs) for p53 transcriptional activity was demonstrated by overexpression of the histone deacetylases HDAC-1, -2, or -3, or hSir2, which deacetylate p53 and inhibit the transcription of p53-target genes [42–46]. These and other recent results suggest an alternative model for p53-mediated transcriptional activation in which p53 serves to target HATs to the promoters of p53 activated target genes (Fig. 2, bottom). Likewise, the targeting of HDACs by p53 to p53 repressed genes has been suggested as a mechanism of p53-mediated gene repression [47]. The mechanisms by which HATs (and HDACs) associate with p53 are not completely understood. As noted earlier, we previously proposed that DNA damage-induced N-terminal phosphorylations promote the association of p300/CBP with p53 [36]. However, other targeting mechanisms may exist. Recently it was suggested that acetylated residues in the p53 C terminus may serve to recruit coactivators [48].

A role for p53 in targeting the coactivator p300 to chromatin is consistent with recent *in vitro* transcription studies by Espinosa and Emerson [39]. They found that a plasmid containing the *CDKN1A* (*p21*) promoter, which contains two p53 binding sites located 2.3- and 1.5-kb proximal to the transcription start site, was not transcribed efficiently when assembled into chromatin. Transcription was strongly enhanced, however, by addition of both p53 and p300 but not by either factor alone. Importantly, unacetylated p53 bound efficiently to the plasmid DNA and with even higher affinity to the nucleosomal template, but not to a 25-bp oligonucleotide containing the 5′ *p21* promoter consensus site. Transcriptional activation required the C terminus of p53 but not acetylation of C-terminal sites. Furthermore, p53 was shown to induce acetylation of nucleosomal histones preferentially in the promoter region of the chromosomal template, consistent with the model depicted in Fig. 2 (bottom). The authors [39] suggested that binding of p53 to consensus sites in long DNA might be facilitated by the ability of long DNA, but not short oligonucleotides, to form non-B-DNA structures [49] or to bend DNA [50], as suggested previously. Surprisingly, however, several p53 activated promoters including *KARP1* [51], p21, and 14-3-3σ [51a] were found to be in an "open" conformation as judged by the presence of DNAse I hypersensitive sites in these promoters prior to p53 activation by treatment of cells with DNA damage-inducing agents. DNAse I chromatin hypersensitivity is a hallmark of promoter regions of actively transcribed genes, whereas the promoter regions of inactive genes usually are insensitive to DNAse I.

Activation of p53 by Genotoxic Stresses

Mammalian cells appear to have at least two largely independent signaling pathways for activating p53 in response to genotoxic stress; one is activated by the presence of DNA double-strand breaks, the other in response to bulky lesions such as pyrimidine dimers and base adducts.

Ionizing Radiation

Treatment of cells with ionizing radiation or several radiomimetics (e.g., neocarzinostatin, bleomycin) activates several kinases that phosphorylate p53 at multiple sites (Fig. 3). Although the molecular mechanism(s) by which DNA strand breaks are recognized are still obscure, key among the kinases activated in response to DNA breaks is ATM, a protein kinase member of the phosphatidylinositol-3-kinase (PI3K) kinase family encoded by the gene responsible for the human genetic disorder ataxia-telangiectasia (A-T). ATM directly phosphorylates p53 at Ser15 and activates several other protein kinases that phosphorylate the N-terminal transactivation domain including Chk1 and Chk2, which

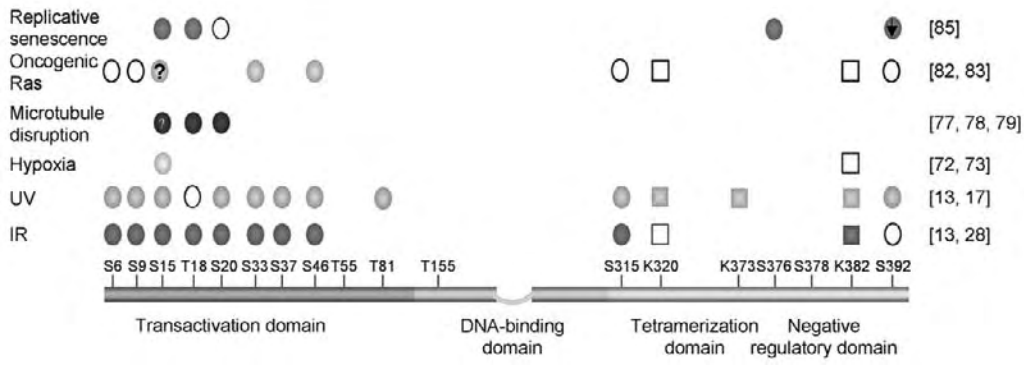


Figure 3 Posttranslational modifications to p53 in response to genotoxic and nongenotoxic stress. The bar at the bottom represents the human 393-amino-acid p53 polypeptide; functional regions are indicated. Reported posttranslational modification sites are indicated above the bar; S, serine; T, threonine; K, lysine. Filled circles (phosphorylation) or squares (acetylation) indicate modification in response to the indicated stress (left); open symbols indicate no change in modification in response to stress. No symbol indicates the site has not been examined. Selected references are given at the right. A "?" indicates conflicting literature reports; a down arrow indicates that treatment induced a decrease in site modification. Thr55 [14] and Ser376 and 378 [15] were reported to be constitutively phosphorylated; Thr55 and Ser376 are dephosphorylated in response to DNA damage. Ser6, 9, 33, 315, and 392 may be constitutively phosphorylated at low levels in some cell lines (Saito *et al.* unpublished).

phosphorylate p53 at Ser20 and perhaps other residues (see Fig. 1). ATM appears to activate other, unidentified protein kinases that directly phosphorylated Ser9 and Ser46 [28]. Protein kinases that directly phosphorylate Ser6, Ser33, Ser37, and Ser315 appear to be activated in response to DNA double-strand breaks by ATM-independent pathways, although inactivation of possible site-specific phosphatases for these residues in response to DNA strand breaks cannot be ruled out. Induction of phosphorylation through substrate modification also is possible. Casein kinase 1 (CK1) phosphorylates sites two residues distal to a previously phosphorylated serine or threonine. *In vitro* p53 previously phosphorylated at Ser6 and Ser15 is phosphorylated by CK1 at Ser9 or Thr18, respectively [22,52]. Thus, CK1, or a similar activity, may provide a mechanism for phosphorylation site signal amplification, at least in some cells. Several kinases, including CAK (Ser33), CDK (Ser315), PKA, and PKC (Ser376, Ser378), which are capable of phosphorylating p53 *in vitro*, have been identified (see Fig. 1), but if and how these might be activated in response to DNA strand breaks is unknown (reviewed in [13]).

DNA-PK, another PI3K family member that phosphorylates p53 at both Ser15 and Ser37 *in vitro*, is directly activated by DNA strand breaks through targeting to DNA ends by its Ku subunit, but its role *in vivo* remains uncertain. A recent study reported that p53 is found in a complex that contains nucleotide analog-modified DNA and activated DNA-PK [53]. Treatment of cells with the analog induced DNA-PK protein accumulation phosphorylation of p53 on Ser15, resulting in its stabilization and activation; and triggered apoptosis. Consistent with this report, Woo *et al.* [54] recently reported that DNA-PK is required for the IR-mediated apoptosis of mouse embryonic fibroblasts. However, DNA-PK is not required for p53-mediated cell cycle arrest in G_1 or the induction of p53 activated transcription in response to ionizing radiation [55].

UV Light

A second DNA damage response pathway is activated in response to bulky lesions, such as the pyrimidine dimers caused by UV-C (see Fig. 3). As for IR, the molecular mechanism(s) responsible for sensing UV-damaged DNA are not known, although the pathway is thought to involve sensing a block to transcriptional elongation [56] and activation of a third PI3K family member, ATR (A-T and Rad3-related) [57]. Recently, it was reported that ATR exhibits preferential binding to UV-damaged DNA; thus, under some circumstance, it may act directly as a sensor of UV damage [58]. *In vitro* ATR phosphorylates p53 on Ser15 and Ser37. UV-C also induces p53 phosphorylation on Ser6, 9, 20, 33, 46, 315, 392, and on Thr18 and 81 (see Fig. 3); thus exposure of cells to UV light must activate or induce a number of other kinases that phosphorylate p53. Among these are p38 MAPK, which targets Ser33 and Ser46 [59]; HIPK2, which also targets Ser46 [60,61]; JNK, which phosphorylates Thr81 [17]; and a complex containing casein kinase 2 (CK2) and the transcription elongation factor FACT that phosphorylates Ser392 [62]. In response to UV light, each of these sites is phosphorylated in cells lacking ATM ([28]; Saito *et al.,* unpublished).

The dependence of phosphorylation at other sites on ATR has not been carefully investigated since ATR-deficient cells are not viable. Phosphorylation at Ser46 correlates with the induction of apoptosis and the transcriptional induction of *AIP1* (p53 regulated *a*poptosis *i*nducing *p*rotein *1*) by UV-C [63]. Substitution of Ser46 with alanine inhibited p53-mediated apoptosis and the induction of *AIP1*. Evidence has been presented that the majority of p53 protein can be phosphorylated at Ser315 by cdc2/Cdk2 in response to UV-induced DNA damage and that the cyclin-dependent kinases play a role in stimulating p53 function [64]. In contrast to UV-C, UV-A triggers activation of p53 through activation of the ATM kinase activity, apparently through the production of reactive oxygen species [65].

Carboxy-Terminal DNA Damage-Induced p53 Modifications

As noted earlier, two C-terminal PKC sites, Ser376 and 378, were reported to be constitutively phosphorylated in the absence of DNA damage [15]. Thr55 also may be constitutively phosphorylated and then dephosphorylated in response to DNA damage. Treatment of MCF7 cells with IR led to the ATM-dependent dephosphorylation of Ser376 and to the association of p53 with a 14-3-3 protein, which increased its *in vitro* affinity for sequence-specific DNA. A confounding observation is the fact that p53 from unstressed cells is recognized by the monoclonal antibody PAb421, which recognizes an epitope including Lys372 to Lys382, but recognition is inhibited by phosphorylation of Ser376 or Ser378 (and acetylation of Lys382). Recently H7, an inhibitor of PKC, was reported to prevent p53 ubiquitination and to stimulate p53 accumulation in cells without inducing its activation, suggesting that the constitutive phosphorylation of p53 at Ser376 or Ser378 contributes to its degradation [66]. The function of UV-mediated phosphorylation of Ser392 is not clear, because previously Ser392 was shown not to be required for the suppression of cell growth or the activation of transcription [67]. *In vitro*, phosphorylation of Ser392 stimulated formation of p53 tetramers, while phosphorylation of Ser315 reversed this effect [68]; however, these effects are seen at low p53 concentrations and may have been masked in cells overexpressing exogenous p53.

The acetylation of several C-terminal p53 lysines is stimulated by several forms of stress including the genotoxic damage caused by both UV and IR [36,69]. These residues include Lys320, which is acetylated by PCAF *in vitro*, and Lys373 and Lys382, which can be acetylated by p300/CBP. Prior to the discovery of DNA damage-mediated p53 acetylation, CBP/p300 and PCAF were shown to be coactivators of p53-mediated transcription. As noted earlier, C-terminal acetylation is promoted by the phosphorylation of N-terminal sites, especially Ser15, but acetylation at Lys382 also was

enhanced by phosphorylation at nearby residues, including Ser6, Ser9, and Thr18 [28]. However, in A549 cells acetylation was not induced equivalently by IR and UV. Acetylation, especially of Lys320, was much more strongly induced by UV than IR. Potential roles for p53 acetylation include contributions to p53 stability through interference with C-terminal ubiquitination and the modulation of p53's interactions with other proteins and DNA. Possible roles for p53 in targeting HATs and HDACs to chromatin were discussed earlier and illustrated in Fig. 2.

Other Genotoxic Agents

Because of their convenience, ionizing radiation and UV light are commonly used in the laboratory to produce two different forms to genotoxic damage: DNA double-strand breaks and pyrimidine dimers. Many other environmental, physiological, and therapeutic agents cause genotoxic damage that activate p53 through one or more signaling pathways. These include anticancer drugs such as adriamycin; topoisomerase inhibitors such as camptothecin, etoposide, and quercetin; DNA synthesis and transcription inhibitors including aphidicolin, actinomycin D and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB); DNA cross-linking agents, such as cisplatin and mitomycin C; and environmental chemicals including arsenite, cadmium, and chromate. Each of these agents have been shown to induces p53 accumulation and its phosphorylation on Ser15 except DRB, which interferes with phosphorylation of the CTD domain of RNA polymerase II rather than with elongation [70]. For most of these agents, however, modifications to p53 at other sites have not been investigated.

Activation of p53 by Nongenotoxic Stresses

p53 activation occurs in response to several physiological processes that are not associated with frank DNA damage, including hypoxia, nucleotide deprivation, microtubule inhibitors, oncogene activation, and senescence potentiated by telomere erosion. Some of these processes may be mimicked by pharmacological agents that may or may not induce identical responses.

Hypoxia

Tumor hypoxia occurs in most solid tumors from abnormal vasculature development; hypoxia is also an important pathophysiological feature of ischemic disorders. Hypoxia and several hypoxia mimetics have been shown to induce p53 accumulation as a result of the down-regulation of MDM2 [71] with concomitant phosphorylation of Ser15, but not acetylation of Lys382 [72] (see Fig. 3). However, in contrast to IR, hypoxia treatment failed to induce the transcription of downstream effector mRNAs including GADD45, Bax, and p21 [72]. Hypoxia does not induce detectable DNA damage, and, in contrast to DNA damage-inducing agents, primarily caused an association of p53 with mSin3A rather than p300. Consistent with this finding, p53-mediated transrepression was induced. In hypoxia-treated human papillomavirus HPV-16 transformed cells, p53 was resistant to E6-mediated degradation, and its association with E6AP was reduced [71]. Interestingly, a recent study has shown that inhibition of ATR kinase activity reduced the hypoxia-induced phosphorylation of p53 protein on Ser15 as well as p53 protein accumulation [73]. These data suggest that hypoxia could select for the loss of ATR-dependent checkpoint controls, thus promoting cell transformation.

Ribonucleotide Depletion

Studies by Linke *et al.* [74] showed that p53 is activated in normal human fibroblasts by the *N*-phosphoacetyl-L-aspartate-induced (PALA) depletion of ribonucleotides in the absence of detectable DNA damage. In contrast to the G_1 arrest induced by DNA damage, that induced by PALA was readily reversible. PALA treatment induced a pattern of gene expression that was distinct from that induced by IR. Some of these gene expressions, such as *MDGI*, a mammary-derived growth inhibitor gene, were induced independent of p53, whereas for others, such as *TSG6*, a tumor necrosis factor stimulated gene, induction was p53 dependent [75]. The modification status of p53 after PALA treatment has not been addressed.

Microtubule Disruption

Activation of p53 also occurs in response to factors such as colcemid, nocodazole, and taxol that deregulate cell adhesion or microtubule architecture and dynamics. Taxol (Paclitaxel), which inhibits microtubule depolymerization, is one of the newer chemotherapy drugs commonly used to treat ovarian, breast, and head and neck cancers. After nocodazole treatment, which depolymerizes microtubules, quiescent human fibroblasts accumulated transcriptionally active p53 and arrested in G_1 with a 4 N DNA content [76]. Activation of p53 after colcemid treatment was accompanied by a moderate increase in phosphorylation at Ser15 and correlated with activation of Erk1/2 MAP kinases and the development of focal adhesions rather than disruption of the microtubule system [77]. Curiously, murine fibroblasts did not undergo the same response. Taxol and vincristine, but not nocodazole, were found to induced multisite phosphorylation of p53 in several tumor-derived human cell lines, including HCT-116 and RKO cells, and the pattern of p53 phosphorylation was distinct from that observed after DNA damage [78]. Nevertheless, both nocodazole and taxol increased phosphorylation at Ser15 (Fig. 3).

Interestingly, microtubule inhibitor-induced p53 stabilization and Ser15 phosphorylation did not occur in ATM-deficient fibroblasts nor in normal human dermal fibroblasts. Studies with ectopically expressed p53 phosphorylation site mutants indicated that several p53 amino-terminal residues, including Ser15 and Thr18, were required for the taxol-mediated

phosphorylation of p53 [78]. In contrast, Damia *et al.* [79] reported that taxol induced p53 phosphorylation at Ser20 but not at Ser15 in HCT-116 cells. Phosphorylation at Ser20 was accompanied by increased Chk2 activity and was not inhibited in A-T cells lines nor by wortmannin treatment. Thus, the signaling pathways that impinge on p53 after hypoxia, ribonucleotide depletion, or microtubule disruption, although still not well defined, appear distinct from those induced by genotoxic stresses.

Oncogene Activation

Oncogenes, such as Ras, c-Myc, or E1a, when activated or overexpressed, stabilize and activate p53, and, depending on the cell type, induce senescence (Ras) or apoptosis (c-Myc or E1a) through hyperproliferative signaling pathways that activate ARF, the product of the alternative reading frame of the cell cycle regulatory gene INK4a/CDKN2a [30]. ARF, in turn, inhibits p53 degradation by MDM2. Based on the observation that p53 Ser15 was not phosphorylated in response to adenovirus E1A expression, it was concluded that oncogenic activation of p53 occurs in the absence of DNA damage [80]. However, in normal fibroblasts brief c-Myc overexpression induced DNA damage prior to S phase that correlated with the induction of reactive oxygen species [81], raising the question of whether oncogenes activate p53 through DNA damage and whether the ability of oncogenes to promote either apoptosis or senescence correlates with different p53 posttranslational modifications. Ferbeyre *et al.* [82] reported that expression of oncogenic Ras induced phosphorylation of Ser15 in IMR90 cells. In contrast, Bulavin *et al.* [83] found that p53 was phosphorylated at Ser33 and Ser46 but not at other N- or C-terminal sites, nor was it acetylated at Lys382 (see Fig. 3). Interestingly, a similar induction of permanent cell cycle arrest resembling cellular senescence was produced in murine fibroblasts engineered to express the MAP kinase Mek1 [84]. The induction of senescence by Ras required wild-type p53 and ARF, but p53 was not required to maintain the senescent state. These data indicate that other signals may influence the outcome of p53 activation, likely by changing its association with various coactivators; however, whether this leads to the expression of different p53 target genes remains to be determined.

Replicative Senescence

Replicative senescence in human fibroblasts correlates with activation of p53-dependent transcription and was shown to be associated with increased phosphorylation at Ser15, Thr18, and probably Ser376, and decreased phosphorylation at Ser392 [85] (see Fig. 3). It was inferred from the finding that no change occurred in staining with the DO-1 monoclonal antibody, the epitope for which includes Ser20, that phosphorylation on Ser20 was not induced. These results, in conjunction with findings showing that changes in p53 phosphorylation are abrogated in cells that have been immortalized by overexpression of telomerase, indicate that the preceding modifications may be the product of telomere erosion. Shorten or disrupted telomere structures may signal to p53 via pathways partially shared with DNA damage responses.

Conclusions

Multiple, distinct signal transduction pathways clearly activate and modulate p53-dependent transcription in response to both genotoxic and nongenotoxic stresses. Although key protein kinases that are likely to phosphorylate p53 in response to DNA damage have been identified, the identities of kinases that phosphorylate several important sites are still unknown. Furthermore, several sites may be phosphorylated by more than one protein kinase. This complexity is augmented further by the facts that signaling pathway activation may be cell-type and cell-cycle dependent and that many signaling initiation events activate more than one pathway.

A fundamental question that remains unanswered is what mechanism(s) contribute to the ability of different cells to interpret p53 activation in different ways. The activation of p53 by hypoxia or oncogenes clearly induces different effects than the response to genotoxic stresses. Therefore, the pattern of posttranslational modifications may determine the selection of the subsets of target genes regulated in response to p53 activation, but a precise understanding of the mechanisms is not yet in hand. It is clear that the p53 protein forms complexes with many other cellular components and with particular nuclear structures. This characteristic may influence the degree of its activation and contribute to the heterogeneity of p53-dependent responses observed within a specific tissue. The analysis of the modification patterns in different mouse tissues of knock-in mutants should give insights as to the role played by individual phosphorylations and acetylations sites in eliciting a molecular signaling outcome. While there is still much to learn, substantial progress in understanding the causes and effects of p53 responses is being made.

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CHAPTER 307

Abl in Cell Signaling

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Introduction

Abl encodes a nonreceptor tyrosine kinase that is conserved in metazoan. The c-*abl* gene was discovered by virtue of its identity to the oncogene of Abelson murine leukemia virus, which expresses a Gag-Abl oncoprotein. The oncogenic potential of Abl is also activated in the BCR-ABL fusion protein of human chronic myelogenous leukemia (CML). The oncogenic function of Gag-Abl and BCR-ABL requires the Abl tyrosine kinase activity. This is best demonstrated by the clinical success in treating CML with an Abl kinase inhibitor, GLEEVEC [1]. The BCR-ABL tyrosine kinase can activate many signaling pathways in transformed cells. However, because overproduction of c-Abl does not transform cells, the signaling capabilities of BCR-ABL may not be identical to those of c-Abl. This chapter focuses on the signaling functions of mammalian c-Abl.

In the mouse and human genome, an *abl-r*elated *g*ene (*arg*) has been identified. In mice, mutation of the c-*abl* gene causes neonatal lethality and several low-penetrant phenotypes including lymphopenia and osteoporosis [2,3]. In addition, splenocytes from *abl*-knock-out mice do not respond to bacterial lipopolysaccharide [4]; and retinas from *abl*-knock-out mice are resistant to hyperoxia-induced retinopathy [5]. The *arg*-knock-out mice, by contrast, are healthy and fertile [6]. The double knock-out of *abl* and *arg* causes early embryonic lethality [6]. Thus, *abl* and *arg* have redundant functions during early embryonic development, whereas *abl* has unique functions later in development. The present understanding of *abl* and *arg* cannot provide a coherent explanation for the various knock-out phenotypes. Nevertheless, these phenotypes suggest that *abl* is required for the proper development and function of different tissues and cell types.

Functional Domains of Abl

N-Terminal Region: Kinase Function

The mammalian c-Abl protein contains modular functional domains, consistent with its role as a signal transducer (Fig. 1). The N terminus of c-Abl is variable, encoded by two alternative 5′-exons. This variable domain is followed by the Src homology (SH) domains 3, 2, and the tyrosine kinase domain. The Abl SH3 domain exerts a negative effect on the catalytic activity. This inhibitory effect is mediated by an intramolecular interaction between the SH3 and the kinase domains [7], similar to that found in the inhibited conformation of the Src tyrosine kinase [8]. The inhibited conformation of Src involves a second intramolecular interaction between its SH2 domain and a C-terminal phosphotyrosine [8]. An SH2-dependent intramolecular interaction with the kinase domain also exists in Abl. This SH2/kinase interaction does not involve an internal phosphotyrosine ligand for the SH2 domain. However, this interaction depends on myristate binding to the kinase C-lobe [7]. The Abl SH3 and SH2 domains can also engage in intermolecular interactions with other cellular proteins, which are activators, inhibitors, or substrates of the Abl kinase (see later discussion).

C-Terminal Region: Localization Cues

The C-terminal region of c-Abl specifies its subcellular localization. The mammalian c-Abl contains three nuclear localization signals (NLSs) [9]. Mutations of all three NLSs are required to prevent c-Abl from entering the nucleus [9]. The mammalian c-Abl also contains a nuclear export signal (NES) [10]. Mutation of the NES causes the nuclear accumulation of c-Abl [10]. In proliferating fibroblasts, c-Abl shuttles between the cytoplasmic and the nuclear compartments [10].

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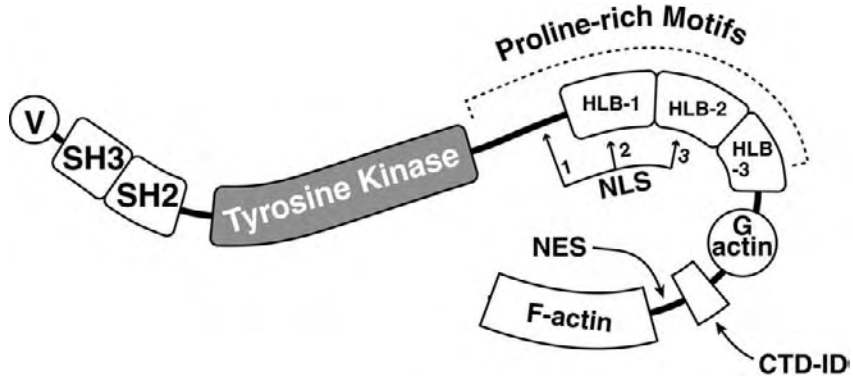


Figure 1 Functional domains of mammalian Abl. The variable N terminus (V) is encoded by two different exons. The murine type I (human Ia) exon encodes 26 amino acids. The murine type IV (human Ib) exon encodes 45 amino acids. SH3, Src homology 3; SH2, Src homology 2; NLS, nuclear localization signal; NES, nuclear export signal; HLB, high-mobility group (HMG)-like box. The three HLBs cooperatively bind to A/T-rich DNA with a distorted structure. Proline-rich motifs that bind to SH3 domains of several adaptor proteins are distributed in the C-terminal region. G-actin, binding site for actin monomers; CTD-ID, binding site for the CTD of RNA polymerase II. The CTD-ID is conserved in mammalian Abl and Arg. An F-actin binding consensus sequence is located at the very C terminus of Abl and this sequence is conserved in mammalian and *Drosophila* Abl.

In the cytoplasm, c-Abl associates with the actin cytoskeleton because it contains binding sites for F-actin and G-actin [11,12]. The C terminus of Abl contains an F-actin binding consensus sequence, which is conserved in Arg and the *Drosophila* Abl [11]. Purified mammalian c-Abl protein is an active kinase, which can be inhibited by F-actin *in vitro* [13]. This inhibition is abolished by mutation of the F-actin binding site [13]. The cytoplasmic c-Abl plays a role in actin-dependent cellular processes, including cell adhesion, cell migration, and axon guidance [14], possibly because Abl can regulate the actin dynamics [15].

In the nucleus, c-Abl associates with the chromatin [16]. The mouse and human c-Abl protein contains three HMG-like domains that bind to A/T-rich DNA with distorted structures [17,18]. The nuclear c-Abl plays a role in the regulation of transcription. This is suggested by the interaction between c-Abl and several transcription factors, including E2F-1 [19], p53 [20], p73 [21,22], c-Jun [23], RXFI [24], and the catalytic subunit of RNA polymerase II [25].

Functional Interactions between the N- and C-Terminal Regions of Abl

The C-terminal region plays an important role in the biological function of Abl kinase. A mutant mouse strain that expresses a C-terminal truncated Abl protein exhibits phenotypes similar to the Abl-null mice [26]. A functional interaction between the N- and the C-terminal regions of Abl is also supported by other observations. For example, F-actin binding to the C terminus can exert a negative effect on Abl kinase activity, suggesting an interaction between the C terminus with the kinase domain [13]. The C-terminal region of Abl also contains a binding site for the C-terminal repeated domain (CTD) of mammalian RNA polymerase II [25,27]. This CTD-binding site is required for Abl to phosphorylate RNA polymerase II [27,28], again, suggesting juxtaposition of the C-terminal region to the kinase domain.

The C-terminal region of Abl is not well conserved through evolution. Consequently, the C-terminal region of the murine Abl cannot substitute for that of the *Drosophila* Abl; however, the kinase region could be exchanged between mouse and fly [29]. Taken together, these observations support the notion that interaction between the two regions of Abl is important to the biological function of the Abl kinase.

Proteins that Interact with Abl

The multiple functional domains of Abl direct its interaction with a large number of proteins, which are listed in Table I.

SH3 Binding Proteins

The SH3 domain binds proteins with the PXXP motif [30]. Most of the Abl SH3-binding proteins are substrates of the Abl kinase (Table I). Some of these substrates were thought to be "inhibitors" of Abl kinase. Those conclusions could have been biased by the experiments, which were based on kinase assays where substrates competed with each other for Abl. Another cause for the biased conclusion might be the use of truncated proteins that could have exerted a dominant negative effect.

As mentioned earlier, the SH3 domain engages in an intramolecular interaction to impose an inactive conformation on Abl kinase [7]. Intermolecular interaction through the SH3 domain should disrupt this intramolecular interaction to cause an increase in catalytic activity. Thus, SH3 binding by a substrate may promote its phosphorylation by the Abl kinase. It is conceivable that a protein may bind to the SH3 domain without disrupting the intramolecular interaction.

Table I Proteins that Bind to the Mammalian c-Abl

Binding domain in Abl	Protein	Function	Functional interaction with Abl	Ref.
SH3	3BP-1	GAP of Rac	?	[62, 63]
	3BP-2	adaptor	?	[62]
	Abi-1, Abi-2	adaptor	Abl substrate	[64, 65]
	APP-1	unknown	Abl inhibitor	[66]
	ATM	DNA damage signaling	Abl activator	[50]
	Cables	adaptor	Abl substrate	[67]
	Cbl	adaptor	Abl substrate	[68]
	DNA-PK	DNA repair	Abl substrate	[69]
	NMDA receptor NR2D subunit	neuronal signaling	Abl inhibitor	[70]
	Mena	F-actin assembly	Abl substrate	[71]
	PAG	peroxiredoxin	Abl inhibitor	[39]
	Scramblase-1	lipid bilayer remodeling	Abl substrate	[72]
	WAV E-1	F-actin assembly	?	[42]
SH2	Ptyr-Cbl	adaptor	Abl substrate	[68]
	Ptyr-Eph receptor	axon guidance	Abl substrate	[37]
	Ptyr-p130cas	adaptor	Abl substrate	[36]
	Ptyr-CTD of RNA polymerase II	transcription	Abl substrate	[35]
Kinase	PAG	peroxiredoxin	Abl inhibitor	[39]
	RB	transcription co-repressor	Abl inhibitor	[38]
Proline-rich motifs	Abi-1, Abi-2	adaptor	Abl substrate	[64, 65]
	Crk, CrkL	adaptor	Abl substrate	[40, 73]
	Grb2	adaptor	?	[40]
	Nck	adaptor	Abl activator	[68]
	PSTPIP1	adaptor	Abl substrate	[43]

If this SH3-binding protein further stabilizes the inactive conformation, then it would qualify as an inhibitor of Abl kinase. Inhibitors of Abl should not become phosphorylated, despite their binding to the SH3 domain. Given these considerations, proteins that interact with the Abl SH3 domain can be substrates, activators, or inhibitors of Abl kinase.

The SH3 domain is deleted in the Gag-Abl oncoprotein, and it does not contribute to the oncogenic function of BCR-ABL. Mutation of the SH3 domain can convert c-Abl into an oncogenic kinase, albeit of weaker potency than Gag-Abl and BCR-ABL [31,32]. Thus, the SH3 domain is not required for the signaling functions of oncogenic Abl tyrosine kinase. Instead, the Abl SH3 domain is likely to constrain the kinase function toward specific substrates.

SH2 Binding Proteins

The SH2 domain of Abl interacts with phosphorylated tyrosine in the consensus sequence (P) YXXP [33]. The kinase domain of Abl also prefers the YXXP motif in its substrates [34]. The coordinated recognition of substrates by the kinase and the SH2 domain is important for catalysis for two reasons. First, the SH2 domain can mediate a continued association of Abl with a substrate. This is particularly important in the processive phosphorylation of the multiple tyrosines in the p130cas adaptor protein or the CTD of RNA polymerase II [35,36]. Second, the SH2 domain may increase the catalytic efficiency by extracting the product from the kinase domain [35]. The Abl SH2 domain plays an essential role in the oncogenic functions of Gag-Abl and BCR-ABL, in keeping with its positive role in catalysis. In addition, the Abl SH2 domain could serve as an adaptor to bring Abl kinase to specific signaling complexes. For example, the Abl SH2 domain binds to tyrosine phosphorylated EphB2 receptors [37]. Thus, Abl SH2-binding proteins can be substrates or recruiters of Abl kinase in signal transduction.

Kinase Domain Binding Proteins

The substrates of Abl are expected to bind the kinase domain. In addition, two protein inhibitors of the Abl kinase have been shown to bind Abl through its kinase domain. These are the retinoblastoma tumor suppressor (RB) and PAG [38,39]. RB binds to the ATP-binding lobe of the Abl

kinase domain, and this interaction inhibits Abl kinase activity [38]. *In vitro*, RB can inhibit Abl, Gag-Abl, and BCR-ABL kinase [38]. *In vivo*, only the nuclear Abl is inhibited by RB, most likely because RB is an exclusively nuclear protein [38]. PAG is inducibly expressed under oxidative stress, and it catalyzes the destruction of H_2O_2. PAG binds Abl through the SH3 domain and the kinase domain to inhibit Abl kinase [39]. Thus, Abl substrates as well as Abl inhibitors can bind to the kinase domain.

Proline-Rich Motif Binding Proteins

The C-terminal region of Abl contains a series of proline-rich motifs that bind to proteins with SH3 domains [40]. A number of adaptor proteins have been shown to bind Abl through the proline-rich motifs (Table I). Most of these proteins are also phosphorylated by Abl. Thus, the proline-rich motifs appear to function as substrate binding sites. In addition to the recruitment of substrates, the C-terminal region of Abl may have an adaptor function of its own and can contribute to the assembly of signaling complexes. A kinase-defective mutant has been shown to rescue some of the phenotypes of *abl*-deficient *Drosophila* [29]. This observation supports the idea that Abl may have a scaffolding function besides being a tyrosine kinase.

Abl in Signal Transduction

Cytoplasmic Signaling Function of Abl

In the cytoplasm, Abl can transduce extracellular signals to regulate F-actin assembly (Fig. 2). This signaling function is consistent with Abl's ability to interact with G-actin and F-actin [14].

Platelet-derived growth factor (PDGF) activates Abl kinase to stimulate membrane ruffling in fibroblasts [41]. The Src tyrosine kinase and phospholipase C-α1 are involved in the activation of Abl kinase by the PDGF receptor [41,41a]. In response to PDGF, a complex of Abl/WAVE-1/PKA (cyclic AMP-dependent protein kinase) is recruited to the sites of membrane ruffling [42]. PDGF also stimulates the tyrosine phosphorylation of PSTPIP1, a scaffolding protein, and this phosphorylation event requires the Abl kinase [43]. PSTPIP1 can bring Abl to a tyrosine phosphatase (PTP-PEST), which is thought to dephosphorylate and inactivate Abl [43]. PSTPIP1 also binds WASP [44]. Both WAVE-1 and WASP can interact with the Arp2/3 complex to promote cortical F-actin polymerization [45]. Taken together, these observations suggest that Abl can transduce PDGF signal to regulate the assembly of actin filaments (Fig. 2A).

Cell adhesion to fibronectin activates Abl kinase to stimulate the formation of F-actin microspikes [15]. The Abl tyrosine kinase is inactive in cells deprived of extracellular matrix (ECM) signals [13,46]. Upon cell adhesion to fibronectin, Abl kinase is activated to stimulate the formation of F-actin microspikes [15]. Interestingly, Abl-dependent F-actin microspikes can form under conditions when the Rho family of GTPases is inhibited by the bacterial C3 toxin [15]. These observations suggest that c-Abl tyrosine kinase can stimulate F-actin assembly in response to cell adhesion signals through a pathway that is independent of the Rho/Rac/Cdc42 family of small G proteins (Fig. 2A).

Hepatocyte growth factor (HGF) can activate Abl kinase in epithelial cells [47]. Inhibition of Abl kinase by GLEEVEC causes an increase in the chemotaxis response to HGF, suggesting a role for Abl in the reduction of cell migration [47]. The negative effect of Abl kinase on cell motility is also observed in fibroblasts [48]. In transient transfection experiments, Abl is found to reduce motility by phosphorylating and inactivating Crk, which is a rate-limiting factor in the motile response [48].

Taken together, current evidence suggests that the cytoplasmic Abl kinase can transduce a variety of extracellular signals to regulate F-actin assembly [14]. The phenotypic effect of Abl kinase on F-actin appears to be dependent on the signals as well as the cell context. It is interesting to note that F-actin itself can exert a negative effect on the Abl kinase activity [13]. The reciprocal regulation between Abl and F-actin may provide a self-limiting mechanism to control the dynamics of actin filament formation (Fig. 2A).

Nuclear Signaling Function of Abl

In the nucleus, the Abl kinase can transduce cell cycle and DNA damage signals to regulate transcription. The nuclear Abl kinase is inhibited in quiescent and early G_1 cells, through the action of RB [38]. Upon RB phosphorylation, nuclear Abl kinase is released from RB and can be further activated by DNA damage. Ionizing radiation (IR) activates nuclear Abl kinase and this requires the function of ATM, which phosphorylates a SQ motif that is conserved in the kinase domain of mammalian Abl and Arg [49,50]. Cisplatin, a DNA cross-linking agent, also activates the nuclear Abl kinase and this requires the function of mismatch repair (MMR) proteins [22,51]. The DNA lesions induced by IR and cisplatin are different. Hence, nuclear Abl may be activated by different types of DNA lesions through alternative signaling pathways.

As discussed earlier, Abl phosphorylates the CTD of RNA polymerase II [25,28]. Indeed, DNA damage can increase the tyrosine phosphorylation of RNA polymerase II through c-Abl [49,52]. The CTD is composed of heptad repeats with the consensus sequence YSPTSPS [53]. Phosphorylation of the CTD repeats converts the transcription initiation complex to an elongation complex [53]. The Abl-mediated tyrosine phosphorylation of the CTD can be correlated with increased transcription elongation [27]. Moreover, the Abl protein binds A/T-rich DNA with distorted structure, which resembles a transcription bubble [17]. Thus, the nuclear Abl kinase is likely to stimulate transcription elongation during cell cycle progression and following DNA damage (Fig. 2B). In addition to RNA polymerase II, nuclear Abl kinase also phosphorylates p73, a p53-related transcription factor [54]. Activated Abl also contributes to the stabilization of p73 [22],

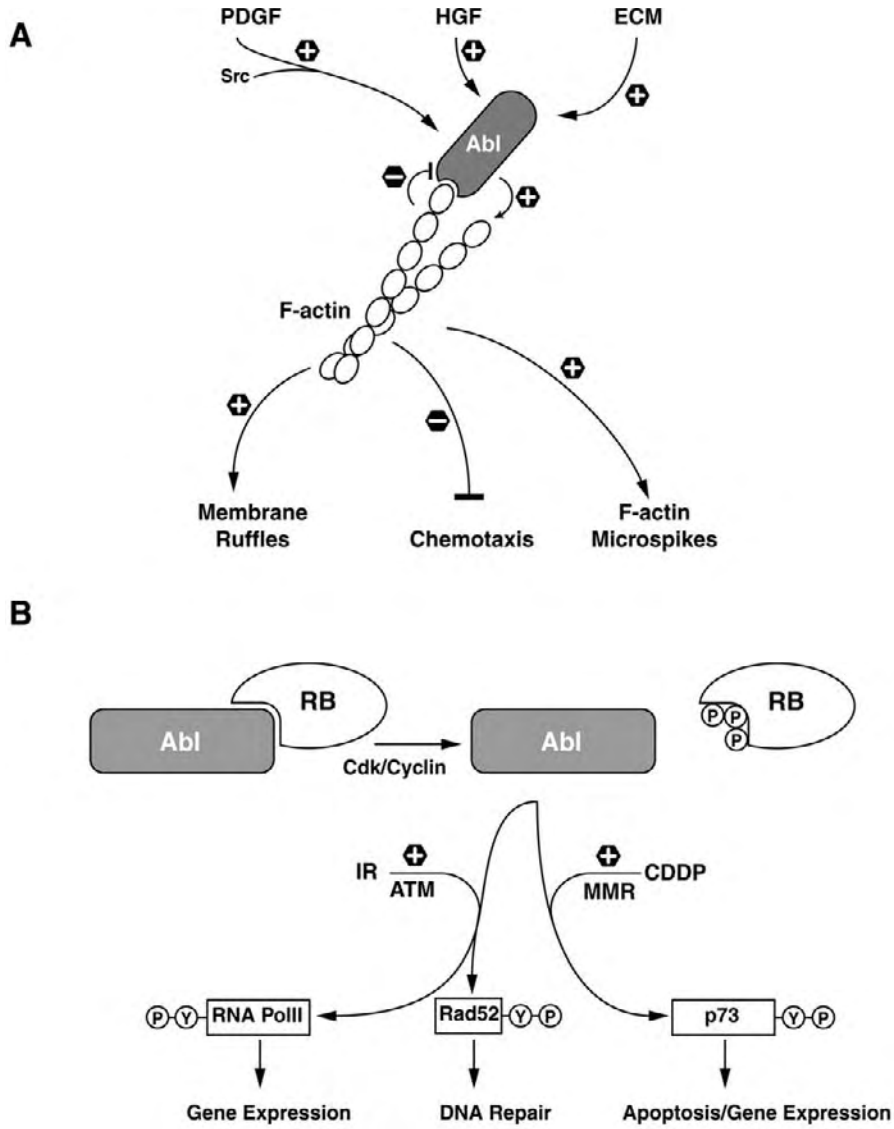


Figure 2 Signaling functions of Abl kinase. (A) Cytoplasmic Abl transduces extracellular signals to F-actin. Growth factors (PDGF, HGF) and extracellular matrix (ECM) can activate Abl kinase to regulate the assembly of actin filaments. Phenotypic outcomes resulting from each signaling pathway appear to be different. Abl stimulates membrane ruffling in response to PDGF. Abl inhibits chemotaxis in response to HGF. Abl stimulates F-actin microspikes in response to ECM. The Abl kinase activity can be inhibited by F-actin. The reciprocal regulation between Abl and F-actin may provide a self-limiting mechanism in regulating the dynamic structure of F-actin. (B) Nuclear Abl transduces cell cycle and DNA damage signals. The nuclear Abl interacts with the retinoblastoma tumor suppressor (RB), which inhibits the Abl kinase activity. Phosphorylation of RB during cell cycle progression releases Abl and allows it to be further activated by DNA damage signals. Ionizing radiation (IR) activates Abl kinase through ATM, leading to an increased tyrosine phosphorylation of RNA polymerase II and Rad52 and contributing to gene expression and possibly DNA repair. Cisplatin (CDDP) activates Abl kinase through the mismatch repair proteins (MMR), leading to the stabilization and activation of p73, a transcription factor related to the p53 tumor suppressor. The interaction between activated Abl kinase and p73 causes apoptosis.

as well as p53 [55], through mechanisms that have not been completely elucidated.

The "biological consequence" of the Abl function in the nucleus is likely to be determined by its interactions with other factors. For example, the nuclear Abl can be recruited to the E2F-1 transcription complex through RB [19]. In this capacity, Abl may stimulate transcription of E2F-regulated genes upon the inactivation of RB. Nuclear Abl has also been detected in transcription complexes that contain c-Jun, p53, p73, or RXF1. Thus, the nuclear signaling function of Abl is likely to be varied and determined by its recruitment to different protein complexes (Fig. 2B).

One of the biological consequences resulting from the activation of nuclear Abl kinase is apoptosis [56]. Inducers of

DNA damage, for example, cisplatin and doxorubicin, can activate nuclear Abl kinase, which in turn activates the pro-apoptotic function of p73 [22,57]. The Abl kinase is an essential upstream activator of p73, because ectopic expression of p73 does induce apoptosis in Abl-null cells [21,22,57]. The reverse is also true, in that the pro-apoptotic function of nuclear Abl kinase is greatly compromised in p73-null cells [74]. Thus, the nuclear Abl kinase can transduce DNA damage signal to p73 to activate apoptosis (Fig. 2B).

The BCR-ABL tyrosine kinase has the ability to inhibit apoptosis. The discrepancy between the anti-apoptotic function of BCR-ABL and the pro-apoptotic function of c-Abl has been resolved [58]. The BCR-ABL tyrosine kinase is an exclusively cytoplasmic protein. Its signaling functions in the cytoplasm culminate in the inhibition of apoptosis. The BCR-ABL protein can enter the nucleus when its tyrosine kinase is inhibited by GLEEVEC [58]. The nuclear BCR-ABL can be trapped by a second drug, leptomycin B, which inhibits the nuclear export of BCR-ABL [58]. Upon removal of GLEEVEC, the nuclear BCR-ABL regains activity to cause apoptosis [58]. These results demonstrate that the pro-apoptotic function of Abl kinase is dependent on it being in the nucleus of a cell. Moreover, these results show that BCR-ABL retains the pro-apoptotic function of Abl. Hence, conversion of Abl into an oncoprotein requires not only the activation of its tyrosine kinase but also the inhibition of its nuclear import to abolish the pro-apoptotic function.

The nuclear Abl can transduce death-inducing signals other than those generated by DNA damage [56]. For example, Abl tyrosine kinase contributes to apoptosis induced by tumor necrosis factor [59]. Abl tyrosine kinase may also contribute to apoptosis caused by oxidative stress [60]. The signaling pathways linking these death inducers to the activation of nuclear Abl kinase are under investigation.

While the nuclear Abl kinase can promote apoptosis, this may not be the only biological consequence of Abl activation. For example, the maximal activation of nuclear Abl tyrosine kinase is achieved by IR at a dose (2 Gy) that does not induce apoptosis. Thus, activation of the nuclear Abl kinase is necessary but not sufficient to cause cell death. The Abl kinase has been shown to phosphorylate Rad52, suggesting a possible role for Abl in DNA repair [61]. Nuclear Abl kinase can also phosphorylate MyoD to inhibit myogeniz differentiation in response to DNA damage [75]. Hence, the signaling functions of the nuclear Abl kinase are likely to be modulated by the nature of the signal and the cell context.

Future Prospects

A framework for the signaling functions of Abl has emerged from (1) the delineation of its modular domains and (2) the identification of cellular phenotypes associated with Abl kinase activation. The cytoplasmic Abl transduces extracellular signals to regulate F-actin. The nuclear Abl transduces cell cycle and DNA damage signals to regulate transcription. With this framework, it is now feasible to further elucidate the mechanistic steps in Abl-dependent signaling pathways.

The accumulated knowledge on Abl has not provided a unifying mechanism for the biological effects associated with Abl kinase activation. Instead, the current evidence suggests a highly context-dependent biological function for Abl kinase. The biological effect of Abl is strongly modulated by its association with other signaling molecules. For example, cytoplasmic Abl kinase stimulates membrane ruffling in response to PDGF, but it stimulates F-actin microspikes in response to fibronectin [14]. Although both phenotypic outcomes may involve the ability of Abl to regulate F-actin assembly, the pathways linking Abl to ruffles or microspikes might not be identical. Likewise, nuclear Abl kinase can be recruited to several different transcription complexes, which are likely to influence the biological output from activated Abl kinase.

Because the signaling functions of Abl may be modulated by the cell context, it is important to study the Abl function in a defined biological system. In addition, a biological effect of Abl found in one system might not be easily extrapolated or generalized to another experimental system. Despite these possible complexities, it is clear that Abl can regulate F-actin and transcription. The precise mechanisms by which these functions of Abl are utilized under specific biological conditions will await further investigation.

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CHAPTER 308

Radiation-Induced Cytoplasmic Signaling

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Introduction

Radiation initiates a plethora of cellular alterations including inflammation, skin reddening, and cancer and also activates several intrinsic cellular programs such as cell cycle arrest, DNA repair, and apoptosis. Many of these changes result from radiation-induced chromosomal damage, while others are a result of lesions in other cellular macromolecules that link into signal transduction pathways. A frequent consequence is the activation of mitogen activated protein (MAP) kinases resulting in phosphorylation of their substrates, for example, transcription factors. In this chapter we summarize our current knowledge of the buildup of the signaling network and of radiation-induced cytoplasmic signaling in mammalian cells.

Cytoplasmic Signaling Network

All living cells require extracellular stimuli for survival, for apoptosis, for differentiation—in essence for any cellular achievement, for any change in gene expression. Physiologically relevant extracellular cues are perceived primarily by cell surface receptors, each of which feeds into signal transduction chains to the nucleus. One class of these signaling chains involves a series of successively acting protein kinases, the most distal of which in the direction of signal flow and the closest to the nucleus are the MAP-kinases. (Fig. 1 shows the principle of a protein kinase cascade.) Some of the upstream protein kinases as well as the MAP-kinases can shuttle between cytoplasm and the nucleus (reviewed in [1]) and phosphorylate transcription and splice regulatory factors directly [2–4] as well as factors that control RNA and protein turnover [5–8]. Some target molecules are addressed by MAP-kinases through still another effector protein kinase (e.g. Msk, Rsk) [9–11].

The linear signal transduction cascades as diagrammed in Fig. 1 are simplified abstractions of reality. In fact, signaling pathways form an elaborate communication network with an enormous degree of cross-talk between pathways (reviewed in [12]). In addition, signaling components are likely ordered in preformed arrays rather than being freely diffusible. To this end, the protein kinases carry, in addition to their catalytic domain, a specific interaction surface. Such interaction domains have been characterized, for instance, for the binding of JNK to its transcription factor substrate c-Jun [13] and for the interaction between MEK and ERK [14]. In addition, specialized scaffolding proteins may contribute to establish preformed arrays of protein kinases [15–17].

A large number of cell surface receptors activate a given MAP-kinase pathway, seemingly to the same extent. How then is specificity established? One possibility is that the combination of several signaling pathways determines the exact phenotypic outcome provided each receptor induces a specific pathway in addition to the common one. Or the duration of signaling, which could be organized by receptor-specific negative regulators such as protein tyrosine phosphatases [18], may be the determining parameter. An interesting aspect concerns the specific adaptation of the signaling components to the receptor. Based on studies of the receptor tyrosine kinase Met, an additional class of factors has been proposed to create specific interactions: A closely associated coreceptor forms a link to the actin cytoskeleton, which is absolutely

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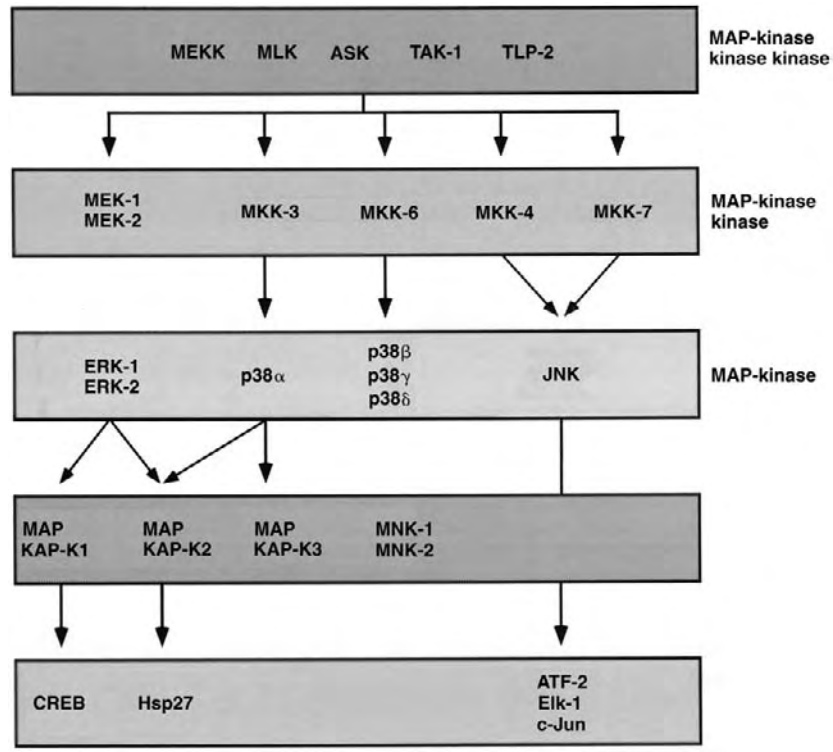


Figure 1 MAP-kinase signaling cascades. MAP-kinases are activated by dual phosphorylation caused by members of the MAP-kinase kinase group, which are in turn activated by phosphorylation mediated by members of the group of MAP-kinase kinase kinases. On activation, MAP-kinases phosphorylate transcription factors either directly or through other effector protein kinases.

required for any signal transfer from Met to the level of the MAP-kinase kinase MEK [19].

As touched on earlier, stimulation of a signaling pathway must be terminated after a "reasonable" period of time. On all levels, means of counterregulation are built in. Protein kinases are associated with protein phosphatases. The stimulation of a pathway often leads to synthesis or activation of a negative terminating factor, for example, of a phosphatase such as MKP-1 [20–22] or of the inhibitor of NFκB, IκB [23]. Inactivation of a phosphatase derepresses signal transfer and enhances transcription [24], suggesting that there is constant firing through signaling components and that specific negative regulators counteract the firing in order to keep the noise level low. Also interference with the actin cytoskeleton, which occurs physiologically during mitosis, disrupts signal transfer from the plasma membrane to the nucleus [19,25–27].

Redox Sensitivity and Metal Toxicity: Toxic Agents Activate Signaling Pathways

Obviously individual cells as well as cells in the multicellular organism are not only exposed to physiological stimuli but also to various adverse agents, ranging from heat and radiation through hypoxia and osmotic shock to many types of toxic chemicals. Low to moderate doses induce elaborate cellular responses—known as UV response, heat-shock response, osmotic shock response, and so on—all of which involve changes in gene expression.

To trigger an active response, the toxic agent is required to find access to the signaling process, which addresses transcription, splicing, or else. Statistically it is by far more likely that a toxic agent destroys the function of a molecule rather than conferring on it a gain of function. The structure and function of most cellular macromolecules depends on charge (and therefore on pH and reductive microenvironment). Moreover, the macromolecules carry reactive chemical groups relevant for function. In particular, redox-sensitive structures can be addressed by adverse agents leading to, for instance, inactivation of an enzyme. A direct gain of function can, in the rare case, be achieved by an induced conformational change or by the generation of a cross-link of two proteins leading to an increase in their activity [28]. More frequently, an indirect gain of function can result from inactivation by the toxic agent of a negative control element and thus derepression of, for example, some step in the signal transduction process.

Indeed, adverse agents of a very different kind have been reported to stimulate signaling in the cell. Here we first briefly review the evidence showing that individual components of the intracellular communication network are activated in response to an insult. This does not indicate that the agent acts directly on the component in question, but rather that a pathway has been addressed into which the component is integrated.

We then ask where the signaling comes from and what the primary target of interaction with the adverse agent is.

Activation of Signaling Components

Adverse agents alter cellular parameters, such as proliferation and gene expression, and induce apoptosis, differentiation, and senescence. Alterations in the program of gene expression are reflected by, for instance, induction of *c-fos*, *c-jun*, uPA, collagenase, metallothionein, and many other genes. These changes in gene expression result from agent-induced signaling activity, which in turn enhances the function of several transcription factors. Indeed numerous components of the signaling network have been found to be activated on cellular contact with adverse agents. Both UV and IR cause activation of IκB kinase and Akt/PKB, or Ras and Raf kinase [29–33]. The predominant link between signal flow and transcriptional activity is provided by the large group of MAP-kinases.

MAP-kinases are parts of five distinct signaling cascades including the mitogenic ERK-1/2 cascade, the stress-activated JNK and p38 cascades, and the ERK-3 and ERK-5 cascades (Fig. 1). These cascades are typically arranged in a three-kinase architecture comprising a MAP-kinase, an activator of the MAP-kinase (MAP-kinase kinase), and an activator of the MAP-kinase activator (MAP-kinase kinase kinase). MAP-kinase cascades have been conserved through evolution and similar cascades are found in yeast, *Drosophila*, and plants [34].

Activation by adverse agents has been reported for the ERK 1/2 branch of the MAP-kinases and for p38 and JNK. Different members of the MAP-kinase family are activated to a different extent. MAP-kinase signaling is habitually transient in nature, because neutralizing principles are addressed by the same signal flow. Indeed, ERK-kinase specific phosphatase (MKP) is rapidly induced following stimuli that provoke MAP kinase signaling [20–22,35]. Activation of JNK (and p38) is counteracted by, for example, heat-shock protein 72 [36] and JIP-1 (JNK-interacting protein). The latter retains JNK in the cytoplasm, hence interfering with subsequent JNK activity [37].

MAP-kinases are normally activated by numerous mitogenic stimuli, and one of the as yet unresolved mysteries of MAP-kinase signaling is that of where the specificity of the response comes from after mitogenic stimuli and cellular stress. One explanation would be that degree or duration of activation accounts for the specificity of the response. p38/JNKs are usually efficiently and stably stimulated in response to cellular stress but only weakly and transiently in response to growth factors such as EGF, PDGF, FGF, and phorbol esters. In contrast, ERKs are heavily activated in response to mitogenic stimuli and marginally after UV or ionizing irradiation. A more convincing, but not well explored idea, as introduced earlier, postulates that the cross-talk between pathways and the actual integration of all of the stimuli hitting cells at a given point in time defines the phenotypic outcome.

Primary Radiation Targets: DNA Damage versus Cytoplasmic Signaling

The previous paragraphs summarized evidence for the activation of signaling chains by adverse agents. For that discussion, we ignored the mechanism of activation. This mechanism is now considered. What are the primary targets that "emit" a signal and lead to activation of, for instance, JNK or ERK?

Radiation injures DNA, RNA, proteins, and other biomolecules. Lesions result either from energy absorption [for example, DNA photoproducts; UVB (280–320 nm) and UVC (200–280 nm) are absorbed by nucleic acid and protein] or from oxidation by free radicals, which are formed by radiation of various wavelengths including UVA (320–400 nm), UVB, UVC, and IR. The finding that treatment of cells with radical scavengers prior to stimulation with adverse agents prevents subsequent signaling and gene transcription provides indirect evidence for the generation of reactive oxygen intermediates [38,39]. Reactive oxygen intermediates such as superoxide anion radicals (O^{2-}), singlet oxygen ($^1O_2$), and hydroxyl radicals (OH^-) and other adverse agents may damage several types of macromolecules, which could be relevant for the subsequent activation of signaling and gene expression. Ionizing radiation may cause damage to DNA and inactivate numerous proteins in the cytoplasm at the same time. With increasing dose, certain targets may be damaged earlier than others. For instance, arsenite has been found to activate AP-1 by inhibiting JNK-phosphatase [40], although it is clear that arsenite must interact with numerous proteins.

With the notion in mind that adverse agents target numerous macromolecules at the same time, dissecting the pathways and defining the primary signal-generating targets is not a trivial task. To find the primary target molecules relevant for signal transduction, "retrograde" analyses were performed. Interestingly, both nuclear DNA damage as well as oxidative lesions of cytoplasmic macromolecules have been found to activate preexisting signaling chains.

Activation of JNK appears to reveal the complexity of finding the source of radiation-induced signaling. There is good evidence that JNK activation is triggered by prior membranal receptor tyrosine kinase activation in that it works in cytoblasts [41] and is addressed by the TNFR activated by UV [42]. Others have, however, reported on JNK activation by damaged DNA [43]. In addition, JNK is activated during the ribotoxic stress response. This illustrate the point that adverse agents have usually targets.

DNA Damage-Dependent Signaling

In recent years it turned out that nuclear and cytoplasmic signaling cascades are not completely separated branches of the cellular signaling network. Signals go in and out of the nucleus and influence the signal flow in both compartments after exposure to adverse agents. Transcription of collagenase, metallothionein, and plasminogen activator is enhanced in response to UV-DNA lesions in transcribed regions of

the genome [44,45]. If these are repaired proficiently, higher doses of UV are required to obtain the same response, suggesting strongly that the lesions generate signals to the transcription factors acting on these genes. The relevant factors are probably members of the AP-1 family. The DNA-lesion-dependent signals thus likely feed into MAP-kinase pathways. How the DNA lesion is recognized and how a signal is generated is processes linked to not precisely understood. However, as lesions in transcribed units provoke collagenase and metallothionein induction, one might speculate that signaling originates from stalled RNA-polymerase [44].

As mentioned earlier, it has been reported that JNK activation comprises a DNA-damage-sensitive component in that irradiated or sonicated DNA added to lysates of UV-treated cells stimulated JNK activity in a dose-dependent manner [43]. An alternative mechanism has been proposed by the finding that JNK can be activated by binding of the protein GADD45 to its NH_2-terminal domain [46]. The synthesis of GADD45 is induced in response to DNA damage and GADD45 may mediate sustained JNK activation. A third example is provided by the activation of NFκB in response to IR. Activated IKK phosphorylates IκB in the cytoplasm. This activity appears to depend on DNA double-strand breaks and ATM-kinase whose activity is regulated by double-strand breaks [47].

Activation of Receptor Tyrosine Kinases

One of the earliest measurable responses of mammalian cells to UV irradiation of all wavelengths is tyrosine phosphorylation of membrane-bound growth factor receptors [24,42,48,49]. The autophosphorylation of growth factor receptors is ligand independent and is an essential condition for transcription of the downstream genes *c-fos* and *c-jun,* which is completely ablated on inhibition of tyrosine kinase activity [24,49]. Activators of the receptors for EGF, IL-1, and bFGF account for most of the UV response in HeLa cells [49]. UV also activates the receptor for tumor necrosis factor (TNF) in a ligand-independent manner and to an extent comparable to that induced by the natural ligand TNF-α [50]. Fas (CD95) can serve as the third example of ligand-independent receptor activation by UV [51] or by the organometal TBT (Own unpublished data).

Growth factor receptors are usually phosphorylated in response to growth factor binding. The interaction with their cognate ligand animates the receptors to adopt a phosphorylated state achieved by the mutual action of two neighboring receptor molecules exhibiting intrinsic receptor tyrosine kinase activity. UV-mediated receptor phosphorylation occurs in the absence of ligand [49], yet is the result of protein tyrosine phosphatase (PTP) inhibition. PTPs are particularly sensitive to oxidative stress because each carries a conserved cysteine in its catalytic center, which is prone to oxidation [18,52]. The UV-induced inhibition of membrane-associated PTP activity [24] and of individual candidate PTPs (SHP-1, RPTPα, RPTPσ, and DEP-1; [48,53]) could account for the increase in receptor phosphorylation after UV irradiation provided that the intrinsic receptor tyrosine kinase is constitutively active. PTP inhibition is also achieved by treatments of cells in metal toxins, for example, vanadate [54], arsenite [40], or the organometal TBT (Unpublished data). Although plausible, PTP inactivation must not be the only mechanism responsible for RTK activation. Inactivation of other negatively controlling components and possibly a direct stabilization of receptor oligomers are conceivable options.

Phosphorylation of growth factor receptors upon UV irradiation seems to be sufficient to render them fully functional. Proteins such as Grb-2, phospholipase C-γ and Shc coprecipitate with the receptors after UV irradiation, similar to their response after growth factor stimulation [24,55,56]. Translocation of Grb-2/Sos complexes from the cytoplasm to activated growth factor receptors at the cell periphery leads to the activation of Ha-ras. UV and IR exposure increases the low basal activity of Ha-ras and enhance its association with Grb2 and Sos, followed by activation of Raf-1 and Raf-2 [33,38,57].

Ribotoxic Stress Response

The ribotoxic stress response is a cellular reaction to toxic agents that is initiated by modification or damage to the functional center of the 28S ribosomal RNA. This response is characterized by three features: (1) interference with translational elongation, (2) interference with enzymatic activities of the 28S ribosomal RNA (aminoacyl-tRNA, peptidyl transfer, or ribosomal translocation), and (3) activation of stress kinases [58]. UV irradiation provokes all of these features, presumably elicited by UV-mediated damage to the 28S ribosomal RNA. Lesions in the ribosomal RNA after UV irradiation resemble UV-induced photoproducts in the DNA, and they likewise involve adjacent pyrimidine nucleotides [59]. UV-induced inhibition of translational elongation results in activation of JNK-1 and p38 [60].

Other Signaling-Initiating Principles

Signaling initiated at the cell periphery can also be based on the activation of voltage-gated K^+ ion channels. Changes in K^+ channel activity are normally used to stabilize the membrane potential and maintain the salt and water balance in the cell. Accordingly, channels are regulated by growth factors, but they also respond to a variety of chemical and physical stresses. By using the patch-clamp technique, UVC irradiation as low as 45 J/m^2 was found to increase the K^+ current within seconds by an as yet unknown mechanism [61]. When K^+ channel activity was suppressed by specific inhibitors, UV-dependent activation of MKK-4 and JNK was ablated and apoptosis largely abrogated, at least in myeloblastic cells. The degree of JNK inhibition correlated with the potency of the K^+ channel inhibitors [61].

UV and ionizing irradiation also enhance phospholipid turnover in the plasma membrane and stimulate the generation of diacylglycerol (DAG) and ceramide. Both DAG and

ceramide are potent activators of PKC, and several isoforms of PKC are activated in response to UV and ionizing irradiation [62,63]. Ionizing irradiation-mediated JNK activation was furthermore prevented by induced PKC degradation or by treating cells with the kinase inhibitor H7, implying that induction of JNK in response to IR depends at least partially on PKC [64].

Conclusions

Radiation affects the function of numerous macromolecules. Among these are components that result in elevated signaling and subsequent changes in gene expression. Extranuclear signals are predominantly generated by the inactivation of negative control elements, for example, PTPs. They are, however, supplemented by stimuli generated by nuclear DNA damage. How nuclear DNA damage is converted into signal transduction is as yet not well understood. The final phenotype of irradiated cells results from the mix of stimuli—radiation-induced intranuclear and extranuclear signals and physiological stimuli—that address cells at the same time. Radiation of cells induces apoptosis, triggered either through MAP-kinases, or through DNA damage and p53, or through direct Fas (CD95) activation. Modulated by the sum of all stimuli, radiation can act as a tumor promoter, is mitogenic, or induces differentiation apoptosis and senescence.

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CHAPTER 309

Endoplasmic Reticulum Stress Responses

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Introduction

Eukaryotic cells must adapt to wide variation in the load of client proteins that their endoplasmic reticulum (ER) must process. They do so by modulating the synthesis of client proteins and the expression of genes whose products determine the folding capacity of the organelle. The signaling pathways activated to effect these adaptations are part of the unfolded protein response (UPR), which is similar in many ways to the cytoplasmic heat-shock response. In mammalian cells, signaling is initiated by three known ER resident transmembrane proteins (IRE1, ATF6, and PERK) that can sense the stress caused by imbalance between capacity and demand in the ER. IRE1 and PERK are transmembrane protein kinases whose activity is repressed by free chaperones in the ER lumen.

During ER stress, as free chaperones are recruited to serve client proteins, these transducers are activated, resulting in downstream signaling. IRE1 effector functions include endonucleolytic processing of a substrate mRNA (HAC1 in yeast and XBP-1 in metazoans) and activation of JUN N-terminal kinase, resulting in activated gene expression. PERK phosphorylates the translation initiation factor eIF2 and mediates both translational repression of client protein biosynthesis and activation of specific mRNA translation. The latter culminates in increased expression of the transcription factor ATF4 and activates a gene expression program that is also accessed by other forms of stress and is referred to as the *integrated stress response.* ATF6 activation is mediated by proteolytic processing and liberation of an active N-terminal protein fragment that serves as a transcription factor for activating gene expression in the UPR.

ER Stress Defined

Proteins destined for secretion and membrane insertion are synthesized on ribosomes associated with the endoplasmic reticulum. Such client proteins of the ER are translocated into the organelle's lumen where they undergo posttranslational modifications specific to that environment. Chaperone proteins specific to the ER assist in the folding and posttranslational processing of the client proteins, and quality-control mechanisms operating within the organelle clear properly folded client proteins for transport to the Golgi apparatus or earmark others for degradation by the ER associated degradation apparatus (ERAD).

The ER is thus a dynamic cellular compartment whose activity level varies among different cell types and between physiological states. In complex metazoans it is most developed in cells devoted to secretion, such as those of the exocrine and endocrine glands, immunoglobulin-secreting plasma cells, and cells with extensive membranous processes such as neurons and myelin-producing glial cells. In other words, the quantity and intrinsic properties of client proteins synthesized in response to developmental programs and physiological states set the level of *demand* made of the ER. The ability of the ER to *supply* this demand is impacted by the size of the organelle, by the complement of chaperones and other intrinsic protein and lipid components at its disposal, and by the physiological state of the cell. The latter is particularly important in pathological states associated with nutrient limitation, hypoxia, or toxins that degrade the ER's ability to process client proteins (reviewed in [1–3]).

Specific signaling pathways are activated by an imbalance between the demand on the ER and the ability of the organelle

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to meet the demand, so-called "ER stress." Activation of these pathways occurs under both pathological conditions and in response to normal physiological demands. However, much of what we have learned about them is derived from experiments in which cells were treated with toxins, for example, tunicamycin, an inhibitor of N-linked glycosylation. Because such toxins cause ER stress by interfering with protein folding, the signaling pathways that they activate came to be known as the *unfolded protein response* (UPR) [4,5]. This is a somewhat misleading term; because the pathways in question appear to be activated by the threat of malfolded proteins and do not necessarily require the accumulation of measurable amounts of malfolded proteins.

The UPR in Yeast

The UPR was first studied in yeast where treatment with tunicamycin or mutations that affect protein folding in the ER were noted to activate transcription of the *KAR2* gene encoding the yeast homolog of the mammalian ER chaperone BiP [6,7]. Genetic analysis of signaling from the yeast ER to the *KAR2* promoter unveiled a truly novel pathway. Signaling in response to ER stress is initiated by a type 1 ER resident transmembrane protein encoded by the *IRE1* gene [8,9]. The N-terminal lumenal domain of Ire1p responds to the ER stress signal and the signal is transmitted to the C-terminal, cytoplasmic effector domain in which two distinct activities reside: a protein kinase activity for which the only known substrate is Ire1p itself [8,9] and, remarkably, an endonuclease activity that removes an intron from the *HAC1* mRNA [10–12]. Ire1p activation thus entails transautophosphorylation that unmasks the endonucleolytic activity [13]. The cleaved ends of the *HAC1* mRNA are joined by tRNA ligase to generate an activated, spliced form of the *HAC1* mRNA [14]. The unspliced *HAC1* mRNA is poorly translated, whereas the protein encoded by the spliced form is well expressed. Thus, noncanonical splicing controls expression of Hac1p, a transcription factor that activates downstream target genes of the yeast UPR [15,11].

In yeast the IRE1->HAC1 pathway is a simple linear one that is required for activation of all known target genes of the UPR. These have been the subjects of detailed analysis by expression microarrays. Surprisingly, the genes induced by the UPR in yeast include not only the expected ER chaperones but also components of the apparatus for degrading ER proteins, the machinery required for membrane biosynthesis and non-ER components of the secretory apparatus [16,17]. The yeast UPR, therefore, appears to up-regulate the capacity of the entire endomembrane system to deal with client proteins. In this context the UPR should be viewed as a pathway concerned both with adapting to stress in the short term and with growth and anabolism [18]. It is also clear, however, that the ability to up-regulate target genes of the UPR is an important adaptation, because yeast defective in this function are hypersensitive to ER stress [16,17,19].

IRE1 proteins are conserved in higher eukaryotes [20,21] and their mechanism of action appears to be conserved too (see later discussion), however their preeminent role in regulating all aspects of the UPR has been superseded by new components that are lacking in yeast. One important difference between higher eukaryotes and yeast is that the former rapidly and reversibly attenuate protein biosynthesis in response to ER stress. This adaptation decreases the quantity of client proteins that are delivered to the stressed ER and reduces the demand on the organelle.

The UPR is Metazoans

Translation control by ER stress is mediated by PERK (also known as PEK or EIF2AK3), a type 1 transmembrane ER resident protein kinase related to IRE1 in its stress-sensing lumenal domain. The C-terminal, cytoplasmic effector domain of PERK is a protein kinase that phosphorylates the alpha subunit of translation initiation factor 2 [22]. Phosphorylation of eIF2α inhibits nucleotide exchange on the eIF2 complex and blocks the initiation step of translation. An important component of the regulation of PERK and IRE1 activity depends on an interaction between their related N-terminal lumenal domains and the ER chaperone BiP. In unstressed cells, dispensable BiP protein is available to bind the lumenal domains of PERK and IRE1. BiP binding maintains these proteins in a monomeric, inactive state. When the folding reserve of the ER is challenged, either by increased demand or reduced function, levels of dispensable BiP decrease and PERK-BiP and IRE1-BiP complexes dissociate. Freed from the inhibitory effects of BiP binding, PERK and IRE1 oligomerize, transautophosphorylate, and initiate downstream signaling [23,24].

Cells lacking PERK are unable to reduce translation when experiencing ER stress and consequently are exquisitely sensitive to agents and conditions that cause such stress [25]. Humans and mice with *PERK* mutations develop early onset diabetes mellitus caused by destruction of the insulin-producing pancreatic β cells [26,27]. Protein synthesis in β cells is strongly activated by plasma glucose; PERK modulates this increase, ensuring that the quantity of proteins translocated into the ER does not exceed the capacity of the organelle to fold them. In $PERK^{-/-}$ islets one finds cells with distended ER, and the presence of these correlates with high rates of programmed cell death in the mutant islets. It is clear, therefore, that PERK signaling is activated under normal physiological circumstances, notably in professional secretory cells.

The hypothesis whereby loss of translational control leads to stuffing of the ER with client proteins goes a long way toward explaining the phenotype of the *PERK* mutation. However, this simple hypothesis does not take into account the role of PERK in regulating gene expression in the UPR. eIF2α phosphorylation decreases the efficiency of translation of most mRNAs; however, some mRNAs are translated more efficiently in such circumstances. The mechanism for

this has been worked out in great detail in the case of the yeast *GCN4* mRNA, which encodes a transcription factor that activates genes involved in amino acid biosynthesis [28]. Translation of the mammalian *ATF4* mRNA is similarly up-regulated by eIF2α phosphorylation [29]. Thus, ATF4 translation during ER stress and up-regulation of its downstream target genes require PERK. Because this signaling pathway is activated both by ER stress and other conditions associated with eIF2α phosphorylation (e.g., amino acid starvation, arsenite treatment), we propose referring to it as the *integrated stress response* (ISR). Preliminary results suggest that the ISR may contribute substantially to cell survival during ER stress and to developmental programs [30].

GADD34 is an interesting target gene of PERK (and the ISR). The encoded protein binds the catalytic subunit of protein phosphatase 1 and recruits it to dephosphorylate eIF2α. Thus, the integrated stress response induces a negative feedback loop that serves to terminate the response [31]. It seems likely that eIF2α dephosphorylation plays a role in the recovery of protein biosynthesis, which, in turn, is required for the translation of mRNAs that are up-regulated as part of the gene expression program activated during the UPR (and other stresses associated with an ISR). According to this model, the first line of defense against ER stress in complex metazoans is translational repression. It is followed, soon thereafter, by remodeling of the internal milieu; the latter process requires new protein synthesis that is favored by GADD34.

Higher eukaryotes possess a third signaling pathway from the ER to the nucleus (Fig. 1), which is mediated in mammals by two ATF6 genes, α and b, encoding very similar type 2 transmembrane ER resident proteins. The N-terminal cytoplasmic portion of these proteins is a bZIP transcription factor that binds and activates ER stress response elements (ERSEs) in the promoter of such classic UPR target genes as BiP and CHOP [32–34]. In unstressed cells ATF6 is maintained in an inactive state by being tethered to the ER membrane. In response to ER stress, the protein is proteolytically cleaved, generating a free N-terminal fragment that migrates to the nucleus and activates transcription through ERSEs [35,33]. This mode of activation resembles that of another ER-tethered bZIP protein, the sterol-regulated transcription factor SREBP. This is more than a superficial resemblance, because the proteases that process and activate SREBP were found to be essential also for the activation of ATF6 [36]. These proteases are found in the Golgi apparatus. It is likely, therefore, that ATF6 activity is controlled by ER retention, and migration of ATF6 to the Golgi apparatus during ER stress likely leads to processing and liberation of the active transcription factor.

The comprehensive analysis of the yeast UPR, carried out by expression microarrays, has not yet been extended to mammalian cells. Consequently, we do not have detailed information on how the different signaling components of the UPR interact to control gene expression in higher eukaryotes. However, based on even the limited information available, it is clear that the mammalian UPR has diverged considerably from that of the yeast. The ATF6 pathway appears to have assumed a major role in controlling chaperone gene expression. This is revealed by the severe defect in *BiP*

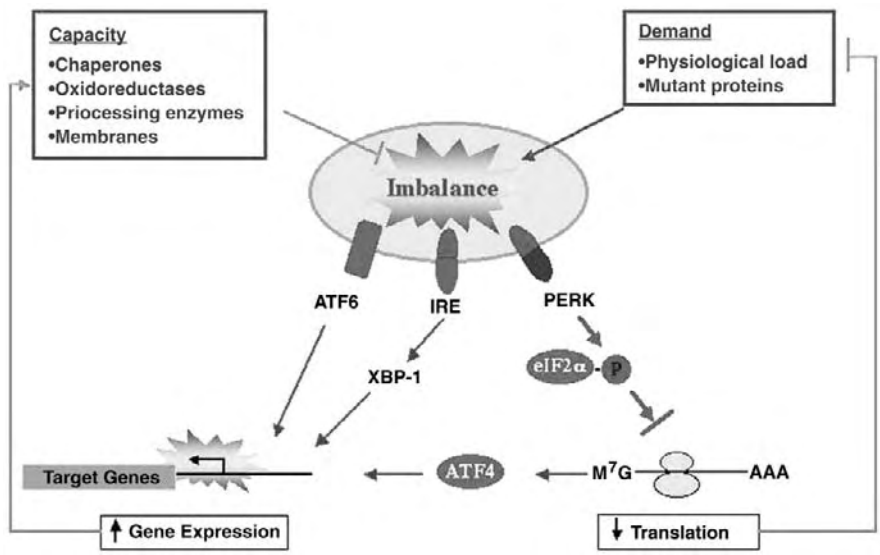


Figure 1 Depiction of the major aspects of the endoplasmic reticulum stress response and its mediators in mammalian cells. An imbalance between the load of client proteins imposed on the ER (demand) and capacity of the ER to fold client proteins is sensed by the three ER stress transducers: ATF6, IRE1, and PERK. These coordinate two functional responses: activation of the expression of genes whose products increase ER folding capacity and inhibition of protein synthesis that attenuated demand on the ER. IRE1 activity is transduced though XBP-1 to activate gene expression, whereas ATF6 directly mediates ER to nuclear signaling. PERK signaling is mediated by eIF2α phosphorylation modifying translation initiation rates. The latter results in a general decrease in demand on the ER and at the same time activates the translation of transcription factors such as ATF4 that contribute to activated gene expression.

expression manifested by cells that lack ATF6-processing protease activity [36]. PERK and the integrated stress response also contribute significantly to gene expression in the UPR [29,30]. Some target genes of the UPR, such as *GADD34* and *CHOP*, are highly dependent on the ISR, whereas other genes, such as *BiP*, are less dependent on PERK signaling and the ISR. IRE1 genes, of which mammals have two, appear to have been diverted from exclusive control of a broad set of UPR genes (as is the case in yeast) toward certain specific cellular functions.

We have found that mouse cells lacking *IRE1* are virtually unimpaired in activating most target genes of the UPR. Furthermore, such mutant cells are not manifestly hypersensitive to pharmacological manipulations that cause ER stress. One measure of the divergence of IRE1 function from yeast to higher eukaryotes is the finding that mammalian IRE1 activates stress-activated protein kinases during ER stress. It does so by recruiting TRAF2 to the ER membrane [37]. The physiological significance of this signaling pathway is not fully understood, but it may link ER stress and programmed cell death. The association between ER stress and programmed cell death is a poorly understood but potentially important subject, particularly in light of recent findings suggesting that ER stress may accompany common pathological conditions [38]. Several signaling pathways leading to CHOP [39], JNK [37], CASPASE-12 [40], and cABL [41] activation may be involved, but the manner in which these are integrated into the UPR remains to be studied in greater detail.

Further clues to the role of mammalian IRE1 have been provided by the identification of a direct downstream target mRNA (analogous to yeast *HAC1*). The *XBP-1* mRNA is processed by mammalian IRE1 in an ER-stress-dependent manner and IRE1 thus controls the expression of this important transcription factor [42–44]. The target genes of XBP-1 have not been identified, but analysis of *XBP-1* knock-out mice reveals its essential role in development of B cells to plasma cells [45]. XBP-1 mediated signaling presumably up-regulates the capacity of the endomembrane system to deal with the load of immunoglobulins that must be secreted by the developing B cell. The finding that XBP-1 is controlled by IRE1 suggests that in mammalian cells the IRE1 genes have specialized to link the load on the ER to certain long-term "developmental" adaptations of the ER. This hypothesis is further supported by the observation that both XBP-1 and IRE1 are enriched in cells that specialize in secretion.

Conclusion

In summary, the mammalian UPR consists of rapid and transient translational repression, which is completely PERK dependent. This short-term adaptation is supplanted by up-regulation of genes that enhance the capacity of ER to deal with increased load. All three known signaling components of the UPR (PERK, IRE1, and ATF6) participate in this latter adaptation. Additional signaling downstream of these and other ER stress responders effects important and poorly understood life and death decisions.

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CHAPTER 310

The Heat-Shock Response: Sensing the Stress of Misfolded Proteins

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Introduction

The heat-shock response is an inducible molecular response to physiological, environmental, and biochemical stress conditions (Fig. 1) that results in the elevated expression of heat-shock genes. These can be classified into four categories: (1) environmental stresses, such as heat shock, amino acid analogs, drugs, oxidative stress, toxic chemicals, heavy metals, and pharmacologically active small molecules; (2) nonstress conditions, such as the cell cycle, growth factors, serum stimulation, development, differentiation, and activation by certain oncogenes; (3) physiological stress and disease states, such as neuroendocrine hormones, tissue injury and repair, fever, inflammation, infection, ischemia and reperfusion, and cancer; and (4) diseases of protein aggregation, such as Huntington's disease, Alzheimer's disease, Parkinson's disease, and ALS. For each of these categories, the various conditions indicated are typically associated with the overexpression of one or more heat-shock proteins through activation of heat-shock factor (HSF) and the heat-shock response.

Common to these stresses are challenges to protein homeostasis that influence folding, translocation, assembly, and degradation events. Consequently, an increased flux of non-native intermediates, if left unprotected, will have an increased propensity to misfold and self-associate to form protein aggregates and other toxic protein species. The heat-shock response, through the elevated synthesis of molecular chaperones and proteases, responds rapidly and precisely to the intensity and duration of specific environmental and physiological stress signals to reestablish protein homeostasis and prevent protein damage [1–4]. Transient exposure to intermediate elevated temperatures or lower levels of chemical and environmental stress has cytoprotective effects against sustained, normally lethal, exposures to stress [5]. This reveals a valuable survival strategy that "a little stress is good."

Transcriptional Regulation of the Heat-Shock Response

The stress-induced regulation of the heat-shock response in vertebrates occurs by activation of a family of heat-shock transcription factors (HSFs) from an inert state under normal steady-state conditions to a transcriptionally competent DNA-binding state [6–9]. Four HSF genes (HSF1–4) are expressed in vertebrates whereas yeast, *Caenorhabditis elegans*, and *Drosophila* encode a single HSF [9–15] with a high degree of conservation in the helix–loop–helix DNA-binding domain [8,16,17], adjacent 80-amino-acid hydrophobic repeat (HR-A/B) necessary for trimer formation [12,18,19], the centrally localized negative regulatory domain, and the carboxyl-terminal transcriptional transactivation domain [20–25].

Stress-induced activation of HSF1 involves a multistep process (Fig. 2) including translocation from the cytosol and nuclear relocalization, oligomerization to a DNA-binding competent state, binding to heat-shock promoter elements, hyperphosphorylation at serine residues, and elevated transcription of heat-shock genes [8,26–32]. These steps can be uncoupled; for example, nonsteroidal anti-inflammatory drugs induce HSF1 trimers that are bound *in vivo* to promoter sites and lack transcriptional activity, yet can be subsequently

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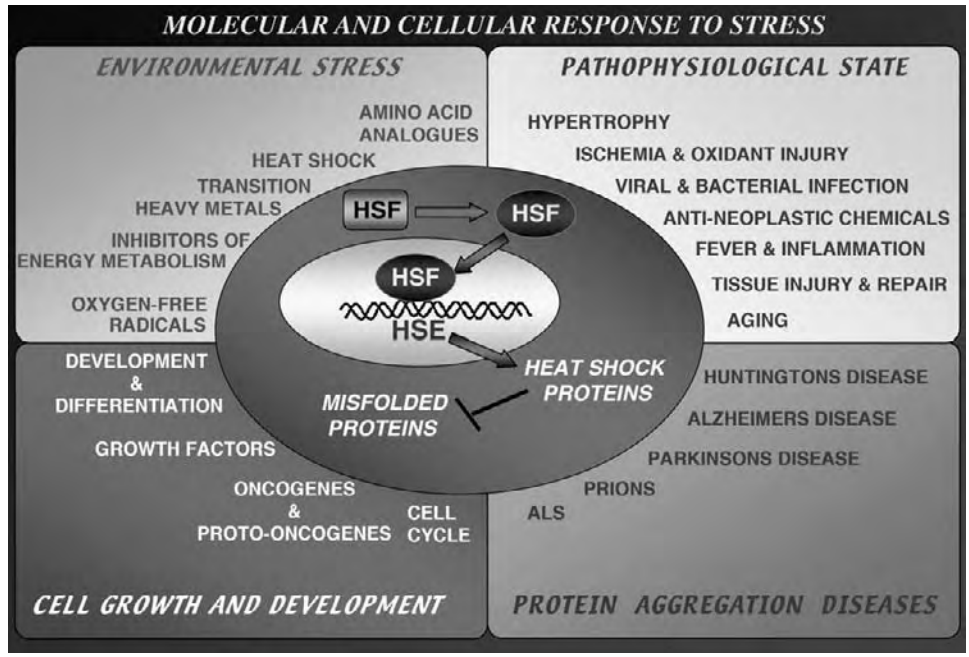


Figure 1 Conditions that activate the heat-shock response. Heat-shock gene expression is induced by environmental and physiological stress, nonstressful conditions including cell growth and development, pathophysiological states, and protein aggregation diseases, that is, conditions that are associated with the appearance of misfolded proteins. Activation of the heat-shock transcription factor leads to the expression of heat-shock proteins, which function to prevent and repair protein damage through interaction with the misfolded proteins.

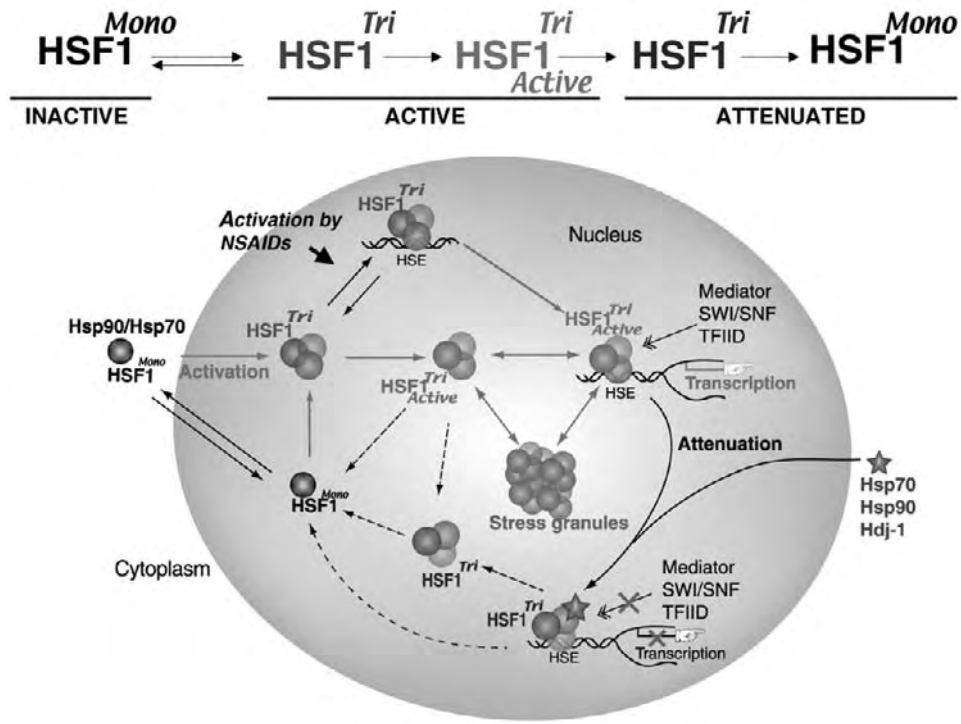


Figure 2 Regulation of the heat-shock response. Activation of HSF1 is linked to the appearance of nonnative proteins and the requirement for molecular chaperones (Hsp90, Hsp70, and Hdj1) to prevent the appearance of misfolded proteins. HSF1 exists in the control state as an inert monomer (shown as intramolecularly negatively regulated for DNA binding and transcriptional activity) and undergoes stepwise activation to a DNA-binding competent state that is transcriptionally inert, acquisition of inducible phosphorylation resulting in complete activation and inducible transcription of heat-shock genes, and attenuation of HSF1 activity. HSF1 activity is negatively regulated by heat-shock factor binding protein 1 (HSBP1), which binds to the region of HSF1 corresponding to the heptad repeat, and by Hsp70 and Hdj1, which bind to the transcriptional transactivation domain thus repressing HSF1 activity.

activated to the fully active HSF1 by subsequent exposures to intermediate levels of stress [33–35]. During continued exposure to heat shock, HSF1 activity attenuates leading to the arrest of heat-shock gene transcription. HSF1 associates with molecular chaperones Hsp70, Hdj1, and Hsp90 during attenuation, and these events correlate with transcriptional arrest, dissociation of HSF1 from DNA, and refolding of the active trimer to the inert monomer [36–42]. Conditional overexpression of Hsp70 is sufficient to inhibit the induction of heat-shock gene transcription with little or no effect on the oligomeric state or level of phosphorylation of HSF1 [38]. These results are also supported by genetic observations that the heat-shock response is autoregulated in the yeast *Saccharomyces cerevisiae*, but interactions of the yeast Hsp70s and HSF [43–46] of HSF1 in a repressed state are delicately balanced and easily disrupted; for example, slight changes in the endogenous levels of HSF1 or expression of point mutants in the negative regulatory domain result in constitutive activity [31,47]. Collectively, these results reveal that multiple chaperones are involved in different aspects of HSF1 regulation and moreover that differences in specificity and kinetics may influence different aspects of the heat-shock transcriptional response.

Another member of the HSF family, HSF2, has been suggested to have a role in heat-shock gene expression during cell growth, differentiation, and upon deregulation of the protein degradative machinery. Activation of HSF2 was first observed in human erythroleukemia (K562) cells exposed to the differentiation agent hemin [48–50], and subsequently detected during murine spermatocyte differentiation [51] and embryogenesis [52,53]. The stress-sensing pathway for HSF2 activation is associated with changes in protein degradation, in a cell-type-independent manner, following exposure to inhibitors of the ubiquitin-dependent proteasome or in cells harboring conditional mutations in components of the proteasome machinery [54]. The function of the ubiquitin-dependent proteasome machinery is to degrade short-lived and misfolded proteins; therefore, the relationship between HSF2 activity and the protein degradative machinery reveals a requirement for heat-shock proteins, perhaps to prevent misfolding and aggregation of non-native proteins targeted for degradation.

Molecular Chaperones: Folding, Misfolding, and the Assembly of Regulatory Complexes

Although some proteins can refold spontaneously, *in vitro*, larger, multidomain proteins often misfold and aggregate. The challenge, *in vivo*, within the densely packed environment of the cell is to ensure that nascent polypeptides fold, translocate, and assemble as multimeric complexes, and that non-native intermediates that accumulate during normal biosynthesis or are enhanced due to mutations or environmental stress are efficiently captured, refolded, or degraded. Molecular chaperones of the Hsp104, Hsp90, Hsp70, Hsp60, and small Hsp class accomplish these activities by capturing non-native intermediates and, together with cochaperones and ATP, facilitate the folded native state (Fig. 3) [3]. The Hsp70 chaperones recognize hydrophobic residue-rich stretches in polypeptides that are transiently exposed in folded intermediates and typically confined to the hydrophobic core in the native state [55]. This contrasts with the Hsp60/GroEL chaperonin, which creates a protected environment with the properties of a "protein-folding test tube" in which non-native proteins undergo rounds of binding and release to acquire the native state [56,57]. Common to these chaperone interactions is their ability to shift the equilibrium of protein folding toward on-pathway events and to minimize the appearance of off-pathway, aggregation-prone species.

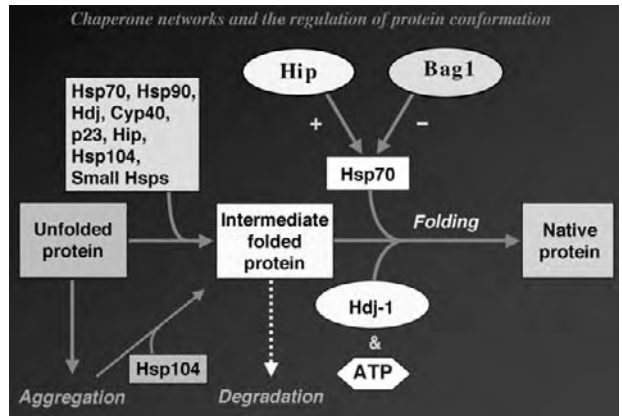


Figure 3 Chaperone networks and the regulation of protein conformation. The biochemical fate of an unfolded protein is schematically presented. In absence of chaperones, unfolded proteins are prone to aggregation. The presence of the molecular chaperones Hsp70, Hsp90, Hdj, Cyp40, p23, Hip, Hsp104, or small Hsps results in an intermediate folded state. The chaperones prevent aggregation or target the unfolded proteins for degradation. Proteins in an intermediate folded state can be refolded to the native state by Hsp70, ATP, and the cochaperone Hdj-1. Refolding can be positively (+) or negatively (–) influenced by Hsp70 cochaperones such as Hip and Bag1.

The family of molecular chaperones is large, diverse in size and apparent structure, yet highly conserved [1,3,58]. Chaperones are abundant in growing cells and can attain concentrations of 5–20% of total cell protein. They are classified according to molecular size, that is, Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small Hsps distributed in all subcellular compartments (Table I). Biochemical studies on chaperones have established common properties of Hsp104, Hsp90, Hsp70, the small Hsps, immunophilins (FKPB52 and CyP40), the steroid aporeceptor protein p23, and Hip (Hsp70 and Hsp90 interacting protein) to prevent the *in vitro* aggregation of model protein substrates and to maintain the substrate in an intermediate folded state competent for subsequent refolding to the native state [59–62]. A distinction among proteins that exhibit the properties of chaperones is that refolding to the native state requires the activity of a specific subset of chaperones such as Hsp90, Hsp70, or Hsp60/GroEL and nucleotide (Fig. 2). Cycles of nucleotide

TABLE I Brief Summary of the Nomenclature, Location, and Function of the Major Heat-Shock Protein and Molecular Chaperone Families

Family	Organism	Chaperones	Location	Functions
HSP 100	*E. coli*	ClpA,B,C	Cytosol	Role in stress tolerance; helps the resolubilization of heat-inactivated proteins from insoluble aggregates
	S. cerevisiae	HSP104	Cytosol	
HSP90	*E. coli*	HtpG	Cytosol	Role in signal transduction (e.g., interaction with steriod hormone receptors, tyrosine kinases, serine/threonine kinases); refolds and maintains proteins *in vitro;* autoregulation of the heat-shock response; role in cell cycle and proliferation
	S. cerevisiae	HSP83	Cytosol	
	Mammals	HSP90	Cytosol	
		GRP94	ER	
HSP70	*E. coli*	DnaK	Cytosol	Roles in lambda phage replication; autoregulation of the heat-shock response; interaction with nascent chain polypeptides; functions in interorganellar transport; roles in signal transduction; refolds and maintains denatured proteins *in vitro;* role in cell cycle and proliferation; anti-apoptotic activity; potential antigen-presenting molecule in tumor cells
	S. cerevisiae	Ssa 1-4	Cytosol	
		Ssb 1,2	Cytosol	
		Kar2	ER	
		Ssc1	Mitochondria	
	Mammals	HSC70	Cytosol/nucleus	
		HSP70	Cytosol/nucleus	
		BIP	ER	
		mHSP70	Mitochondria	
HSP60	*E. coli*	groEL	Cytosol	Refolds and prevents aggregation of denatured proteins *in vitro;* may facilitate protein degradation by acting as a cofactor in proteolytic systems; role in the assembly of bacteriophages and Rubisco (an abundant protein in the chloroplast)
	S. cerevisiae	HSP60	Mitochondria	
	Plants	Cpn60	Chloroplasts	
	Mammals	HSP60	Mitochondria	
HSP40	*E. coli*	dnaJ	Cytosol	Essential co-chaperone activity with HSP70 proteins to enhance rate of ATPase activity and substrate release
	S. cerevisiae	Ydh1	Cytosol/nucleus	
	Malmmals			
Small HSPs	*E. coli*	lbp A and B	Cytosol	Suppresses aggregation and heat inactivation of protein *in vitro;* confers thermotolerance through stabliization of microfilaments; anit-apoptotic activity
	S. cerevisiae	HSP27	Cytosol	
	Mammals	αA and αB-crystallin	Cytosol	
		HSP27	Cytosol	

binding and hydrolysis are regulated by a large family of cochaperones such as p23 or the immunophilins which enhance Hsp90, dnaJ/Hsp40, and Hip, or Bag proteins which associate with Hsp70, or Hsp10/groES which stimulates Hsp60/groEL [59,63,64]. The association of chaperones with different partner proteins thus influences the folded state of the substrate and consequently its activities (Fig. 2).

The ability of a cell to know whether to grow, divide, differentiate, or die depends on extracellular signals and the ability to properly recognize and respond to these signals. The cell may receive these extracellular signals in different forms such as soluble hormones, small peptides, or proteins attached to neighboring cells. Cellular receptors receiving the signals transmit the extracellular information to the nucleus through cascades of protein–protein interactions and biochemical reactions. Chaperones of the Hsp90 and Hsp70 family and their cochaperones have been found to interact with key cell signaling molecules, including intracellular receptors, tyrosine- and serine/threonine kinases, cell cycle regulators, and cell death regulators [42,65–67]. Decreasing the levels of functional Hsp90 in *Drosophila,* by genetic mutation or by treatment with Hsp90 inhibitor geldanamycin, causes developmental abnormalities [68]. Likewise, increasing the levels of Hsp70, by overexpression or upon heat shock, has growth inhibitory effects on mammalian tissue culture cells and in *Drosophila* salivary gland cells, whereas expression of a dominant-negative form of Hsp70 causes developmental defects in *Drosophila* [69–71]. However, what remains less well understood is whether this represents a general strategy of the cell to link specific signaling pathways with cell stress-sensing events.

Interestingly, cells that have lost their ability to properly regulate cell growth, such as tumor cells, often express high levels of multiple heat-shock proteins compared to their normal parental cells [72]. Depletion of Hsp90 by geldanamycin, or of Hsp70 by antisense methodology in transformed cells, but not in their nontransformed counterparts, causes either an arrest of cell growth or progression into cell death [73,74]. Apparently, tumor cells have become dependent on increased levels of Hsps, although the beneficial reasons for this have yet to be clearly established. One possibility is the ability of chaperones to suppress and buffer mutations that accumulate during the transformation process, thus allowing cell viability and even enhanced cell growth of otherwise mutant cells. This is exemplified by the relationship between p53 and Hsp90, where mutant forms of p53, but not wild-type p53, depend on Hsp90 for their normal levels and function [75].

As a consequence of stress and physiological change, the equilibrium between substrates, heat-shock proteins, and cochaperones within a cell may be perturbed, with potentially profound consequences for the cellular phenotype. Changes in the abundance and relative levels of chaperones

and cochaperones could result in novel combinations of heat-shock proteins, which, in turn, could redirect information flow through the intracellular pathways and change the overall response to signals. Whereas some pathways may become favored because of an increase in a particular cochaperone that is specifically required for its regulation, other pathways might be shut down entirely by the lack of critical chaperone components. The overall effect of changes in chaperone or cochaperone levels on cellular and organismal phenotypes will depend on which chaperone or cochaperone is affected. For example, changes in the levels of Hsp90 by exposure of cells or organisms to geldanamycin can have strong pleiotropic effects on development [68,76]. Likewise, the Ras/Raf-1 signaling pathway in mammalian cells is arrested when the levels of Hsp70 increase in response to stress. The inhibition of MAP kinase is due to the sequestration of Bag1, a Raf1 positive effector, by Hsp70, thus disrupting cell growth [71,77].

Altogether, heat-shock proteins have evolved as integrated components of signal transduction networks, in which they can function in the maturation, activation, and inactivation of signaling molecules. Their involvement in a particular pathway within the network is determined by the availability and relative abundance of partner specific cochaperones, which will influence, in a cell-type-specific manner, the natural response to physiological intracellular and extracellular signals. Consequently, we suggest that altered levels of heat-shock proteins and cochaperones in response to stress or disease states alters how organisms integrate and respond to the flow of their normal physiological signals. Future studies in multicellular model systems will help to elucidate with greater detail the molecular basis for the pervasive role of molecular chaperones in organismal development and disease and how they respond to altered chaperone and cochaperone levels associated with fluctuating environmental conditions and disease.

Neurodegenerative Diseases: When Aggregation-Prone Proteins go Awry

Human neurodegenerative disorders appear to be associated with the accumulation of misfolded proteins and the appearance of protein aggregates, fibrils, or plaques. These include inherited disorders caused by CAG/polyglutamine-expansion as occurs in Huntington's disease (HD), Kennedy disease, the spinocerebellar ataxias SCA1, SCA2, MJD (SCA3), SCA6, and SCA7, dentorubral-pallidoluysian atrophy (DPRLA), Alzheimer's disease, and prion disease [78]. A hallmark of these diseases is the expression of abnormal proteins that form a predominant β-sheet conformation that self-associates to form insoluble aggregates in neuronal cells or in the extracellular space [79–81].

Prion diseases from yeast to humans are associated with changes in the folded state of the prion, which correlates with conversion from a noninfective to an infective state. Among the fascinating features of prion diseases is the epigenetic transmission of an altered protein conformation, thus endowing the infectious prion with unique biological properties. The association of chaperones with prion disease has been suggested based on studies in the yeast *S. cerevisiae*. A subunit of a translation termination factor, Sup35p, can form self-seeded fibers in [psi+] strains, in a prion-like manner which is prevented if Hsp104 is deleted or overproduced [82]. Hsp104 can directly interact with the prion-like domain of Sup35p and also with the human prion protein, which is suggestive for a conserved mechanism [83]. The conserved properties of heat-shock proteins and the striking similarity in the characteristics of *in vitro*-formed fibers of Sup35p, polyglutamine repeat, and prion proteins have further strongly suggested the involvement of heat-shock proteins in aggregate formation in other neurodegenerative disorders.

Indeed the heat-shock response and heat-shock proteins have been implicated in these polyglutamine-expansion misfolding diseases. Studies with mammalian tissue culture cells and the nematode *C. elegans* have established that the heat-shock response is activated in cells expressing polyglutamine-expansion-containing proteins [84,85]. Moreover, the colocalization of several heat-shock proteins, including the Hsp40 family members Hdj-1 and Hdj-2, Hsp70, and ubiquitin, with polyglutamine aggregates in mouse tissues and tissue culture cells has suggested a direct relationship between these heat-shock proteins and polyglutamine diseases [86]. Recently, evidence has accumulated that heat-shock proteins could play important roles in cytoprotection based on observations that overexpression of Hdj-1, Hdj-2, or Hsp70 reduces polyglutamine aggregate formation and prevents cellular degeneration [87–89]. These observations offer interesting possibilities to develop therapeutic strategies based on activation of the stress response or selectively increasing the levels of individual chaperones.

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CHAPTER 311

Hypoxia-Mediated Signaling Pathways

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Introduction

Oxygen homeostasis is a tightly regulated process. Disruption of oxygen delivery pathways can lead to cardiovascular disease, cerebrovascular disorders, and chronic pulmonary disease. Within solid tumors, poorly oxygenated regions develop because the tumor vasculature is unable to meet the demands of tumor expansion, and because of aberrant blood vessel formation within the tumor. Numerous clinical studies have documented the presence of low oxygen levels within most solid tumors and correlated the presence of these hypoxic regions with poor clinical outcome [1–4].

Hypoxic cells are relatively resistant to conventional radiation therapy and chemotherapy. When extremely hypoxic cells are irradiated, three times the radiation dose is required to achieve the same level of killing as required under fully aerobic conditions. Furthermore, most hypoxic cells are not actively undergoing cell division, thus impeding the efficacy of conventional chemotherapeutic agents that are targeted to actively dividing cells [5]. Numerous studies have indicated that hypoxia selects for tumors with an increased malignant phenotype by promoting angiogenesis [6], increasing metastases [7], and decreasing apoptotic potential [8].

HIF-1 Signaling

At the molecular level, transcriptional control of hypoxia-regulated genes occurs most prominently through hypoxia-induced factor (HIF-1). Although other transcription factors have also been reported to be activated by hypoxia (AP-1 [9–11], NFκB [12,13], Egr-1 [14,15], and NF-IL6 [16,17]), HIF-1 controls the majority of hypoxia-regulated genes. HIF-1-regulated genes play critical roles in a variety of cellular processes including angiogenesis, energy metabolism, iron homeostasis, vascular regulation, cell proliferation/survival, and erythropoiesis. To date, more than 30 genes have been reported to be transcriptionally regulated by HIF-1 [18]. Analysis of HIF-1 staining from clinical specimens suggests a relationship between HIF-1 protein accumulation, malignant progression, and outcome after therapy [19–22].

HIF-1 is a heterodimer consisting of HIF-1α and the aryl hydrocarbon receptor nuclear translocator (ARNT or HIF-1β). HIF-1 belongs to the basic helix–loop–helix (bHLH) family of transcription factors in which the HLH motifs mediate dimerization and the basic regions bind to DNA. In contrast to the constitutively expressed HIF-1β subunit, HIF-1α is an oxygen-labile protein that becomes stabilized in response to hypoxia, iron chelators, and divalent cations. These inducers of HIF-1α are all related to the metabolism of heme, leading some investigators to postulate that the cellular oxygen sensor is a heme-associated protein [23,24]. To date, no definitive identification of such a protein has been made. Interestingly, under hypoxic conditions, HIF-1a mRNA levels do not change in most cell lines, but HIF-1a protein levels increase [25].

The stability of HIF-1α protein is one of the critical elements in its regulation. HIF-1α interaction with the von Hippel-Lindau tumor suppressor gene product targets it for ubiquitin-mediated proteosomal degradation under normoxic conditions. Under hypoxic conditions, HIF-1α degradation is inhibited, and it translocates to the nucleus where it dimerizes with HIF-1β to form an active HIF-1 complex. The HIF-1 heterodimer then binds to the promoter region of target genes that contain specific recognition sequence(s) known as the hypoxia response element (HRE).

Tumors with mutations in the Von Hippel-Lindau (VHL) gene, such as renal cell carcinomas, exhibit high levels of HIF-1α protein under normoxic conditions. When wild-type

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VHL is reintroduced into these cells, the expression of HIF-1α protein significantly decreases during normoxia. VHL binds to a region in HIF-1α known as the *oxygen dependent degradation domain*. Several groups have reported that HIF-1α binding to VHL is regulated by proline hydroxylation at residue 564. This enzymatic modification is dependent on oxygen and requires iron as a cofactor. Epstein *et al.* [26] demonstrated that HIF-1α posttranslational modification is mediated by a novel prolyl-4-hydroxylase that they have termed HIF-α prolyl-hydroxylase (HIF-PH). Under hypoxic conditions or in the presence of an iron chelator (desferrioxamine, DFO), this interaction is disrupted and HIF-1α is stabilized (Fig. 1).

Direct phosphorylation of HIF-1α also appears to play a role in the regulation of HIF-1 activity. Wang and Semenza [27] were the first to report that HIF-1 was a phosphoprotein. They demonstrated by electrophoretic mobility shift assays that the HIF-1/DNA complex could be disrupted by treating nuclear extracts of hypoxic cells with a phosphatase [27]. More recently, Suzuki and coworkers [28] reported that the phosphorylated form of HIF-1α is the predominant form found to associate with HIF-1β, whereas the dephosphorylated form of HIF-1α mediates binding to p53. Although the phosphorylation sites for HIF-1α are not known, these investigators correlated dephosphorylation of HIF-1α with increased apoptosis. These studies suggest that HIF-1α may have different cellular functions depending on its phosphorylation status.

The most widely studied downstream target gene of HIF-1 is vascular endothelial growth factor (VEGF), a potent angiogenic factor that is involved in the progression of a variety of cancers. Transcriptional regulation of VEGF is mediated by binding of HIF-1 to an HRE within the VEGF promoter [29]. In addition, under hypoxic conditions when cap-dependent translation may be limited, several groups have reported indirect evidence that VEGF gene induction may be mediated by an internal ribosome entry site mechanism to maintain a high level of gene expression during stress conditions [30,31].

Several lines of evidence suggest that the PI3-kinase/Akt pathway is involved in hypoxia-regulated VEGF expression and that the tumor suppressor gene PTEN can function as a negative regulator of the PI3-kinase/HIF pathway (Fig. 2). PTEN is mutated or inactivated in a large percentage of glioblastomas [32] and direct measurement of pO_2 with the Eppendorf microelectrode within these tumors demonstrates that they are hypoxic relative to normal tissue. Using PTEN-deficient glioblastoma cell lines, Zundel *et al.* [33] demonstrated that PTEN inhibits HIF-1 activation and VEGF induction by hypoxia to the same extent as wortmannin, a potent PI3-kinase inhibitor. Interestingly, PTEN overexpression inhibited the hypoxia-stimulated accumulation of HIF-1α, indicating that PTEN regulates growth factor signaling to HIF-1. Similar observations have been made in other cell types such as human breast cancer cells [34,35] and human prostate cancer cells [36,37]. These observations suggest that tumor cells deficient in PTEN tend to have elevated HIF-1 activity and thus increased production of pro-angiogenic factors.

Downstream of PTEN, Mazure and colleagues [38] demonstrated that in *ras*-transformed cells, transcriptional induction of VEGF is mediated by HIF-1 and occurs through the activation of the PI3-kinase/Akt pathway. Furthermore, the PI3-kinase/Akt pathway preferentially regulates HIF-1α without apparent effect on HIF-2α [34] or HIF-1β [37]. However, another report [39] indicated that the effect of the PI3-kinase/Akt pathway on hypoxia-induced activation of HIF-1α may be cell-type specific. For example, in some cell types (HepG2 and HEK293T), no correlation is seen between the activation of the PI3-kinase/Akt pathway and HIF-1α activity during hypoxia. Taken together, these seemingly inconsistent findings suggest that multiple and perhaps redundant regulatory mechanisms modulate HIF-1α activity

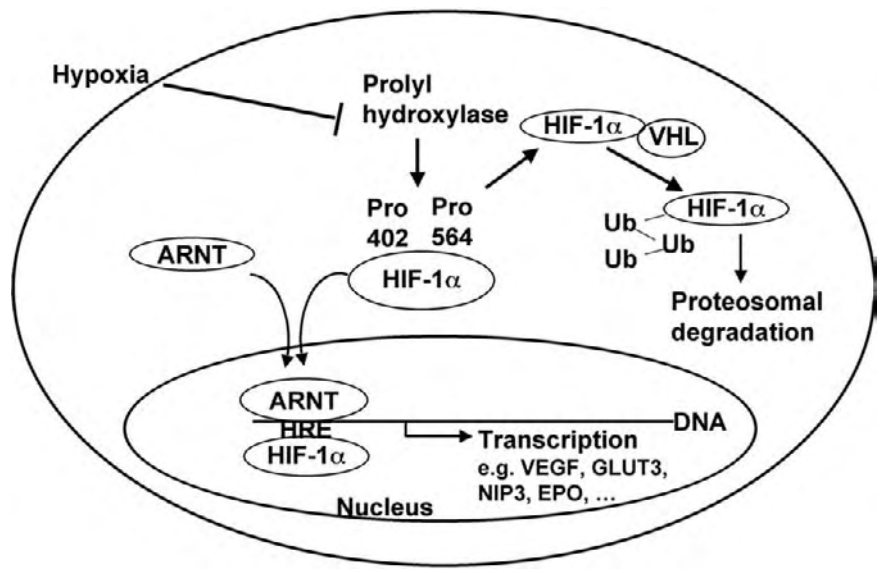


Figure 1 Diagram depicting our current understanding of HIF-1 regulation.

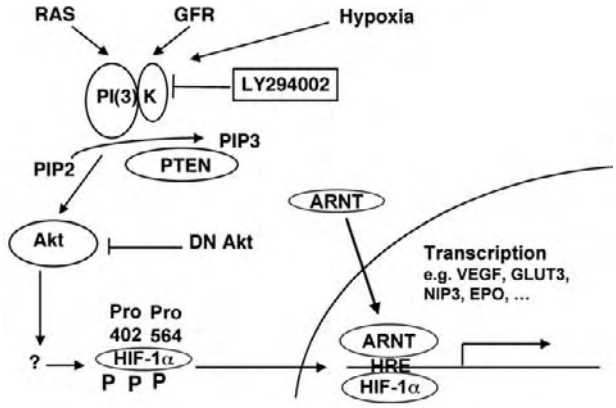


Figure 2 Involvement of a growth factor receptor/PI3-kinase/Akt pathway in HIF-1 regulation. In this figure, an activated growth factor receptor (GFR) binds the lipid kinase PI3K and results in the activation of Akt. Akt itself does not phosphorylate HIF-1α but through a presumed intermediate kinase results in HIF-1α phosphorylation (P). The phosphorylated form of HIF-1α then binds to ARNT and activates transcription.

during hypoxia. Therefore, it will be important to delineate specific pathways leading to the modulation of HIF-1α activities under hypoxia in any given cell type.

Involvement of the Raf-1/ERK pathway has also been implicated in the hypoxic induction of VEGF. With the use of dominant negatives of c-*src* and Raf-1 in 293 cells, Mukhopadhyay and coworkers [40] inhibited VEGF induction by hypoxia. However, this observation may also be dependent on the cell type, because other investigators do not report involvement of the Raf-1/ERK signaling cascade in hypoxia-mediated VEGF induction in *ras*-transformed NIH3T3 cells [38]. Similarly, Gleadle and colleagues [41] did not observe any modulation of HIF-1 DNA binding or downstream effector gene activation (GLUT-1, VEGF) using *src* dominant negatives or $src^{-/-}$ fibroblasts. More recently, however, Pal *et al.* [42] demonstrated increased c-*src* activity in breast cancer cells after exposure to hypoxia and inhibition of VEGF induction with exposure to antisense *src*. This area remains controversial and further work will help to delineate the role of *src* in the regulation of HIF-1/VEGF.

Unfolded Protein Response

Although HIF-1 is the major transcriptional regulator of the hypoxic stress response, during exposure to chronic hypoxia, other non-HIF-1 signaling pathways are also activated. For example, induction of the glucose regulated proteins (GRP78, GRP94, GRP170, GRP58) protects cells against hypoxia and other environmental stresses. The function of these endoplasmic reticulum (ER) resident proteins is to aid in protein folding, trafficking, and secretion. These chaperone proteins are induced during ER, stress in some cell types to help maintain proteins in their native conformation. The process, referred to as the *unfolded protein response* (UPR), involves the activation of a signaling cascade from the ER to the nucleus. Although this pathway has been well studied in yeast, many of the mammalian signaling pathways leading to the UPR have only recently been elucidated.

With the use of microarray technology, Travers and colleagues [43] have characterized global transcriptional changes during exposure to UPR-inducing agents in yeast. In this study, they found that the genes responsible for the yeast UPR were involved not only in protein folding in the ER but also in regulating secretion. They concluded that activation of the UPR results in a complete remodeling of the secretory pathway to minimize the accumulation of unfolded proteins in the ER [43]. Some of the mammalian orthologs to these yeast genes are known and characterization of the mammalian response to ER stress is an area of active investigation.

In yeast, the Ire1p and Hac1p genes are essential elements of the UPR. Ire1p, an ER transmembrane protein, has been implicated as a sensor for the accumulation of unfolded proteins. The exact role of Ire1p in the UPR is unknown, but in response to ER stress, Ire1p is autophosphorylated, leading to activation of its endonuclease activity. Activated Ire1p cleaves a 252-nucleotide inhibitory intron from HAC1 mRNA, resulting in an efficiently translated protein that up-regulates transcription of ER chaperone proteins [44,45].

The mammalian orthologs of Ire1 were identified as Ire1α and Ire1β [46,47]. Ire1α is ubiquitously expressed and Ire1β is expressed predominantly in the gut. Two different groups have independently shown that Ire1α endonuclease activity is required for proper processing of XBP1 mRNA, which results in transcriptional activation of the UPR [48,49]. Furthermore, during ER stress, ATF6 cleavage is mediated by site-2 protease (S2P), releasing a transcriptionally active 50-kDa protein. UPR target genes are then up-regulated by these transcription factors.

Analysis of mammalian GRP promoters revealed a novel consensus sequence responsible for the induction of these genes. This element, designated as the ER stress response element (ERSE), consists of $CCAATN_9CCACG$ [50]. Previously, Li *et al.* [51] showed that the CCAAT portion of the ERSE is constitutively occupied, and later studies by Yoshida and coworkers [52] identified the general transcription factor NF-Y (also known as CBF) to bind constitutively to this portion of the ERSE. Activated transcription factor 6 (ATF6) and X-box binding protein 1 (XBP1) were identified in a yeast 1 hybrid screen as two inducible transcription factors that can bind to the ERSE [50]. The transcriptional activity of activated ATF6 is enhanced by interactions with TFII-I [53], NF-Y [54], and YY1 [54].

The current model proposes that in response to ER stress, the cytoplasmic domain of ATF6 is cleaved, translocates to the nucleus, binds to the ERSE, and promotes the induction of UPR target genes. XBP1 also contains an ERSE in its promoter and is regulated by ATF6 activation, which ultimately leads to increased transcription of XBP1 mRNA (Fig. 3). During normal growth conditions, unspliced XBP1 mRNA is produced at low levels. However, during ER stress, XBP1 mRNA is spliced and the spliced form of XBP1 enhances transcription of ERSE-regulated

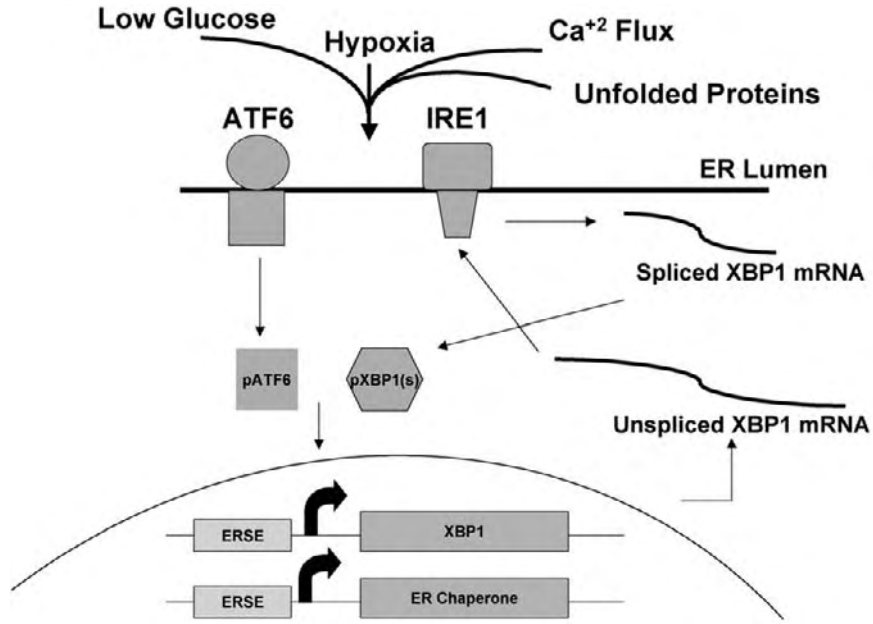


Figure 3 Model depicting the relationship between hypoxia and ER stress.

ER chaperone proteins. XBP-1 is postulated to be responsible for maintaining a prolonged UPR during exposure to chronic ER stress [48].

Song and coworkers [55] reported that GRP78 induction by hypoxia is mediated through a protein kinase C (PKC) epsilon/ERK/AP-1 signaling cascade. They showed that transfection with a dominant negative of PKC epsilon blocks the hypoxia-regulated signaling pathway leading to GRP78 production. GRP78 induction by hypoxia can also be blocked by pretreating cells with PKC inhibitors such as staurosporine. Furthermore, as evidenced by gel mobility shift assays, hypoxia activated the binding of a protein complex to a specific sequence in the promoter region of GRP78 containing the ERSE, and this binding was abolished in the presence of PKC inhibitors [56]. These results suggest that PKC mediates a portion of the signaling cascade, leading to induction of GRP78 during hypoxia. ER stressors such as glucose deprivation, protein accumulation, glycosylation inhibition, and calcium flux are all initiating agents of the UPR. Similarly, hypoxia is another stress that can illicit the UPR through similar and overlapping pathways. The chaperone proteins appear to serve a protective function during ER stress. Experiments have shown that antisense inhibition of GRP78 during hypoxia results in decreased cell survival under these conditions [57]. Another important function of the ER chaperone proteins was demonstrated by Ozawa and colleagues [58], who showed that antisense inhibition of oxygen-regulated protein (ORP150), also known as glucose-regulated protein 170 (GRP170), resulted in intracellular accumulation of VEGF within the ER and decreased VEGF secretion. These results suggest that the chaperone proteins may be an important component of angiogenesis.

Conclusions

Much progress has been made in characterizing the signaling pathways that are activated during hypoxia; however, many questions remain unanswered. Ultimately, by understanding the signaling pathways that are regulated by hypoxia, novel anticancer therapies may be developed to selectively target hypoxic tumor cells.

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CHAPTER 312

Regulation of mRNA Turnover by Cellular Stress

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Introduction

Changes in gene expression occurring in response to stressful stimuli are controlled at multiple levels, through a variety of regulatory mechanisms. Although we have extensive knowledge of the transcriptional mechanisms involved in altering gene expression, the posttranscriptional regulatory events are poorly understood. Alterations in mRNA stability constitute a major mechanism of posttranscriptional gene regulation, effecting rapid changes in gene expression (reviewed in [1–3]). In mammalian cells, the stability of mRNA fluctuates widely, ranging from 20 min to 24 hr [4]. In the absence of altered gene transcription, even small differences in half-life provide a very effective means of dramatically altering the abundance of a given mRNA and consequently the amount of protein expressed [2]. Different types of mRNA turnover have been identified, some promoting mRNA stability, others enhancing mRNA degradation. Among the genes whose expression is regulated through altered mRNA turnover are many encoding T-cell activation molecules, cell cycle regulators, cell survival proteins, and stress-response factors. Given their pivotal role in many physiological and pathological conditions, including conditions of stress, there is increasing interest in understanding the mechanisms that govern mRNA stability and mRNA degradation.

Messenger mRNA stabilization is indeed emerging as a major mechanism of *coordinate* regulation of stress-response gene expression. For example, the growth arrest and DNA-damage-inducible (*gadd*) genes are synchronously up-regulated in response to genotoxic stress, at least in part, through mRNA stabilization [5]; likewise, mRNAs encoding immediate-early genes (c-fos, c-myc, c-jun, IκB, etc.) are coordinately stabilized following irradiation with short-wavelength ultraviolet light [6]. Although the precise mechanisms determining mRNA turnover during stress are poorly understood, they are generally believed to involve RNA-binding proteins recognizing specific RNA sequences [7] (Fig. 1). During the past two decades some of the *cis* elements (RNA sequences) and *trans*-acting factors (participating proteins) involved in these processes have been described. However, the specific stress-triggered events that regulate the process of mRNA turnover are only now beginning to be characterized. In this chapter, I focus on our current understanding of how stress-triggered signaling pathways influence mRNA stability and regulate the expression of labile mRNAs.

mRNA Stability

Cis Elements Regulating mRNA Turnover

Certain mRNA stability-promoting structures are ubiquitous, like the 5′ *end cap*, a 7 methylguanosine triphosphate (m^7GpppG) structure that protects messages from general 5′→3′ exonucleases, and the 3′ *end polyadenylate* or *poly(A) tail*, which protects the 3′ terminus from degradation by 3′→5′ exonucleases. Other mRNA stability determinants are specific and they can be found throughout the 5′ untranslated region (UTR), as described for interleukin 2 (IL-2) mRNA [8], and the coding region, as shown for the c-fos

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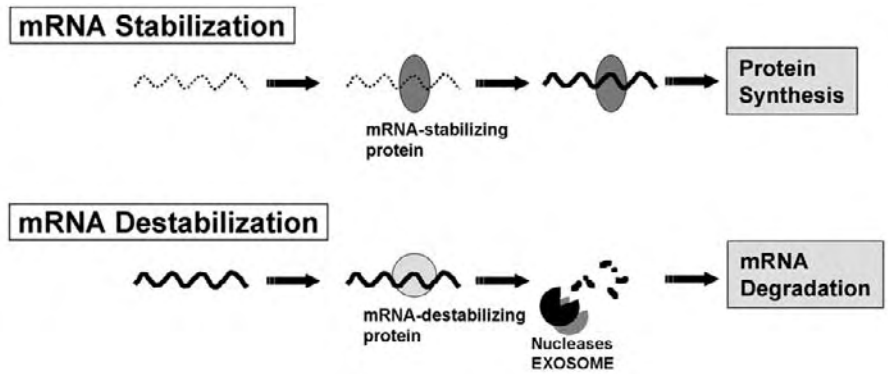


Figure 1 Schematic of mRNA Turnover. *mRNA Stabilization*: Labile mRNAs can be stabilized through mechanisms involving their association with proteins that promote enhanced transcript integrity. Examples include mRNAs encoding p21, VEGF, c-Fos, TNFα, and several interleukins. *mRNA Destabilization*: Labile mRNAs can be destabilized through mechanisms involving their association with proteins that promote transcript degradation, including RNases. Examples of mRNAs whose stability can decrease in a regulated manner include cyclin D1, cyclooxygenase-2, and APP.

and c-myc mRNAs [9,10]. However, the majority of specific regulatory sequences reside within the 3′UTR. The best understood examples of 3′UTR-mediated stability are the IRE (iron-responsive element), which confers stability to transferrin mRNA, and the ARE (AU-rich elements) present in the 3′UTR (and occasionally the 5′UTR) of many mRNAs, such as those encoding cytokines (interferons, interleukins, tumor necrosis factor [TNF] α), cell cycle regulatory proteins (p21, cyclin A, cyclin B1, cdc25), growth factors (GM-CSF, VEGF), and oncoproteins (c-fos, c-myc) [11–13]. AREs consist of one or several copies of the pentamer AUUUA, generally within a U-rich sequence.

Trans Factors Regulating mRNA Turnover

Many RNA-binding proteins have been described that selectively recognize and bind to AU-rich regions of labile mRNAs, including AU-A, AU-B, AU-C, AUBF, AUF1 (hnRNP D), AUH, GAPDH, Hel-N1 (HuB), hnRNP A0, hnRNP A1, hnRNP C, HuR (HuA), tristetraprolin, and TIAR [14–25]. Among these, the influence of HuR and AuF1 on the stability of ARE-containing mRNAs has been studied most extensively.

HuR is an ubiquitously expressed member of the *elav* family of RNA-binding proteins, which also comprises the neuronal-specific proteins HuD, HuC, and Hel-N1 [23,26–29]. Elav proteins contain three RNA recognition motifs (RRM) and bind with high affinity and specificity to AREs in a variety of mRNAs, among which are those encoding VEGF, p21, Id, cyclin A, cyclin B1, GLUT-1, and c-Fos [20,26,30–37]. Although HuR is believed to increase the stability and/or translation of target mRNAs, the precise mechanism(s) regulating HuR function remain largely unknown. HuR can shuttle between the nucleus and the cytoplasm through a motif named HNS [38]. Because HuR is predominantly localized in the nucleus of resting cells, the stabilizing influence of HuR is believed to require its translocation to the cytoplasm [38–40].

HuR's mRNA stabilizing effect is illustrated in two recent studies from our laboratory. Exposure of cells to short-wavelength ultraviolet light (UVC) resulted in stabilization of the p21 mRNA, increased HuR abundance in the cytoplasm, and increased binding of HuR to the p21 ARE [41,42]. In another study, HuR was shown to stabilize target mRNAs encoding cyclin A and cyclin B1. In synchronous cultures, HuR was found to be almost exclusively nuclear during early G_1 phase, but increased in the cytoplasm during the S and G_2 phases. This cell-cycle-dependent HuR localization correlated closely with HuR's increased binding to, and stabilization of, target mRNAs encoding cyclin A and cyclin B1 [43]. In both studies, cells expressing reduced HuR levels exhibited decreased HuR binding to target transcripts and diminished half-lives for the corresponding mRNAs.

AUF1 consists of four alternatively spliced isoforms: p37, p40, p42, and p45 [44]. All isoforms contain two RRMs and can be found in both the nucleus and the cytoplasm [18,45]. Overexpression of AUF1 accelerates the decay of ARE-containing mRNAs [46–49], although the specific pathways and/or cellular events that regulate AUF1 activity remain to be defined. We recently reported that prostaglandin A_2 (PGA_2), a model stress agent and experimental chemotherapeutic compound capable of eliciting a potent inhibition of cell growth, decreased cyclin D1 expression by rendering the cyclin D1 mRNA unstable. PGA_2 treatment also increased the association of AUF1 with an instability element within the cyclin D1 3′UTR, suggesting that AUF1 may participate in the degradation of cyclin D1 mRNA [50,51].

Generally speaking, the RNA sequence requirements for recognition by a given RNA-binding protein appear to be substantially more flexible than those governing, for example, transcription factor–DNA interactions. A given RNA-binding protein can bind to a collection of RNA substrates of related sequences with varying relative affinities, at least *in vitro*. Conversely, a given mRNA sequence can be the target of many RNA-binding proteins *in vitro*. These observations have led several groups to postulate that the half-life of a

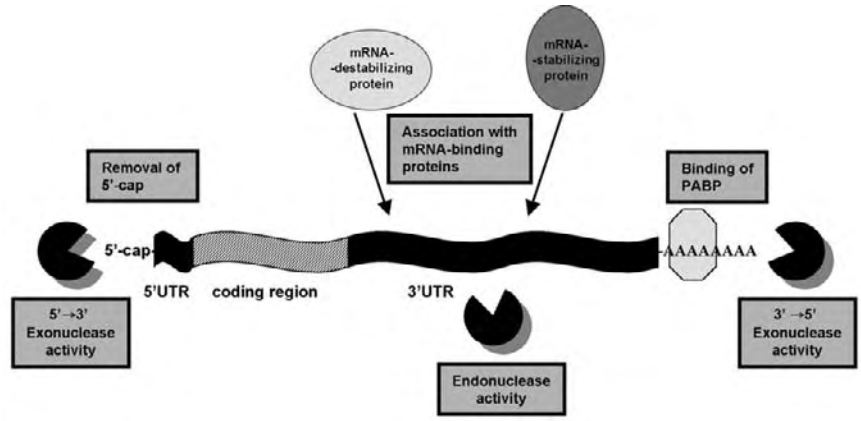


Figure 2 Potential levels of regulation of mRNA turnover. mRNA turnover can be influenced by a number of events, such as the enzymatic action of endonucleases and exonucleases, the association of PABP with the transcript's poly(A) tail, the cleavage of the 5′ cap, and the formation of complexes with RNA-binding proteins that either enhance or reduce transcript stability. Each of these regulatory mechanisms, indicated in shaded squares, is a potential target of stress-modulated events.

given labile mRNA is likely to reflect the *net* effect of its interaction with RNA-binding proteins that promote stabilization and with RNA-binding proteins that promote degradation. Although this hypothesis remains to be proven experimentally, we anticipate that the relative association of proteins that alter mRNA turnover will depend on whether cells are proliferating or not, and on the type of stress to which they are exposed.

Models of Regulation of mRNA Turnover

Although we have a considerable amount of information concerning the *cis* and *trans* determinants of mRNA turnover, we know relatively little about the different levels at which mRNA stability can be regulated. For example, the 5′ end cap that renders the mRNA resistant to general 5′→3′ exonucleases can be removed in a potentially regulatable fashion by a decapping nuclease (DCP1), an enzyme activity that has been purified from yeast and mammalian cells. The 3′ poly(A) tracts can be bound, and therefore protected, by a poly(A) RNA-binding protein (PABP), with the steps leading to poly(A) removal also being subject to regulation. Moreover, poly(A) shortening appears to be a rate-limiting step in the decay of many mammalian mRNAs. Nevertheless, regulation of the turnover of specific mRNAs is largely exerted through AREs. Among the plausible mechanisms whereby associating RNA-binding proteins, with target AREs (or with other stability-influencing mRNA sequences) can affect mRNA turnover are the following: (1) RNA-binding proteins may mask/unmask sites of endonucleolytic cleavage; (2) they may enhance or reduce the activity of potential decapping nucleases; (3) they may elevate or diminish the protective influence of PABPs; (4) they may direct mRNAs to other subcellular locations, such as polysomes, which can also influence mRNA translation and turnover, although the precise influence that these two processes exert on each other remains controversial; and (5) they may target given mRNAs to the *exosome*, a cellular complex of proteins with exonuclease activities (similar to the proteasome, but specialized in degradation of nucleic acid). Such possible regulatory events are shown in Fig. 2.

These possible mechanisms remain to be investigated in depth, but two general mRNA turnover pathways have been described. *Deadenylation-independent mRNA decay* is initiated through the endonucleolytic cleavage of a mRNA, followed by subsequent exonuclease digestion of each fragment through the combined action of 5′→3′ and 3′→5′ exonucleases. *Deadenylation-dependent decay*, believed to be the major pathway, is initiated by the removal of the poly(A) tract, followed by decapping and degradation by both 5′→3′ and 3′→5′ exonucleases. This is the pathway of degradation of most ARE-containing mRNAs. For a review on this topic, see [52].

Stress-Activated Signaling Molecules that Regulate mRNA Turnover

Only a limited number of studies reported to date have addressed specifically how stress stimuli and stress-triggered signaling influence mRNA turnover. However, the available evidence provides support for the notion that mRNA turnover is regulated through mechanisms akin to those controlling gene transcription, that is, by triggering signal transduction pathways that involve phosphorylation cascades. I next discuss our current knowledge of stress-triggered signaling events that regulate mRNA turnover, including those altering the levels of mRNA-binding proteins, their association with target mRNAs, and the degradation rates of labile mRNAs. Importantly, mRNA turnover changes occurring during other cellular processes, such as T-cell activation, neoplastic transformation, and cell cycle progression, may be regulated through the very same stress- and/or growth factor-activated pathways discussed here. A schematic representation of

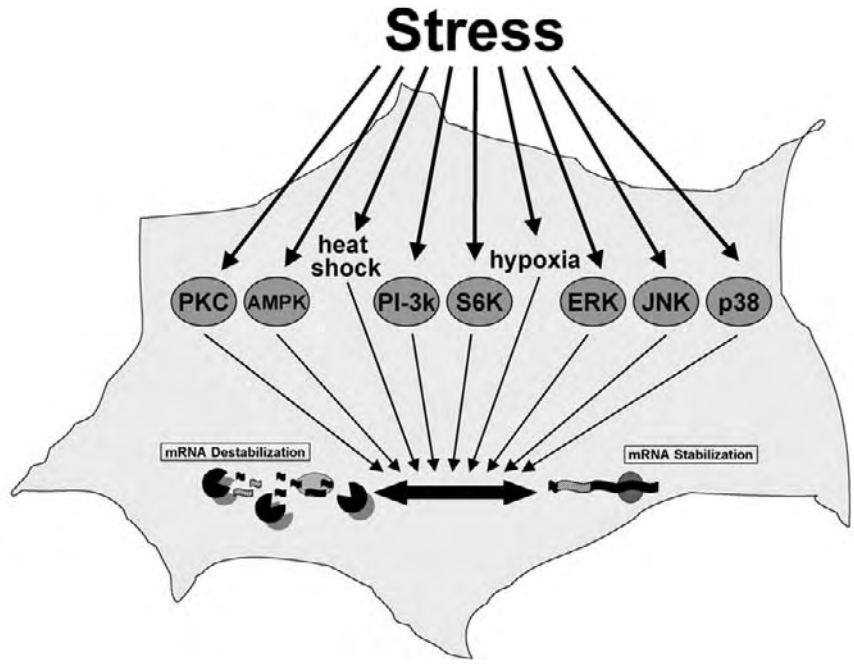


Figure 3 Stress-triggered signaling pathways that modulate mRNA turnover. Schematic representation of stress-activated signaling events that either enhance or reduce mRNA stability. Detailed knowledge of the molecules and mechanisms that influence mRNA turnover along each stress-triggered pathway awaits further investigation. However, we anticipate that the precise regulatory events will vary depending on factors such as the type of stress agent, the particular labile transcript targeted, the cell type, and the cell's metabolic status.

stress-regulated pathways known to influence mRNA stability, either positively or negatively, is shown in Fig. 3.

Protein Kinase C

Among the earliest reports describing regulated mRNA stabilization were several studies describing the role of protein kinase C (PKC) in the stabilization of ARE-containing mRNAs. In response to a variety of stimuli, including phorbol esters, calcium ionophores, diacylglycerol, antibodies recognizing CD3/CD28 surface receptors, TNFα, oxidants, NGF, and interleukins, PKC was implicated in the enhanced stability of many labile mRNAs, such as those encoding IL-1, IL-2, interferon γ, nitric oxide synthase, GAP-43, GM-CSF, HSP70, HSP90, ribonucleotide reductase R1, bcl-2, uPA, and p21 [53–66]. PKC activity was implicated in the stabilization of TNFα mRNA by preventing removal of its poly(A) tail [67]. PKC activity was also shown to influence binding of an adenosine-uridine binding factor (AUBF) to target labile mRNAs, leading to their enhanced stability [16]. More recently, PKC was proposed to increase binding of hnRNP C to the uPA mRNA ARE [64]. In addition, PKC-dependent neurite outgrowth was shown to require HuD-mediated stabilization of the GAP-43 mRNA [68].

Mitogen-Activated Protein Kinases (MAPKs)

Stress-Activated Protein Kinases (SAPKs)

In mast cells treated with ionomycin (a Ca^{2+} ionophore), the c-Jun N-terminal kinase (JNK) pathway was found to participate in the stabilization of the IL-3 mRNA through an ARE present in its 3′UTR [69]. The JNK pathway was also found to be involved in the stabilization and increased translation of IL-2 mRNA in Jurkat cells. In this paradigm, the JNK-regulated response was mediated through a JNK-response element (JRE) present in its 5′UTR, and involved the proteins Nucleolin and YB-1 [8,70]. In response to anisomycin, an inhibitor of protein translation, the JNK pathway was also found to be involved in stabilizing VEGF mRNA, through an ARE in its 3′UTR [71].

Another SAPK, p38, was also shown to participate in the anisomycin-induced stabilization of VEGF mRNA [71]. Exposure to stress stimuli including LPS, cytokines, dexamethasone, and serum deprivation similarly revealed a requirement for p38 and its downstream target MAPK-activated protein kinase 2, in the ARE-dependent turnover of mRNAs encoding IL-8, c-fos, GM-CSF, TNFα, and cyclooxygenase-2 [72–76].

Extracellular Signal-Regulated Kinases (ERKs)

ERK proteins have also been implicated in the stabilization of mRNAs encoding p21, VEGF, and nucleolin [53,71,77,78]. The ERK-controlled up-regulation of nucleolin has been proposed to mediate the destabilization of the β-amyloid precursor protein (APP) mRNA [79].

Heat-Shock-Regulated Events

The heat-shock pathway has been linked to changes in mRNA turnover at many levels. The mRNA encoding the major heat-shock protein, hsp70, has long been known

to be stabilized by heat shock [80]. Laroia and colleagues [49] showed that heat shock also stabilizes mRNAs encoding cytokine and protooncogene mRNAs. This stabilization mechanism involves hsp70-mediated sequestration of the AUF1 protein, which would otherwise promote the degradation of ARE-containing cytokine and protooncogene mRNAs [49]. These studies also directly link the rapid degradation of cytokine mRNAs to the ubiquitin-proteasome system. Two additional roles for heat shock and hsp70 have been postulated, although they have not been fully explored: hsp70 can influence mRNA turnover by modulating mRNA decapping in yeast [81], and hsp70 has been found to preferentially bind to, and protect, ARE-containing mRNAs [82].

Heat shock was also shown to decrease HuR's ability to bind poly(A)-containing RNA in the cytoplasm and increase it in the nucleus [83], suggesting that heat shock causes HuR to retain the bulk of its target mRNAs in the nucleus. Interestingly, however, HuR-mediated nuclear export of hsp70 mRNA, and consequently hsp70 expression, increases with heat shock, revealing the specific influence of HuR on the transport and enhanced expression of a particular target mRNA [84]. The authors of this study propose that heat-shock-triggered specific binding of HuR to hsp70 mRNA involves the association of HuR with protein ligands pp32, SETα, and SETβ [84].

AMP-Activated Protein Kinase

Recent studies from our laboratory have identified signaling events that regulate HuR function. Because HuR activity appears to depend largely on its subcellular localization, with binding to target mRNAs occurring in the cytoplasm, we sought to identify signaling events regulating its nucleocytoplasmic transport. Exposure to many different stresses, including UVC, H_2O_2, and PGA_2, elevated HuR's presence in the cytoplasm. Surprisingly, however, neither the major stress-activated signaling cascades (ERK, JNK, p38 pathways) nor other more general signaling pathways (phosphatidylinositol 3-kinase [PI-3K], PKA, PKC) appeared to influence HuR's subcellular localization. Instead, we found evidence that the subcellular compartmentalization of HuR is potently influenced by the activity of a lesser known kinase that is primarily involved in sensing and responding to metabolic stresses: the AMP-activated protein kinase (AMPK). Specific AMPK activators, including treatment with A1CA riboside and infection with an adenovirus that expresses constitutively active AMPK, promoted the nuclear localization of HuR. By contrast, inhibition of AMPK activity, including that achieved through infection with adenoviruses expressing dominant-negative AMPK, caused cells to elevate cytoplasmic HuR levels [85]. Whereas the precise mechanisms of AMPK-regulated subcellular localization of HuR remains to be identified, *in vivo* phosphorylation experiments indicate that HuR itself is not phosphorylated. Instead, we propose that one or several of the HuR protein ligands [87] may be phosphorylated, directly or indirectly, by AMPK-triggered mechanisms, and that these phosphorylation events, in turn, affect the transport of HuR to the cytoplasm [86].

Hypoxia-Triggered Events

Among the response mechanisms implemented by the cell in response to low oxygen levels (hypoxia) is the stabilization of critical mRNAs (reviewed in [88]). VEGF, a key hypoxia-induced angiogenic factor, contains many instability sequences (generally AU-rich) in its 5′UTR, coding region, and 3′UTR. Several hypoxia-inducible proteins that bind to the VEGF mRNA and appear to enhance the stability of its mRNA have been described, among them HuR and hnRNP L [89,90]. However, sequences in the 5′UTR, coding region and 3′UTR have been found to be required for fully effective mRNA degradation under normoxic conditions, and for mRNA stabilization during hypoxia [91]. The mRNA encoding erythropoietin (EPO), another major hypoxia-regulated gene, also contains many turnover determinants. The EPO mRNA is targeted by several cytoplasmic proteins, including the poly(C)-binding proteins $PCBP_1$ and $PCBP_2$, although these associations are not hypoxia inducible [92]. Tyrosine-hydroxylase (TH) mRNA is also stabilized by hypoxia. A pyrimidine-rich sequence in the TH 3′UTR that was identified as important for basal and hypoxia-regulated mRNA stability constitutes a binding site of PCBPs. Significantly, hypoxia induces the stability of the mRNA encoding the hypoxia-inducible factor 1α (HIF-1α), a protein pivotal for the transcriptional up-regulation of hypoxia-inducible genes [93]. Less well understood are the mechanisms controlling the turnover of other mRNAs regulated by hypoxia, including Glut-1, PAI-1, eNOS, MnSOD, Cu, ZnSOD, and the iron-regulatory proteins IRP1 and IRP2.

Other Signaling Molecules and Pathways

PHOSPHATIDYLINOSITOL-3 KINASE

The PI3K pathway was recently implicated in the stability of cyclin D1 mRNA [94]. However, the precise cyclin D1 mRNA elements and RNA-binding proteins remain to be identified.

S6 KINASE

The stability of cyclin D1 mRNA is also dependent on the activity of the S6 kinase pathway, because treatment with the S6 kinase inhibitor rapamycin stabilizes the mRNA and enhances cyclin D1 expression [95].

Conclusions

Given that steady-state mRNA levels are critically important for determining cellular protein levels, the regulation of mRNA turnover is crucial in determining the cell's ability to respond to situations of stress. The importance of mRNA turnover in determining the expression of individual genes is being investigated on a case-by-case basis. My group recently initiated efforts to investigate the "global" contribution of mRNA turnover to the stress-induced changes in expressed gene patterns by using cDNA arrays. Following treatment of cells with stress agents (heat shock, PGA_2, or UVC),

we compared *changes in total mRNA levels* (after hybridization of arrays with standard radiolabeled cDNA probes obtained through reverse transcription of total cellular RNA) with *changes in gene transcription* using nascent, radiolabeled RNA from nuclear run-on reactions to hybridize the same cDNA arrays. We estimated that approximately *one-half* of all transcripts whose expression was altered by stress was subject to a significant change in mRNA turnover, either mRNA stabilization or destabilization [96].

The role that cellular stress plays in regulating mRNA half-life is thus gaining increasing attention. Some common mechanisms regulating mRNA stability are currently emerging in the form of related *cis* elements and even shared *trans*-acting factors. However, the complete elucidation of how mRNA turnover is controlled requires that complex events regulating the expression and function of RNA-binding and RNA-degrading proteins be thoroughly investigated. Considering the great relevance of many stress-regulated genes for cellular growth and homeostasis, it is not surprising that such regulatory events have begun to be the focus of intense investigation in recent years. It is widely anticipated that an in-depth understanding of the stress-triggered processes that control mRNA stability will contribute novel insight into basic mechanisms serving to regulate gene expression and will help identify new targets of clinical intervention.

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SECTION D

Post-Translational Control

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CHAPTER 313

RNA Localization and Signal Transduction

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Introduction

Sorting of mRNA to specific compartments of the cell determines cell asymmetry. This sorting occurs in oocytes and embryos as well as somatic cells such as fibroblasts and neurons (for reviews see [1–6]). Translation of localized mRNAs spatially directs protein synthesis. The cellular signals that direct specific RNA sequences to particular cellular compartments have recently been examined in fibroblasts, neurons, and *Drosophila* embryos. This chapter examines the regulation of mRNA localization through signal transduction pathways in organisms and their tissues.

Growth Factors Induce mRNA Localization

In chicken embryo fibroblasts (CEFs), β-actin mRNA has been shown to be localized toward the leading edge where it plays a role in cell motility and asymmetry [7,8]. A specific sequence in the 3′ untranslated region (UTR) of the β-actin mRNA, termed the *zipcode* [9], is necessary for the localization of the mRNA and specific *trans*-acting factors, termed *zipcode binding proteins* [10]. Growth factors can affect the site of synthesis of β-actin in the cytoplasm by inducing rapid localization of β-actin mRNA toward the leading edge in CEFs [11,12]. This localization can be induced after serum starvation by serum as well as growth factors such as LPA and PDGF and is inhibited by tyrosine kinase inhibitors herbimycin and genistein [11].

β-Actin mRNA localization can also be induced in growth cones of forebrain neurons by neurotrophin-3 (NT-3), forskolin, or db-cAMP [13]. Growth factors can act on growth cones to induce path finding in neuronal development [14]. In another study, NT-3 also induced localization of mRNA granules into dendrites and was inhibited by K252a, an inhibitor of tyrosine kinase receptors [15]. Brain-derived neurotrophic factor (BDNF) induced dendritic mRNA localization and translation of a reporter GFP with the 5′ and 3′UTR of CAMKII-α [16]. Growth factors such as NT-3 and BDNF may induce the movement of mRNAs into dendrites and induce the localized translation of these mRNAs at their target cellular compartment and affect changes in actin cytoskeletal reorganization within growth cones.

Tetanic stimulation of hippocampal neurons induces an increase in the concentration of CaMKII-α in the dendrites of postsynaptic neurons within 5 min after a tetanus [17]. This is the result of local synthesis on CaMKII mRNA within dendrites rather than the slower transport of CaMKII protein and was blocked by protein synthesis inhibitors [17–19]. In the case of *Arc* mRNA being targeted to active synapses, NMDA receptor activation is necessary for this localization [18,20]. The expression of *Arc* mRNA was induced by electroconvulsive seizure, and newly synthesized *Arc* mRNA was targeted to synapses in the dentate gyrus, but not when NMDA receptor antagonists were present [20]. Ca^{2+} signaling may be also be involved in localization and subsequent translation as binding of the growth factors to their receptors induced tyrosine phosphorylation [21] and subsequent increase in intracellular Ca^{2+} [22]. Increased [Ca^{2+}] may induce stability of some mRNAs and subsequent translation [23,24].

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In *Drosophila* oogenesis the localization of mRNA when translated is a signal to surrounding cells. *Gurken* mRNA is localized in the posterior oocyte cortex and then translated into an EGF similar growth factor, which then induces the follicle cells to form the polarity of the egg chamber [25,26]. This localization of *Gurken* mRNA is necessary for signaling via the EGF receptor pathway, as *maelstrom* mutants that do not localize the mRNA develop follicle cells with anterior rather than posterior fates [27]. A similar localization of the mRNA of *wingless* determines the apical localization of the signaling protein [28,29].

Signaling from the Extracellular Matrix Induces mRNA Localization

Another cellular signaling system is that from the extracellular matrix, usually integrins [30]. A study using microbeads coated with focal adhesion complex (FAC) proteins such as β_1 integrin talin, vinculin, and the RGD peptide induced "halos" of poly $(A)^+$ RNA and ribosomes within a few minutes [31]. These are presumably the mRNAs of FAC proteins although this was not determined. These halos also formed when tension was exerted on adhered beads.

mRNAs Localized via the Cytoskeleton

Numerous investigations have examined which cytoskeletal components are involved in localizing mRNA. In fibroblasts it appears that the actin cytoskeleton is involved in localizing β-actin mRNA. Early work demonstrated that β-actin mRNA was not localized in the presence of cytochalasin D, but was localized with colchicine [32]. More recently the rapid (<5 min) localization induced by serum or growth factors was shown to be inhibited by cytochalasin D, but not nocodazole [33]. Conversely, in neurons, mRNA localization is predominantly microtubule based and is inhibited by colchicine but not cytochalasin D [4].

mRNA Granule Movement in Neurons

Movement of mRNA in neurons was first tracked with ^{3}H-uridine in pulse-chase experiments [34]. More recently a membrane-permeable nucleic acid stain, SYTO-14, has been used to track mRNA movement in living cells [35]. Messenger RNA granules move into neurites within 15 min after NT-3 stimulation [15]. These granules are enriched in the RNA binding protein Staufen [36,37] and are complexed with polyribosomes [38]. After depolarization, mRNAs shift to a less dense granule fraction but are not translationally competent [38]. The movement of mRNA granules is responsive to neurotrophins in neurons [39] and growth factors in fibroblasts [11,12].

Regulation of mRNA Localizing Proteins

Many proteins necessary for localization of mRNAs such as ZBP1 [10], ZBP2 [40] in fibroblasts and neurons, and Egalitarian (Egl) and BicaudalD (Bic D) in *Drosophila* [41] may be targets of signal transduction pathways regulating mRNA localization. These proteins may be directly phosphorylated through signaling pathways or through their interactions with motor molecules such as myosins, dyneins, or kinesins. One such protein, zipcode binding protein 1, is required for the localization of β-actin mRNA to growth cones [39]. Neurons transfected with EGFP-ZBP exhibited rapid bidirectional movements of granules that required ZBP1 binding to RNA.

GTPase Signals Regulating Actomyosin Interactions Are Involved in mRNA Localization

β-Actin mRNA localization near the leading edge in CEFs is dependent on the actin cytoskeleton and not the microtubule-based cytoskeleton [32]. Similarly the rapid induction of β-actin mRNA localization by serum and growth factors is also dependent on actin and not microtubules [33]. Consistent with the Rho GTPases regulation of the actin cytoskeleton [42,43], β-actin mRNA localization at the leading edge has been shown to be regulated by the Rho GTPase [33]. This signaling pathway goes through the Rho-associated kinases, because the specific inhibitorY-27632 inhibited localization and transfected Rho-kinase constructs induced localization. Because β-actin mRNA localization can lead to the development of cell polarity [7,44], this would allow for signaling regulation of cell polarity. Similarly in *Drosophila*, Rho-associated kinase (Drock) regulates planar cell polarity through the actin cytoskeleton [45]. Also in *Drosophila*, Rho regulatory proteins such as rhoGEF may play a role in the cytoskeletal changes during development [46] and may be involved in mRNA localization. The Rho signaling pathway may involve the formin protein p140mDia [47]. Because mDia can regulate the cytoskeleton through stabilization of microtubules [48] and the actin stress fibers [49,50] it could participate in mRNA localization on either actin or microtubules perhaps via anchoring of RNA by EF1α [51].

Cytoskeletal motors involved in mRNA localization have been demonstrated during development in *Xenopus* [52], and Drosophila and more recently a myosin V has been found to be involved in *ASH 1* mRNA localization in budding yeast [53–58]. Some studies using pharmacological inhibition of myosin showed inhibition of β-actin mRNA localization to the leading edge [33], and the inhibition of poly$(A)^+$ RNA movement into "halos" around integrin-coated beads with myosin ATPase inhibitor BDM [31] and the myosin light chain kinase inhibitor ML-7 [33]. Because Rho and Rho-kinase can regulate myosin phosphatase by inhibiting the phosphatase in the presence of ongoing myosin light chain kinase (MLCK) activity [59,60], the induction of mRNA localization by activation of this

pathway acts through myosin. Recently mouse embryo fibroblasts (MEFs) from myosin IIB knock-out mice were shown to be deficient in localizing β-actin mRNA to the leading edge [33]. MEFs from homozygous knock-out mice showed no movement of β-actin mRNA to the leading edge, whereas wild-type and the heterozygous MEFs responded within minutes [33]. The two-headed myosin II filaments can translocate on polarized bundles of actin filaments or stress fibers toward the leading edge associated with an mRNA complex that can bind the myosin. These actin bundles have a polarity with barbed ends increasingly directed toward the lamellipodium and thus could constrain activated myosin II-B movement only toward the leading edge [61,62]. Rho kinase can lead to phosphorylation of the light chains internally near the nucleus, where myosin filament assembly, stress fiber formation, and motility occur [63]. Growth cones from IIB knock-out mice neurons have been analyzed and showed differences in growth cone shape, actin organization, and reduced filipodial traction force [64], as does a localized knockout of myosin IIB using lazer inactivation.

Conclusion

There is no question now that motors move localized RNAs to their ultimate destination in the periphery of the polarized cell. This then allows proteins to be sorted asymmetrically to the specific region of the cell where they can exert their functionality. But this movement of the RNA is in response to the polarized nature of the asymmetric cell and is not causal for the asymmetry. The synthesis of specific protein components at the periphery enhances and stabilizes the polarity of the cell. How this polarity all gets established is a result of the signaling molecules impinging on the cell directionally from the outside. The transduction of these signals into polarity, through actin polymerization, for instance, provides a substrate for the trafficking of the RNA. In this way, the extracellular environment determines the distribution of specific protein synthesis within the cell. Figure 1 summarizes this process for asymmetric cells.

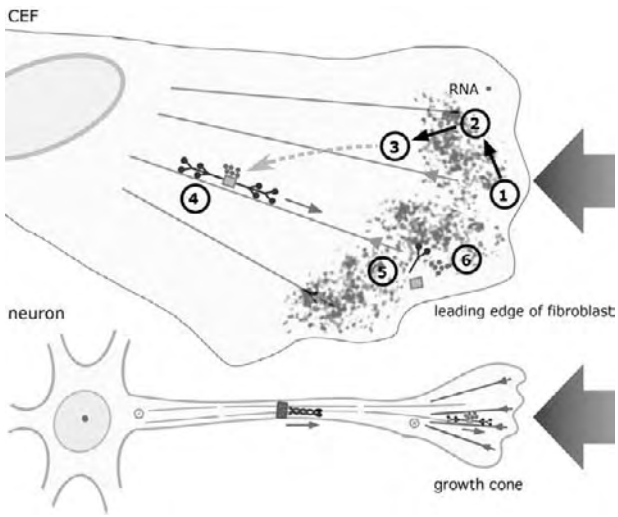


Figure 1 Model for how mRNA may move in response to a signal transduction mechanism on polarized actin bundles, associated with myosin II-B in fibroblasts (top) or with kinesin in neurons (bottom). Incoming signals (arrow) binding receptors (1) activate the RhoA cascade (2) through Rho-kinase (3), which activates myosin II-B light chains and myosin assembly with an mRNA complex. (4) The complex moves over polarized actin bundles to disassemble toward the leading edge as a result of heavy chain phosphorylation (5) and the RNA then anchors (6). The speed of myosin movement predicts that the mRNA would only be transported for 20 sec before it becomes anchored. Hence the steady-state distribution of the mRNA population would be at the lamella [33]. For neurons, an analogous cascade would be initiated by neurotrophins, possibly leading to the activation of the kinesin light chains. Kinesin would then move the ZBP1-RNA complex down the process and then onto microfilament bundles in the growth cone where myosin would take over [39].

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CHAPTER 314

Translational Control by Amino Acids and Energy

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Introduction

Translation is possibly the most energy-consuming process of the cell. Organisms have therefore developed broad control mechanisms that adjust the rate of translation to available nutrient and energy resources. The control of translation rapidly and directly sets a cell growth rate that is appropriate to prevailing environmental conditions. In general, translational control involves the transduction of a signal through a pathway that at one end senses environmental conditions and at the other end impinges on the translational machinery. Among many other signals, translation is controlled by amino acids—the building blocks that are required for the translation process itself—and by the energy status of the cell. Here we summarize recent findings on translational control by amino acids and energy, focusing on two signal transduction pathways—the GCN system and the TOR signaling pathway—that control at least four different steps of protein synthesis. The yeast GCN system, in response to amino acid availability, controls the translation initiation factor eIF2. The mammalian TOR (mTOR) signaling pathway, in response to amino acids and energy, controls translation by regulating the initiation factor eIF4E, ribosomal protein S6, and elongation factor eEF2. mTOR controls the latter two via p70 S6 kinase (S6K) (Fig. 1). Depending on the signal and the specific control mechanism, the global rate of protein synthesis or only the translation rate of specific mRNAs is affected. In general, the changes in global translation are rather modest, but the effects on specific mRNA populations are dramatic.

GCN System

The GCN signaling pathway has been characterized in yeast. The kinase GCN2 responds to amino acid or purine deprivation by phosphorylating the initiation factor subunit eIF2α at Ser51. This results in a down-regulation of initiation-competent 40S ribosomal subunits, which in turn leads to increased translation of a specific mRNA encoding GCN4 (see later discussion). GCN4 is a transcriptional activator of several genes encoding amino acid biosynthetic enzymes [1,2]. Thus, as a consequence of GCN-mediated down-regulation of translation, amino acid synthesis is transiently boosted to compensate for a limited supply of exogenous amino acids. Mammalian cells also make extensive use of eIF2α Ser51 phosphorylation to down-regulate translation initiation, employing different kinases in response to different stresses. These kinases include PKR (dsRNA viral infection), HRI (heme/iron metabolism), PERK (unfolded protein response in the ER), and a mammalian GCN2 homolog. It remains to be determined if mGCN2, like its yeast counterpart, is activated by amino acid deprivation.

How is GCN2 activated in response to amino acid deprivation? GCN2 contains a domain that is similar to histidyl-tRNA synthetase (HisRS) and which interacts with a tRNA acceptor stem. The activating ligand for GCN2 is therefore likely uncharged (deacylated) tRNA—especially since levels of uncharged tRNA significantly increase upon amino acid limitation. In mammalian cells, the phosphorylation of eIF2α typically observed upon amino acid depletion can also be induced by treatment with histidinol, a competitive inhibitor

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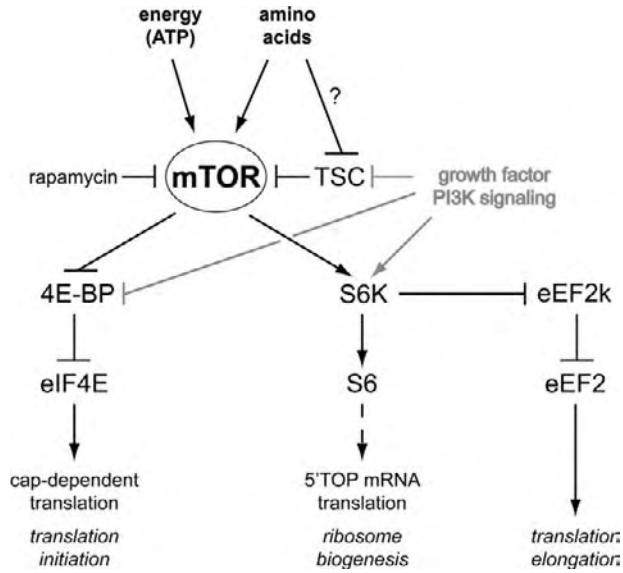


Figure 1 Model of mTOR controlling translation in response to amino acids and ATP. mTOR controls translation in conjunction with growth factor signaling via PI3K. The input of amino acids into mTOR may be direct or via inhibition of the TSC complex. The dashed arrow between S6 and 5′TOP mRNA translation reflects the uncertainty of this link (see text). TSC indicates the TSC1-TSC2 complex. Arrows indicate activation; bars indicate repression.

of HisRS, indicating that the mechanism of GCN2 activation may be conserved [2,3].

How does eIF2α Ser51phosphorylation lead to a down-regulation of translation initiation? As one of the early steps of initiation, GTP-loaded eIF2 (of which eIF2α is a subunit) delivers Met-tRNA$_i$ to the 40S ribosomal subunit. GTP loading and activation of the eIF2 complex is catalyzed by the GDP/GTP exchange factor eIF2B. Phosphorylation of Ser51 interferes with the activation (GTP-loading) of eIF2 by eIF2B. This results in decreased formation of the eIF2-GTP-Met-tRNA$_i$ ternary complex required for assembly of initiation-competent ribosomes and, thus, a global down-regulation of translation initiation [2]. Despite the global down-regulation of translation initiation, translation of GCN4 is up-regulated due to a mechanism that depends on the presence of short open reading frames (uORFs) in the leader region of the *GCN4* mRNA [1,2].

TOR Signaling Pathway

In mammalian cells, amino acid deprivation causes rapid dephosphorylation and activation of eIF-4E binding protein(s) (4E-BP; an inhibitor of eIF4E, also known as PHAS-I) and dephosphorylation and inhibition of the kinase S6K. This results in a down-regulation of translation initiation (see the following two sections). There are several 4E-BPs, but most experimental work has focused on 4E-BP1. Similarly, S6K comprises S6K1 and the more recently discovered S6K2, but most studies have involved only S6K1. The effect of amino acids on 4E-BP and S6K phosphorylation is mediated by the kinase mTOR (Fig. 1). TOR, initially identified in yeast, is the target of the immunosuppressive and anticancer drug rapamycin. The conserved TOR kinases, found in yeast, plants, worm, fly, and mammals, control a large and diverse set of cell growth-related readouts, including translation initiation [4,5]. TOR positively controls translation initiation and is thus essential for growth. This is in contrast to the nonessential GCN system, which negatively regulates translation as a homeostatic response to fluctuations in amino acid availability.

Inactivation of mTOR by treatment with rapamycin elicits the same changes in 4E-BP and S6K phosphorylation as amino acid deprivation. Furthermore, a truncated version of S6K that is resistant to rapamycin is also resistant to inhibition by amino acid withdrawal [6,7]. These findings indicate that amino acids signal to eIF4E and S6K via mTOR. mTOR phosphorylates 4E-BP and S6K *in vitro*, suggesting that mTOR may act on these two proteins directly. In yeast, TOR controls the phosphorylation of several downstream targets by negatively regulating the type-2A-related phosphatase SIT4. Recent evidence indicates that such a mechanism may also apply in mammalian cells to regulate the phosphorylation and thus the activity of mTOR effectors such as S6K [4].

The phosphorylation of 4E-BP and S6K is also controlled by the phosphatidylinositol 3-kinase (PI3K) signaling pathway, which is activated in response to growth factors such as insulin. Downstream of PI3K, this growth-controlling pathway includes 3′-phosphoinositide-dependent protein kinase 1 (PDK1) and protein kinase B (PKB/Akt). How are mTOR and the PI3K signaling pathway linked? First, mTOR and PI3K signaling are linked via their common targets 4E-BP and S6K, although they respond separately to amino acids (and energy) and growth factors (mitogens), respectively. The inhibition of 4E-BP and the activation of S6K require simultaneous inputs from mTOR and PI3K. The complex and hierarchical phosphorylation of several sites in 4E-BP [8,9] and S6K [10] reflects the dual control of these proteins by mTOR and PI3K (Fig. 1). Second, in addition to mTOR and PI3K controlling common targets in response to different signals, the PI3K pathway may also be linked to mTOR via the TSC complex (see later discussion) (Fig. 1).

Branched-chain amino acids (Leu, Ile, Val) have been shown to play an important role in the regulation of protein synthesis, in particular, in skeletal muscle. Of the branched-chain amino acids, leucine is the most potent in stimulating translation. Leucine as the sole exogenously added amino acid is sufficient to drive phosphorylation of 4E-BP or S6K in diverse cells, such as skeletal muscle, pancreatic β cells, or adipocytes. For leucine to be effective, mTOR must be active, since pretreatment with rapamycin abolishes the leucine-mediated stimulatory effects on the translation apparatus. However, evidence also suggests a so far uncharacterized, mTOR-independent mechanism by which leucine stimulates translation. It is also important to note that the regulatory role of leucine may be tissue specific (reviewed in [11]).

How is mTOR signaling regulated in response to amino acids? For S6K, it has been proposed that, similar to the

GCN system, the aminoacylation state of tRNA may be responsible for the regulation of S6K phosphorylation [12]. It is not known how mTOR would sense the aminoacylated tRNA and, despite the possible similar regulatory mechanism, a connection between GCN2 and TOR has not been demonstrated. Dennis *et al*. [13] argue that amino acid pools rather than the amount of aminoacylated tRNA are important to mTOR signaling. How mTOR would sense amino acid pools is also unknown.

The control of translation initiation by energy is less characterized than the control by amino acids. Moreover, one must define the term *energy*—is it "nutrients" in general (and if so, which?) or is it an "energy-rich" intracellular product? Intracellular levels of ATP or related nucleotides may indicate the energy status of the cell. Along these lines, mTOR has been suggested recently to respond to intracellular levels of ATP. The mechanism by which ATP acts on TOR appears to be different from that used by amino acids, but both ATP and amino acids are required for mTOR signaling [13] (Fig. 1). AMP-activated protein kinase (AMPK) is also involved in controlling cellular metabolism in response to the energy status (AMP concentration or ATP/AMP ratio) of the cell. In contrast to mTOR, AMPK is activated in response to low energy levels, leading to increased glucose uptake and fatty acid oxidation in muscle. AMPK activation by treatment of cells with the compound AICAR (phosphorylated in the cell to form an AMP analog) has recently been demonstrated to result in inhibition of protein synthesis in skeletal muscle and hepatocytes. Interestingly, phosphorylation of the mTOR targets 4E-BP and S6K decreases upon AMPK activation [14,15]. The nature of the relationship between AMPK activity and mTOR signaling, also in terms of mTOR responsiveness to ATP levels (see earlier discussion), remains to be determined.

In terms of energy, another nutrient to be considered is carbohydrates. Patel *et al*. [16] have recently reported that glucose exerts a permissive effect on the ability of insulin to promote the phosphorylation of 4E-BP and the formation of translation-competent initiation complexes. The effect may be mediated by a product of hexose metabolism and seems to be restricted to 4E-BP, since glucose did not allow full activation of S6K. Thus, 4E-BP in particular appears to be regulated in response to the availability of both amino acids (especially leucine) and an energy source, such as glucose [16].

In yeast, TOR signaling controls cap-dependent translation initiation in response to nutrients, such as nitrogen and carbon sources. The mechanism by which TOR controls translation initiation remains to be determined, even though the TOR dependence of cap-dependent translation initiation was first shown in yeast [17]. TOR also controls translation by ensuring the availability of amino acids, via stabilization of plasma membrane amino acid transporters [4]. Recent evidence argues that TOR responds to the amino acid glutamine, suggesting that glutamine is a particularly important indicator of nutrient status [18]. Indeed, glutamine is a preferred nitrogen source and controls TCA cycle-mediated carbon metabolism. TOR must also respond to other yet-to-be-identified nutrients because glutamine starvation affects only a subset of TOR readouts [18]. In mammalian cells, a recent transcriptional profiling has revealed that rapamycin treatment mimics, in part, glutamine or leucine starvation [19]. It is also interesting that, similarly to rapamycin treatment, a decrease in blood glutamine levels causes immunosuppression in humans and mice [20].

The recent identification of TOR interacting proteins as part of large TOR complexes in yeast and mammalian cells may contribute to the understanding of how TOR signaling responds to nutrient availability [21–23]. In particular, the mTOR interactor raptor binds 4E-BP and S6K, and the raptor-mTOR interaction is required for the mTOR-catalyzed phosphorylation of 4E-BP and S6K. Raptor may thus serve as a scaffold linking mTOR to its substrates [21]. Kim *et al*. [22] have proposed that raptor, in a nutrient-sensitive complex with mTOR, has both a positive and a negative role in the control of S6K. Finally, the yeast homolog of raptor, KOG1, interacts with yeast TOR, and depletion of KOG1 mimics rapamycin treatment, including a decrease in protein synthesis [23].

The TSC1–TSC2 complex, composed of the tumor suppressor proteins TSC1 and TSC2 (tuberous sclerosis 1 and 2, also known as hamartin and tuberin, respectively), also physically associates with TOR, as shown in *Drosophila* [24]. Epistasis analysis examining the effects of *TSC* and *TOR* mutations on cell viability and cell size in *Drosophila* places the TSC complex upstream of TOR [24]. Loss of TSC1–TSC2 results in a TOR-dependent increase of S6K activity in both *Drosophila* and mammalian cells and renders these cells resistant to amino acid starvation [24]. Thus, the TSC complex appears to antagonize amino acid signaling to TOR [24]. Recent findings have also shown that TSC2 is phosphorylated and inactivated by the PI3K effector PKB/Akt in response to growth factors [25–27]. Phosphorylation of TSC2 causes dissociation of the TSC complex [25,27] and stimulation of TOR-dependent phosphorylation of 4E-BP and S6K [25,26,28]. Furthermore, TSC1–TSC2 inhibits mTOR kinase activity [25]. These findings suggest that growth factor signaling to mTOR requires PKB-dependent inactivation of the TSC complex, and thereby provide a so far elusive link between growth factor signaling and mTOR. However, because PKB/Akt activity is not controlled by amino acids, it is difficult to reconcile a role for TSC in both amino acid and growth factor signaling to TOR. It would be of interest to determine how amino acid availability inactivates the TSC1-TSC2 complex. The nature of the apparently multiple roles of TSC in TOR signaling remains to be determined.

4E-BP: Regulation of eIF4E

Binding of the 40S ribosomal subunit to mRNA is mediated by the eIF4F complex, which is composed of the mRNA 5′ cap (m^7GpppN, m = methyl, N = nucleotide) binding protein eIF4E, the helicase eIF4A, and the scaffold protein eIF4G. After binding to the 5′ cap, eIF4F (via eIF4A and the associated factor eIF4B) unwinds inhibitory secondary structure

present in the 5′ untranslated leader region (UTR). Growing cells exhibit high eIF4F activity, whereas starved or stressed cells display low eIF4F activity. In mammalian cells, formation of the eIF4F complex is regulated by the 4E-BP family of translational repressors. 4E-BP competes with eIF4G for binding to eIF4E, and binding of 4E-BP to eIF4E is regulated by the phosphorylation state of 4E-BP. Hyperphosphorylation of 4E-BP inhibits the 4E-BP–eIF4E interaction, whereas hypophosphorylation, observed upon amino acid starvation or mTOR inactivation (see earlier discussion), promotes this interaction. Low 4E-BP phosphorylation thus results in the down-regulation of initiation-competent eIF4F complex due to the displacement of eIF4G from the eIF4F complex. Dephosphorylation of 4E-BP occurs not only upon amino acid deprivation, but also in response to a wide variety of other environmental stresses.

Although modulation of eIF4E activity affects global (cap-dependent) protein synthesis, translation of selective mRNAs is particularly affected. For example, the mRNA for ornithine decarboxylase possesses an unusually long 5′UTR predicted to form extensive secondary structure, and therefore falls into a class of mRNAs that are particularly dependent on the helicase activity of the eIF4F complex for their efficient translation. Many mRNAs encoding oncoproteins or other gene products related to cell growth and proliferation display such large, highly structured 5′UTRs and are thus predicted to be particularly subject to control by 4E-BP [5].

S6K: Regulation of S6 and eEF2

The main substrate of the mTOR-controlled kinase S6K is the 40S ribosomal protein S6. Based on its location within the ribosome and its interactions with translation initiation factors, S6 has been proposed to affect protein synthesis at the level of initiation. Increased S6 phosphorylation upon S6K activation may mediate, in particular, the initiation of translation of mRNAs with a 5′ terminal oligopyrimidine tract (TOP) [10,29] (Fig. 1). However, it is controversial whether 5′TOP mRNA translation is indeed controlled via S6K and S6 [30]. Most 5′TOP mRNAs encode components of the translational machinery, such as ribosomal proteins (RP), elongation factors, and the poly(A)-binding protein. Thus, ribosome biogenesis and synthesis of other components of the translational machinery are controlled at the translational level. The biosynthesis of ribosomes consumes a large part of the cell's energy and therefore constitutes an ideal target of translational control by mTOR.

TOR also controls ribosome biogenesis in yeast, but by a mechanism independent of a S6K homolog or 5′TOP regulatory elements because both of these are absent in yeast. Inhibition of TOR leads to a rapid down-regulation of transcription of rRNA (RNA polymerase I [Pol I] and RNA polymerase III [Pol III]) and RP genes, and thus of ribosome biogenesis [31, 32]. Furthermore, treatment of yeast cells with rapamycin blocks tRNA synthesis (Pol III) and processing of 35S rRNA [31,32]. The conserved TOR signaling pathway may thus act in all eukaryotic organisms to control ribosome biogenesis in response to nutrients (amino acids and energy).

mTOR-controlled S6K also positively regulates translation elongation. The eukaryotic elongation factor 2 (eEF2) is phosphorylated and inactivated by the kinase eEF2k. S6K phosphorylates and inhibits eEF2k. Thus, S6K positively controls eEF2, and thus elongation, by inhibiting eEF2k [33] (Fig. 1).

Note Added in Proof

Cherkasova and Hinnebusch have recently demonstrated that the GCN and TOR systems in yeast are functionally connected. Inhibition of TOR by treatment with rapamycin causes dephosphorylation of Ser 577 in GCN2, GCN2-mediated phosphorylation of eIF2α, and induction of *GCN4* translation. Thus, TOR negatively regulates GCN2. TOR controls GCN2 through TAP42, a regulator of yeast type 2A-related phosphatases [34].

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CHAPTER 315

Translational Control and Insulin Signaling

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In mammalian systems the primary function of insulin is to control glucose homeostasis by regulating glucose utilization in peripheral tissues and its own production and secretion in pancreatic β cells. Insulin controls these responses through intracellular signal transduction pathways, which directly modulate major anabolic responses, one of which is the activation and maintenance of elevated rates of protein synthesis. Because protein synthesis is the most energy-consuming anabolic process in the cell, insulin must also serve to sustain elevated levels of energy production required for the continuance of high rates of protein synthesis. To do so insulin acts to stimulate nutrient uptake, to meet the increased demand for energy. Here we describe the insulin-induced signal transduction pathways that coordinate increased protein synthesis rates with increased energy demand.

To maintain glucose homeostasis, insulin must control a number of major anabolic responses in specific target tissues such as liver, adipose tissue, and skeletal and cardiac muscle. Among these responses, the activation and maintenance of high rates of protein synthesis are the most energy-consuming anabolic processes. Therefore, it is not surprising that mechanisms have evolved to precisely modulate translation rates as a function of time, space, and demand [1]. In addition, regulation of gene expression at the level of translation allows the cell to respond more rapidly to sudden changes in the external milieu [2,3]. Insulin controls protein synthesis at the level of both initiation and elongation. Furthermore insulin enhances the cells' translational capacity by stimulating the translation of specific subsets of mRNAs, such as those encoding ribosomal proteins and translation elongation factors [4,5].

The use of mammalian cell culture systems and animal models has led to the identification of a number of signaling components involved in insulin-mediated translational control. In the quiescent state, basal translation rates are required to maintain appropriate levels of housekeeping proteins that turnover at a finite rate. In addition, the translation of certain classes of mRNAs are repressed under these conditions [6,7]. The effects of insulin on the protein synthetic machinery are mediated by signal transduction cascades (Fig. 1), which in turn modulate the specific activity of key translation factors [8]. Moreover, insulin stimulates global protein synthesis by increasing both the rates of translation initiation and elongation as well as by triggering ribosome biogenesis to increase translational capacity [5,9].

Activation of the insulin signal transduction pathway is triggered by the binding of the ligand to its tyrosine kinase receptor, leading to the inter-autophosphorylation of the two β-chains of the receptor at specific tyrosine residues. These phosphorylated residues act as docking sites for proteins containing phosphotyrosine binding domains, such as PTB and SH2 domains. Among these adapter proteins are the insulin receptor substrates (IRSs) [10]. IRSs are recruited to the insulin receptor and phosphorylated on tyrosine residues, which act as docking sites for additional signaling components. One of these is the GRB2/SOS complex, which through the small GTPase Ras stimulates the MAP kinase pathway, a pathway mainly involved in the control of transcription and proliferation [11], but which has a significant impact on translation (see later discussion). Through a distinct set of phosphorylation sites, IRSs recruit the p85 adapter of the p110 catalytic subunit of the phosphatidylinositol 3-kinase (PI3K). PI3K signaling is involved in the regulation of cell

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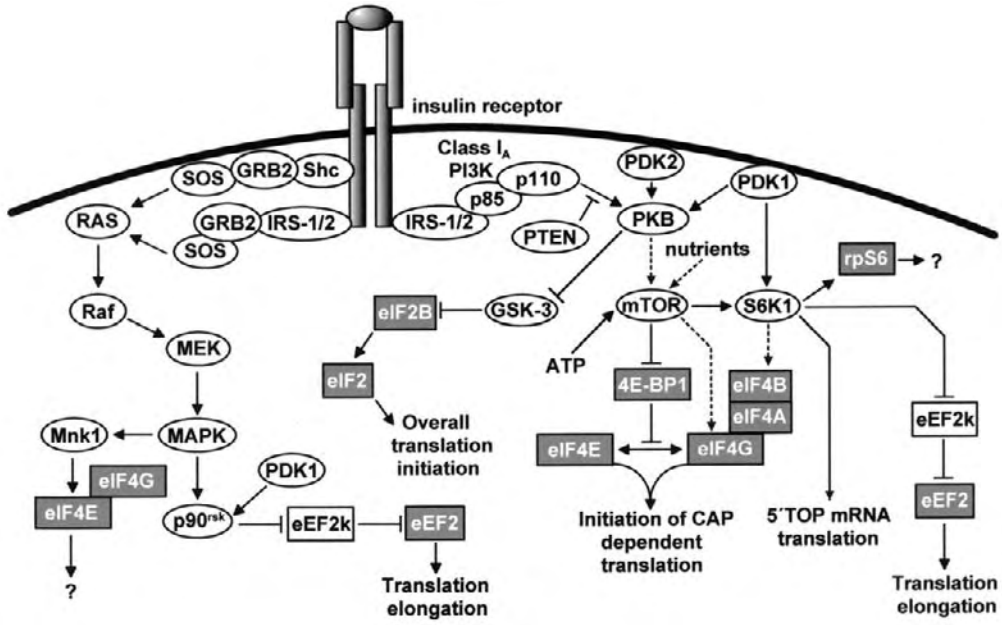


Figure 1 Schematic representation of insulin-induced molecular pathways signaling to the translational control machinery in mammalian cells. Arrows represent a positive input, while bars represent a negative input. Broken arrows indicate unclear and/or possibly indirect relationships. Translation factors targeted by insulin signaling components are depicted as shaded boxes. See text for detailed description of the individual signaling cassettes.

growth, survival, and metabolism and, in the latter case, particularly protein synthesis [12]. The recruitment of PI3K to IRS stimulates the production of the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3] from PtdIns(4,5)P2, leading to the recruitment and activation of protein kinase B (PKB/Akt) by the phosphatidylinositol-dependent kinases, PDK1 and PDK2 [13]. PtdIns(3,4,5)P3 production is counteracted by the lipid phosphatase PTEN (*p*hosphatase and *ten*sin homolog deleted from chromosome 10), a tumor suppressor gene that is either absent or mutated in a large number of cancers [14]. Insulin also leads to the activation of ribosomal protein S6 kinase 1 (S6K1) via *m*ammalian *t*arget *o*f *r*apamycin (mTOR), as well as through PI3K and PDK1 signaling inputs [15].

The primary target of translational control is the initiation step of protein synthesis. The first step of translation initiation is the binding of the ternary eIF2-Met-tRNA$_i$-GTP complex to the 40S ribosomal subunit, containing initiation factors eIF3 and eIF1A, to form the 43S preinitiation complex [16]. After formation of the initiation complex at the translation start site (see later discussion), the GTP bound to the γ subunit of eIF2, is hydrolyzed and eIF2, in a complex with GDP, is released. To begin a new cycle of initiation, eIF2 must interact with the guanine nucleotide-exchange factor, eIF2B, to efficiently exchange GDP for GTP. The guanine nucleotide exchange activity of eIF2B itself is subject to insulin regulation [4]. Insulin treatment promotes eIF2B activation by inducing the dephosphorylation of its ε subunit. This subunit is phosphorylated by GSK-3 [17], a key enzyme involved in regulating glycogen synthase [18]. Insulin induces GSK-3 phosphorylation and inactivation by PKB [19]. Separately, insulin can also promote dephosphorylation of eIF2 α [20]. Phosphorylation of this subunit of eIF2 prevents GDP exchange for GTP, resulting in a strong inhibition of initiation. Despite this knowledge, the insulin signaling pathway controlling eIF2 α dephosphorylation has yet to be elucidated.

Insulin also acts on eIF4F complex formation to exert translational control. The eIF4F complex is composed of three proteins, eIF4G, eIF4A, and eIF4E, which mediate the binding of the mRNA to the 43S preinitiation complex to form the 48S initiation complex [21]. eIF4G is a scaffold protein, which binds eIF4E and eIF4A and directly interacts with eIF3 of the 43S preinitiation complex [3]. eIF4A serves as an ATP-dependent helicase, whereas the function of eIF4E is to bind the 5′ terminal 7-methyl-guanosine, or cap, found on most nuclear encoded mRNAs. eIF4A interacts with a cofactor, eIF4B, which enhances eIF4A helicase activity [22], to drive unwinding of inhibitory secondary structures present within the 5′ untranslated regions (UTRs) of certain mRNAs, until the initiator AUG is encountered during ribosome scanning. eIF4E appears to be the limiting factor in eIF4F complex formation [23] and to be regulated by insulin by two distinct signal transduction pathways. The first involves phosphorylation by MAP kinase activated protein kinase 1 (Mnk1) [24], an event requiring binding of the kinase to the carboxyl terminus of eIF4G [25]. The role of eIF4E phosphorylation is controversial. eIF4E phosphorylation has been reported to increase its affinity toward the mRNA cap structure [26]; however, it has recently been argued that it is neither required for cap-dependent translation nor for eIF4F complex formation [27]. The second involves

a family of small repressor proteins termed the eIF4E binding proteins (4E-BPs), which compete with eIF4G for binding to eIF4E, inhibiting translation [28]. Insulin-induced hierarchical phosphorylation of 4E-BP1 disrupts its binding to eIF4E, favoring eIF4F complex formation [29].

Insulin-induced 4E-BP1 phosphorylation has been proposed to be dependent on PKB activation and to be mediated via mTOR [30]. However, in PDK1$^{-/-}$ embryonic stem cells, which lack PKB activity, growth-factor-induced 4E-BP1 phosphorylation was found to be intact and still dependent on mTOR [31]. eIF4G [32] and eIF4B [3] are also phosphorylated in both mTOR-dependent and -independent manners, however, the functional consequences of these events is yet to be established.

mTOR is emerging as a critical player in insulin-controlled translation, having been placed at the interphase between nutrient and energy sensing to serve as a gatekeeper to balance protein synthesis versus autophagy [2,33], which is a process responsible for bulk lysosomal degradation of proteins and organelles during periods of nutrient limitation. Despite the recent finding that mTOR is phosphorylated by PKB [34], the role of PI3K/PKB in mTOR activation is controversial ([3] and see later discussion). For example, insulin does not cause a change in mTOR kinase activity measured against S6K1 as an *in vitro* substrate [35], suggesting that insulin might instead influence the composition and signaling capabilities of factors that are bound to mTOR rather than mTOR kinase activity itself [35,36].

Interestingly, recent reports have demonstrated constitutive activation of the mTOR/S6K1 pathway in cells lacking tuberous sclerosis complex (TSC) tumor suppressor function [37–39]. Importantly, the TSC tumor suppressor might provide the link between growth factor and nutrient signaling and the mTOR/S6K1 pathway [40,41]. In support of this notion, the PI3K/PKB pathway can promote TSC phosphorylation, which is proposed to lead to inactivation of the tumor suppressor, and overexpression of TSC inhibits nutrient and growth factor induced S6K1 activation and 4E-BP1 phosphorylation [40,42]. However, note that neither Ca^{2+}, amino acids, nor TPA treatment of cells affect PKB activation despite leading to acute S6K1 activation.

Insulin specifically affects the translation of some classes of mRNAs [3]. Among these, are transcripts containing a 5′ oligopyrimidine tract (TOP) that have been shown to encode for components of the translational apparatus, such as ribosomal proteins and elongation factors [7]. The 5′TOP acts as a *cis*-repressing element in quiescent cells [43], with insulin induction relieving this repression by an unknown mechanism that allows efficient translation of 5′TOP mRNAs. The mTOR/S6K1 pathway has been shown to be in part required for recruitment of the 5′TOP mRNAs into polysomes [44,45]. It has been speculated that the effects of S6K1 on 5′TOP translation are mediated through the phosphorylation of the 40S ribosomal subunit protein S6 [46]. However, recent data suggest that S6 phosphorylation may not be required for 5′TOP mRNA translation in response to insulin (S. Fumagalli and G. T., unpublished work), suggesting the involvement of other targets. Alternatively, S6 phosphorylation may protect ribosomes from autophagic degradation [47], which is concomitantly down-regulated following induction of cell proliferation by mitogens [48,49].

Another class of mRNAs, whose translation is strongly induced by insulin, are those that contain long and complex secondary structures within their 5′UTRs. Such transcripts encode for proteins involved in proliferation, including c-Myc, Bcl3, and Cyclin D1 and their translation may involve regulation through the mTOR/S6K/4E-BP pathway [3]. Whether the translation of these transcripts and of 5′TOP mRNAs is constitutively derepressed in cells lacking TSC function remains to be determined.

The elongation phase of protein synthesis is also subject to insulin control. Peptide-chain elongation is mediated by the two elongation factors, eEF1 and eEF2 [1]. In particular, insulin has been shown to regulate phosphorylation of eEF2 [4]. eEF2 forms a complex with GTP and is required for the translocation step during elongation. Phosphorylation of eEF2 by eEF2 kinase occurs within the GTP-binding domain and renders the factor inactive [50]. Insulin induces the rapid inactivation of eEF2 kinase and eEF2 dephosphorylation (by protein phosphatase-2A), leading to a doubling of translation elongation rates [51]. Recently, S6K1 and $p90^{rsk}$ were shown to mediate eEF2 kinase phosphorylation and inactivation [31], providing a link between insulin signaling and the elongation phase of translation.

In parallel with increasing translation rates, insulin also acutely stimulates nutrient uptake [52] to provide the energy required for anabolic responses. Several of the components of the insulin-signaling pathway involved in regulating protein synthesis are also involved in the control of cellular energy homeostasis. For example, insulin-induced glucose uptake by GLUT-4 translocation at the plasma membrane is mediated by PKB, atypical PKCs, and phospholipase D [53–55]. PKB has also been implicated in mediating increased mitochondria-associated hexokinase activity and thus the coupling of glycolysis to oxidative phosphorylation [56]. Moreover, PI3K and PDK1 have been demonstrated to be involved in the activation of wortmannin-sensitive and insulin-stimulated kinase (WISK), which phosphorylates and activates heart 6-phosphofructo-2-kinase [57], leading to increased rates of glycolysis [58].

As with glucose uptake, insulin positively regulates amino acid uptake [59–61]. Interestingly, branched-chain amino acids and, in particular, leucine exert a potent stimulatory effect on protein synthesis, an effect most probably mediated by mTOR [62]. Indeed, regulation by mTOR in response to insulin, of both S6K1 and 4E-BP phosphorylation, requires the presence of amino acids [3,63]. In addition, it has been shown that mTOR activity is directly controlled by homeostatic levels of ATP [35]. Thus mTOR is suggested to regulate protein synthesis according to the level of translational and energy precursors [2,35]. Consistent with this model, mTOR and also PI3K have been proposed to negatively regulate autophagy [47,64,65], whereas PTEN has been suggested to have a positive effect [66].

In conclusion, in the future it will be important to obtain a deeper knowledge of the mechanisms that link the control of translation to metabolism in order to have a clearer understanding of insulin-mediated protein synthesis.

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CHAPTER 316

Unfolded Protein Response: An Intracellular Signaling Pathway Activated by the Accumulation of Unfolded Proteins in the Lumen of the Endoplasmic Reticulum

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Introduction

The endoplasmic reticulum (ER) is a membranous network extending throughout the cytoplasm of the eukaryotic cell and is contiguous with the nuclear envelope. The ER is the site of cholesterol and steroid biosynthesis, lipid biosynthesis, assembly of core-asparagine linked oligosaccharides, and membrane and secreted protein biosynthesis. The ER has evolved as a protein-folding machine and a major intracellular signaling organelle. Numerous posttranslational modification reactions occur at the ER and many of these are required for proteins to attain their final-folded functional conformation. The quality of protein folding is strictly monitored by protein chaperones that prevent aberrant folding and aggregation. These chaperones permit only properly folded proteins to exit the ER, a process termed *quality control.* The ER lumen also contains catalysts of protein-folding reactions, such as *cis-trans* polylisomerases and protein disulfide-bond isomerases (PDIs). The oxidizing environment of the ER provides a constant demand for the cell to monitor and promote correct disulfide bond formation.

Perturbations that alter ER homeostasis disrupt protein folding and lead to the accumulation of unfolded proteins and protein aggregates, which are detrimental to cell survival. These perturbations include disturbances in calcium homeostasis or redox status, an increased demand for chaperone function due to elevated secretory protein synthesis, the expression of mutant, misfolded, or unassembled proteins, and nutrient deprivation [1]. When homeostasis in the ER is altered, signaling pathways are activated to elicit an adaptive response. These pathways are collectively termed the *unfolded protein response* (UPR). If adaptation is not sufficient, then the cell activates a cell death response. It is important to note that all newly synthesized proteins are produced in an unfolded state. Therefore, high-level expression of wild-type proteins can also activate the UPR [2]. One enigma in cell biology is how the cell chooses between survival and death upon activation of the UPR. This chapter summarizes the diverse mechanisms for UPR signaling.

Pastan and colleagues [3] first reported that viral transformation of eukaryotic cells induces a set of genes. Because the transcription of the same set of genes was induced upon

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glucose deprivation, the products of these genes were termed *glucose-regulated proteins* (GRPs) [4]. Many of the GRPs are now known to function as molecular chaperones to either promote productive protein folding or prevent protein aggregation in the ER [5]. Productive protein folding in the oxidizing environment of the ER requires extensive energy, and glucose deprivation reduces the amount of energy that is available for such reactions. A number of ER chaperones, including BiP, use the energy from ATP hydrolysis to promote folding and prevent aggregation of proteins within the ER. In addition, glucose is directly required for the synthesis of asparagine-linked oligosaccharide core structures. The cell monitors the glucosylation status of N-linked oligosaccharides on glycoproteins as a mechanism for quality control. The protein chaperones calnexin and calreticulin bind monoglucosylated asparagine-linked core oligosaccharides on unfolded glycoproteins and prevent their exit to the Golgi compartment [6]. Individual proteins require different levels of ATP for protein folding prior to ER export [7]. It is probable that different levels of glucose deprivation can differentially induce the UPR in different cell types, depending on the amount of secretory proteins they need to fold.

In yeast, the accumulation of unfolded proteins in the ER lumen induces the transcription of 381 genes, many encoding functions to increase the volume or capacity for ER protein folding, or to increase the degradation of misfolded proteins [8]. Transcriptional induction of the ER protein chaperone KAR2/BiP/GRP78 is a classical marker for UPR activation in yeast and mammalian cells [9]. In higher eukaryotic cells, protein synthesis at the level of initiation is inhibited in order to decrease the protein-folding load on the ER [10].

UPR in *Saccharomyces Cerevisiae*

The original description of the UPR signaling pathway in the budding yeast *S. cerevisiae* occurred less than 10 years ago [11,12]. The only ER-stress-sensing protein in yeast *S. cerevisiae* is the kinase/endoribonuclease, IRE1. IRE1 is similar to class I receptor-transmembrane protein kinases consisting of an ER-lumenal extracellular, transmembrane and a serine/threonine kinase intracellular domain. In addition, IRE1 has a carboxy-terminal cytoplasmic endoribonuclease (RNase) domain with strong homology to mammalian RNaseL that is essential to signal UPR transcriptional activation. Accumulation of unfolded proteins in the ER lumen promotes dimerization of IRE1 with subsequent transautophosphorylation to activate its RNase function. Activated IRE1 cleaves the 5′ and 3′ exon–intron splice site junctions in *HAC1* messenger RNA, encoding a basic leucine zipper (bZIP)-containing transcription factor [13,14]. The 5′ and 3′ exons are then joined by transfer RNA ligase [15]. The unconventional *HAC1*-mRNA processing reaction removes a 252-nucleotide intron and thereby replaces the carboxy-terminal 10 amino acids in HAC1 ($HAC1^u$) with a novel 19-amino-acid segment ($HAC1^i$). This splicing reaction regulates UPR transcriptional activation in two ways. First, the new carboxyl terminus on $HAC1^i$ converts $HAC1^u$ into a 10-fold more potent transcriptional activator [16]. Second, removal of the intron by IRE1 and tRNA ligase increases the translational efficiency of *HAC1* mRNA. It was proposed that translation elongation is attenuated by base pairing between the 5′ untranslated region of *HAC1* mRNA and sequences within the intron [17]. Therefore, the *HAC1*-mRNA splicing reaction removes the intron to stimulate HAC1 protein translation. The *HAC1* mRNA splicing reaction occurs independent of *HAC1* transcription, supporting the supposition that it occurs in the cytoplasm [17]. However, mammalian IRE1 was localized primarily to the inner nuclear envelope, suggesting that nuclear splicing may occur in mammals [19,20].

$HAC1^i$ binds to a DNA sequence motif termed the UPR element (UPRE) (consensus motif: CAGCGTG) in the promoter regions of about one-half of UPR-responsive genes in yeast. In addition to activating the transcription of ER-chaperone genes, $HAC1^i$ activates transcription of genes involved in ER-associated protein degradation (ERAD) [8,21,22] and phospholipid biosynthesis (for example, *INO1*) [23,24]. Because there are no readily recognizable UPR elements in the promoter regions of ERAD genes, the molecular basis for their transcriptional activation by $Hac1^i$ is not understood. However, there is a close relationship between the UPR and ERAD. Deletion of the ERAD genes *UBC7*, *DER1*, *HRD1*, and *HRD3* moderately activates the UPR, indicating that the unfolded protein load in the ER of these ERAD-defective cells is increased [8,13,22]. Inositol starvation also activates IRE1-mediated splicing of *HAC1* mRNA [24]. It was proposed that $Hac1^i$ binds the transcriptional repressor OPI1 to relieve repression of *INO1* in order to promote inositol biosynthesis [24].

Not only does the UPR coordinate membrane biosynthesis with ER expansion, it also regulates nutrient-deprivation-induced differentiation responses in yeast. The budding yeast *S. cerevisiae* undergo distinct morphological transitions when starved of nitrogen. Nitrogen starvation in the presence of fermentable carbon sources promotes invasive pseudohyphal or filamentous growth [25,26]. In contrast, nitrogen starvation in the absence of fermentable carbon sources promotes meiosis and sporulation [27]. Genetic and physiological data demonstrated that nitrogen activates IRE1-mediated *HAC1* mRNA splicing to repress both pseudohyphal growth and meiosis [28].

UPR Transcriptional Activation in Metazoan Species

The UPR in higher eukaryotic cells has conserved many of the essential and unique properties from *S. cerevisiae*, but has also superimposed additional sensors and control mechanisms to mediate more diverse responses. In metazoan cells, the complex network of physiological responses to ER stress is regulated by three ER transmembrane proteins: IRE1, PERK, and ATF6 [29]. If these adaptive responses are not sufficient to relieve the unfolded protein load (so-called "ER stress"), the cell enters one of the

cell-death pathways: apoptosis or necrosis. The UPR in metazoan cells not only signals differentiation, but also regulates energy homeostasis and differentiation responses.

UPR Transcriptional Activation by ER Stress-Activated Splicing of *XBP1* mRNA

The mammalian genome contains two homologs of yeast *IRE1: IRE1*α and *IRE1*β. Whereas *IRE1*α is expressed in most cells and tissues, with high-level expression in the pancreas and placenta [19], *IRE1*β expression is primarily restricted to the intestinal epithelial cells [30]. Both IRE1α and IRE1β molecules respond to the accumulation of unfolded proteins in the ER to activate their kinase and subsequent RNase activities (Fig. 1B). The cleavage-site specificities of IRE1α and IRE1β are similar, suggesting they do not recognize different sets of substrates, but rather generate temporal- and tissue-specific splicing [31].

Although overexpression of either *IRE1*α or *IRE1*β activates transcription from a BiP promoter reporter construct [32], deletion of *IRE1*α and/or *IRE1*β in the mouse did not interfere with transcriptional activation of the endogenous *BiP* gene in cultured cells [20,33,34]. Therefore, at least one additional mechanism for UPR transcriptional activation exists. However, deletion of *IRE1*α caused lethality at embryonic day E10.5 [20,33]. In contrast, no developmental defect was detected on deletion of *IRE1*β, although the mice did show increased susceptibility to experimental-induced colitis [35]. These studies demonstrate that although *IRE1*α and *IRE1*β are not essential for the UPR, *IRE1*α is required for mammalian embryonic development.

The only known substrate for mammalian IRE1 is the mRNA encoding the bZIP transcription factor X-box binding protein 1 (XBP1) [20,36–38]. XBP1 was isolated as a protein that interacts with the mammalian ER stress-response element [ERSEI; CCAAT (N_9)CCACG] in a yeast one-hybrid screen [39]. *XBP1* mRNA has two conserved overlapping open reading frames (ORFs). On activation of the UPR, *XBP1* mRNA is cleaved by IRE1 to initiate a splicing reaction that removes a 26-nucleotide intron and generates a translation frame shift to produce a fusion protein encoded from the two ORFs. The new carboxyl terminus on the product from spliced *XBP1* mRNA (XBP1-s) converts XBP1 into a potent transcriptional activator, similar to that described for yeast HAC1. Although deletion of *IRE1*α and/or *IRE1*β did not diminish the transcriptional activation of several UPR-responsive genes, analysis of a minimal UPR reported did uncover a transcriptional defect in *IRE1*α-null mouse embryo fibroblasts (MEFs). This defect was complemented by expression of spliced *XBP1* mRNA [20]. Therefore, it is possible that a subset of UPR targets, those composed of minimal UPR motifs, requires IRE1.

UPR Transcriptional Activation by ER Stress-Induced Proteolysis of ATF6

Activating transcription factor 6 (ATF6) was identified as another ERSEI-binding protein in the same yeast one-hybrid screen that detected XBP1 [39]. There are two ATF6 genes, ATF6α (90 kDa) and ATF6β (110 kDa; also known as CREB-RP), that encode type II ER transmembrane proteins. On activation of the UPR, both forms of ATF6 are processed to generate 50- to 60-kDa cytosolic bZIP-containing fragments that migrate to the nucleus (Fig. 1A). In the presence of the CCAAT-binding factor (CBF; also called NF-Y [40]), both forms bind to the ERSEI in the promoter regions of UPR-responsive genes to activate transcription [41–44]. The DNA-binding specificities of ATF6 and XBP1 are similar; however, XBP1 can bind the ERSEI half-site (CCACG) in the absence of CBF/NFY [20,45,46]. Whereas CBF binds the CCAAT motif, ATF6α/β binds the CCACG motif.

Processing of ATF6 occurs within the transmembrane segment and at an adjacent site that is exposed to the ER lumen. The ATF6 processing enzymes S1P and S2P are the same processing enzymes that are activated upon cholesterol deprivation to cleave the ER-associated transmembrane sterol-response element binding protein (SREBP) [47]. However, only the UPR elicits ATF6 cleavage and sterol deprivation alone induces SREBP cleavage. The mechanism for ATF6 processing is similar to that of SREBP, involving the ER-stress-induced translocation of ATF6 from the ER to the Golgi compartment [48]. The sterol cleavage activating protein (SCAP) confers specificity for SREBP cleavage in response to sterol deprivation [49]. It is possible that another cleavage-activating protein confers S1P and S2P specificity for ATF6 upon activation of the UPR.

IRE1 and ATF6 Signaling Converge at XBP1

Signaling through ATF6 and IRE1 merge to induce *XBP1* transcription and mRNA splicing, respectively (Fig. 1). Transcription of *XBP1* is directly activated by the UPR, as well as by the cleaved form of ATF6. Increased *XBP1* transcription produces more substrate for IRE1-mediated splicing to generate more active XBP1, providing a positive feedback for UPR activation. Cells that lack either IRE1α or IRE1β or are defective for ATF6 cleavage can induce *XBP1* mRNA [20], suggesting that these two pathways provide complementary mechanisms to activate *XBP1* transcription. Alternatively, another pathway—possibly mediated by the ER-localized protein kinase PERK/PEK—may also contribute to *XBP1* mRNA induction.

UPR Transcriptional Activation through Translational Regulation by PERK

Stimuli that activate the transcriptional components of the UPR also transiently attenuate protein translation. This response is coupled with phosphorylation on the α-subunit of heterotrimeric eukaryotic translation initiation factor 2 (eIF2α). eIF2 is a GTPase required for bringing initiator tRNA to the 40S ribosomal subunit and for using GTP hydrolysis to promote 60S ribosomal subunit joining. To perform another round of initiation, GTP exchange for bound GDP is required. Phosphorylation of eIF2α at Ser51 inhibits

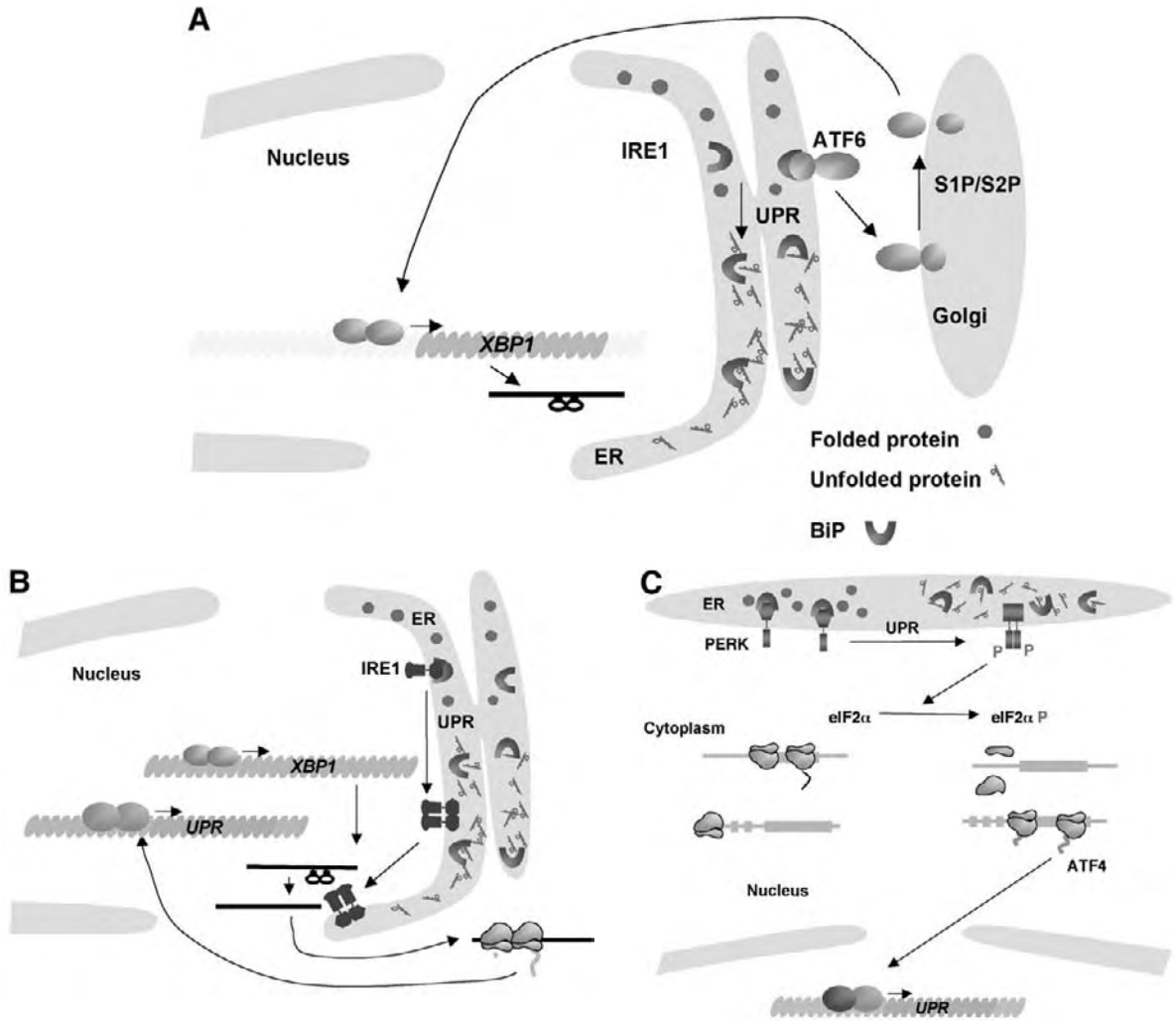


Figure 1 Signaling the unfolded protein response in eukaryotes. The signaling pathways for the three proximal sensors of the UPR are depicted. Whereas only IRE1 is present in *S. cerevisiae*, all three transducers are present in metazoan cells. *Caenorhabditis elegans* only requires IRE1 and PERK for survival to accumulation of unfolded proteins in the endoplasmic reticulum (ER) [37]. Although IRE1 and PERK may be dispensable for most transcriptional regulation of the UPR, ATF6 cleavage is required for UPR transcriptional induction [23,43]. Therefore, ATF6 is the most significant signaling pathway in mammalian cells. BiP negatively regulates these pathways. (A) Upon accumulation of unfolded proteins in the ER lumen, ATF6 transits to the Golgi compartment. In the Golgi, ATF6 is cleaved by S1P and S2P proteases to yield a cytosolic fragment that migrates to the nucleus to activate transcription of responsive genes, including *XBP1*. (B) In parallel, IRE1 protein kinase/RNase dimerizes and activates its RNase activity. (C) Simultaneously, PERK is activated to phosphorylate eIF2 on the α-subunit at Ser51 to reduce the frequency of AUG codon recognition.

this exchange reaction so translation initiation is prevented. The UPR activates a transmembrane ER-localized kinase, PERK/PEK, to phosphorylate Ser51 in eIF2α and to inhibit translation [50,51] (Fig. 1C).

The best characterized translational response to eIF2α phosphorylation is the one that occurs on amino acid starvation in yeast. On amino acid deprivation, uncharged histidyl tRNA activates the eIF2α protein kinase called GCN2. Phosphorylation of eIF2α (yeast SUI2) reduces the rate of polypeptide-chain initiation and induces the transcription of a limited set of genes through preferential translation of *GCN4* mRNA. *GCN4* mRNA encodes a bZIP transcription factor that is required for induction of amino acid biosynthetic genes [52]. The 5′ untranslated region of *GCN4* mRNA contains four upstream ORFs that inhibit the ability of the ribosome to scan through the 5′ end of the mRNA and reach the correct AUG initiation codon. Phosphorylation of eIF2α limits 60S ribosomal subunit joining to allow the 40S ribosomal subunit to scan through the ORFs and initiate polypeptide chain synthesis at the authentic initiation AUG codon for GCN4.

Phosphorylation of eIF2α is a major translational-control mechanism conserved in all nucleated cells. On accumulation of unfolded proteins in the ER, PERK-mediated phosphorylation of eIF2α is required to attenuate translation, to induce transcription of one-third of the UPR-responsive genes, and for survival [53,54]. Interestingly, transcriptional induction of the ubiquitous glucose transporter-1 (GLUT1) by low glucose is coincident with induction of the UPR marker BiP, and may contribute to survival in low glucose [55]. The mechanistic requirement for eIF2α phosphorylation in UPR

gene induction is unknown. However, in the absence of eIF2α phosphorylation or PERK, the mRNA encoding the bZiP transcription factor ATF4 was not translated [53,56]. It was proposed that translation of *ATF4* mRNA, by virtue of ORFs within the 5′UTR, requires phosphorylation of eIF2α. Analogous to the amino-acid-starvation-induced translation of *GCN4* mRNA in yeast, *ATF4* mRNA may be one of many mRNAs that is preferentially translated when eIF2 levels are limiting. ATF4 is another member of the C/EBP family of transcription factors implicated in metabolic control regulated through cyclic AMP [57]. Nutrient-deprivation-induced regulation of translation and transcription of the C/EBP family members may facilitate the interconversion and use of different energy sources; that is, fat, carbohydrate and protein.

Free BiP Inhibits the Activation of IRE1, PERK, and ATF6

One interesting question concerning the UPR is how IRE1, PERK, and ATF6 are simultaneously activated by accumulation of unfolded proteins in the ER. Dimerization and transautophosphorylation are required to activate yeast IRE1 [58,59]. In addition, the lumenal domain of yeast IRE1 is required for UPR signaling and the lumenal domain of IRE1 can dimerize *in vitro* [60,61]. These results support the hypothesis that the lumenal domain promotes dimerization with subsequent kinase activation and autophosphorylation. Interestingly, the amino terminus of the lumenal domain of PERK is homologous to the amino terminus of the IRE1 lumenal domain. Although yeast do not have PERK, the lumenal domain of PERK, as well as the lumenal domain of human IRE1, can functionally replace the lumenal domain of yeast IRE1 to provide UPR-dependent activation in yeast [60]. Therefore, mammalian PERK and IRE1 have conserved the same sensing mechanism as yeast IRE1. Surprisingly, a bZIP dimerization domain can also functionally replace the yeast IRE1 lumenal domain and this chimeric molecule also responds to the UPR [60]. These findings led to the hypothesis that IRE1 is actively held in a monomeric state in the cell and on UPR activation, dimerization is permitted.

What might prevent IRE1 dimerization when the UPR is off? Overexpression of BiP, but not other ER protein chaperones, inhibits PERK and IRE1 activation and also suppresses both the translational attenuation and transcriptional activation that occurs on accumulation of unfolded proteins in the ER [62,63]. Therefore, it was proposed that the level of free BiP negatively regulates UPR activation. In the absence of unfolded proteins, IRE1 and PERK are in a complex with BiP [64]. As unfolded proteins accumulate, their exposed hydrophobic surfaces may bind BiP, thereby reducing the pool of free BiP that is available to bind IRE1 and PERK. As a consequence, PERK and IRE could spontaneously dimerize for transautophosphorylation.

The level of free BiP also regulates ATF6 cleavage. A BiP binding site within the lumenal domain of ATF6 was recently identified that when deleted produced constitutive transport of ATF6 to the *cis*-Golgi compartment [65]. In addition, overexpression of BiP prevents ATF6 transit to the Golgi and subsequent cleavage [65]. Therefore, when the UPR is activated and the level of free BiP decreases, BiP release from ATF6 would permit ATF6 transit to the *cis*-Golgi to gain access to S1P and S2P proteases at the *cis*-Golgi compartment. The BiP negative-regulation model permits the simultaneous activation of all three ER stress sensors on activation of the UPR.

Physiological Role for the UPR in Mammals

The physiological role for UPR signaling has been elucidated through genetic modification in the mouse. To date, mice deleted in *IRE1*α, *IRE1*β, and *PERK* as well as mice deleted of the IRE1 substrate XBP1 and mice with targeted knock-in mutation at the PERK phosphorylation site Ser51 in eIF2α have been analyzed. The observations from these mice support that IRE1/XBP1 provides a major role in cell differentiation, possibly restricted to cells designed to have a specialized secretory compartment. In contrast, PERK/eIF2α may provide a major control over *in vivo* glucose metabolism.

IRE1α/XBP1 Signaling Required for Mammalian Development

Although deletion of *IRE1*α did not interfere with UPR transcriptional activation of many UPR-responsive genes in MEFs, deletion of *IRE1*α caused embryonic lethality at day E10.5. Therefore, although IRE1 is not essential for the UPR, *IRE1*α is required in mammalian development. In contrast, developmental defects were not detected in *IRE1*β-null mice.

Deletion of *XBP1*, encoding the only known mammalian substrate of IRE1, produced embryonic lethality at days 12–14 due to multiple defects in hematopoiesis, cardiomyocyte development, and hepatocyte differentiation [66,67]. However, the *XBP1*-deleted mice studied lack both the unspliced and spliced forms of XBP1 so it is not possible to conclude these defects are due to a loss of IRE1 signaling. Analysis of chimeric mice produced between $XBP1^{-/-}$ mice and immunoincompetent $RAG1^{-/-}$ (i.e., mice deficient in T- and B-cell maturation) demonstrated that *XBP1* is required for plasma cell differentiation and high-level immunoglobulin production [68]. Interestingly, immunoglobulin heavy-chain and light-chain gene rearrangement and surface expression of immunoglobulin μ in B cells occurred normally in *XBP1*-null mice. However, high immunoglobulin-secreting plasma cells were not detected, suggesting a role in plasma cell differentiation and/or survival. On B-cell differentiation into a plasma cell, the ER compartment expands approximately fivefold to accommodate the large increase in immunoglobulin synthesis [68]. Interestingly, plasma-cell differentiation is stimulated *in vivo* by activation of the innate immune response—by treatment with lipopolysaccharide (LPS), for example, or by ligation of CD40 receptors. Treatment of

lymphoid cells with LPS induced *XBP1* mRNA splicing [38], suggesting that activation of IRE1, at least indirectly, might signal an inflammatory response. These observations with the *XBP1*-null mice are consistent with the UPR providing a programmed response to expand ER capacity to accommodate protein secretion needs in highly specialized cells.

PERK/eIF2α Required for Glucose Homeostasis

Strong evidence supports the supposition that PERK signaling through eIF2α phosphorylation provides major control over glucose homeostasis *in vivo*. Two independent human families were identified with an autosomal-recessive infancy-onset insulin-requiring diabetes, termed Wolcott–Rallison syndrome, resulting from loss-of-function mutations in the *PERK* gene [69]. *PERK*-null mice have pancreatic dysfunction and develop diabetes several weeks after birth [70]. Mice with a homozygous Ser51 to Ala mutation in the *eIF2*α gene develop a more severe β-cell dysfunction that appears *in utero* [53]. The more severe phenotype resulting from Ser51Ala mutation in *eIF2*α compared to the *PERK* knock-out suggests that additional eIF2α kinases partially complement for β-cell function in the absence of PERK. In addition, homozygous Ser51Ala eIF2α mice also have a defect in liver gluconeogenesis, suggesting a broader role for eIF2α phosphorylation in glucose production and storage [53].

Why is phosphorylation of eIF2α required for β-cell differentiation and/or survival? Glucose stimulates both the secretion of insulin and the translation of proinsulin [71,72]. It was proposed that blood glucose levels not only affect transcription and translation of glucose-regulated genes but also the protein-folding status in the ER [53]. As glucose levels decline, the cellular energy supply decreases, protein folding becomes inefficient, and IRE1 and PERK are activated to induce UPR transcription and inhibit translation. The downstream effects of activating ER stress kinases in the β cell are largely unknown, but the transcriptional induction of glucose-regulated proteins may have a protective function by increasing the cellular capacity for the uptake and use of glucose. Prolonged activation of IRE1 may activate the c-Jun amino-terminal kinase (JNK) to induce an apoptotic state [33]. PERK activation in response to low blood glucose would inhibit protein synthesis, thereby limiting translation of proinsulin mRNA. In contrast, as blood glucose levels rise, PERK would be turned off so that translation would accelerate, allowing translocation of proinsulin into the ER. In this manner, eIF2α phosphorylation by PERK would mediate glucose regulation of insulin translation. Thus, the signaling mechanisms that β cells use for sensing glucose levels, triggering insulin secretion, and rapidly controlling insulin biosynthesis may have coevolved with the ER signaling pathway to support these specialized functions,

Why do β cells uniquely respond to physiological fluctuations in blood glucose (i.e., between 3 and 10 m*M*)? In contrast to other cell types, β cells express very little, if any, low Km-hexokinases. Therefore, the production of glucose-6-phosphate and glucose utilization for ATP production is controlled by glucokinase, which has a weaker affinity for glucose [73]. Consequently, in β cells the cellular [ATP/ADP] ratio is tightly coupled with blood glucose levels [74]. During intermittent feeding, periodic decreases in the ATP/ADP ratio may compromise protein folding to activate the UPR in β cells. Therefore, the ER lumen of the β cell would be more susceptible to fluctuations in glucose levels within the physiological range. The β cell may require the UPR for survival during intermittent decreases in the blood glucose level. Additionally, the very high level of PERK and IRE1α expression in the pancreas may lower the activation threshold by predisposing them to dimerization.

Future Directions

In conclusion, IRE1, PERK, and ATF6 are proximal sensors that regulate the quantity and/or quality of a class of bZIP-containing transcription factors that may form homo- and heterodimers. Combinatorial interactions of these factors may generate diversity in responses for different subsets of UPR-responsive genes. Each of these pathways contributes to a different subset of responses that are collectively termed the UPR. Future studies should identify under what physiological and pathological states these pathways are activated *in vivo* and how they influence disease outcome. It is likely that under some conditions their activation will be beneficial and under different conditions their activation will be detrimental. As we gain a greater understanding of the three subpathways, it should eventually be possible to target them for intervention in the pathogenesis of different disease states, such as conformational diseases of protein folding (Alzheimer's disease), viral infection, diabetes, cancer, and autoimmune diseases.

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CHAPTER 317

Regulation of mRNA Turnover

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Introduction

Steady-state levels of macromolecules in cells are regulated by the balance of synthesis and breakdown. Although historically the control of mRNA levels by regulation of transcription has received the most attention, in recent years the critical role of mRNA breakdown in regulating transcript levels has been more widely appreciated. The stabilities of different transcripts can vary by several orders of magnitude under normal physiological conditions. In addition, recently there has been increased awareness of the possibility of minute-to-minute regulation of mRNA stability, just as regulation of transcription by signal transduction processes has received intense scrutiny during the past two decades. The purpose of this chapter is to provide an update on some recent research findings on rapid regulation of mRNA turnover in response to extracellular agonists in vertebrate cells. Although much of our knowledge of eukaryotic mRNA turnover comes from studies in yeast, space precludes a review of those studies, which have been reviewed [1,2]. The reader is also referred to other reviews on mRNA stability in mammalian cells [3,4].

Current Models of mRNA Stability in Vertebrate Cells

It is now thought that mRNA stability can be described for most cellular mRNAs in vertebrates in terms of the *closed-loop* model. In this model, the poly(A) tail attached the 3′ end of most mRNAs is physically linked to the mRNA cap structure, through direct binding contacts between a widely distributed poly(A) binding protein and the eukaryotic initiation factor eIF4G. This physical interaction is apparently also able to promote the translation of mRNAs. Thus, under situations in which the mRNA is stabilized, the closed-loop configuration simultaneously inhibits mRNA decay and promotes mRNA translation, leading to net increases in protein synthesis (assuming constant transcription rates).

The biochemical changes that occur when this complex is destabilized and the rate of mRNA degradation is increased are still poorly understood. In vertebrates, these steps are thought to involve the deadenylation of the fully polyadenylated transcripts, followed by further nucleolytic action of endo- and exonucleases. In yeast, the removal of both the poly(A) tail and the 5′ cap structure are key early events in mRNA breakdown, followed by a major pathway of 5′ to 3′ exonucleolytic destruction of the mRNA body, as well as a minor pathway of 3′ to 5′ degradation. Similarly, the breakdown of vertebrate mRNAs also is thought to begin with the deadenylation of the polyadenylated transcript, apparently through a poly(A)-specific exonuclease referred to as PARN. However, a role for a putative mammalian decapping activity in the next steps of the process is not clear, although such an activity has been described recently in HeLa cell extracts [5]. For most mRNAs in vertebrate cells, the initial deadenylation is thought to be followed by further exo- and endonucleolytic breakdown of the completely or partially deadenylated mRNA body. Very recently, it has been shown that the deadenylated mRNA can be physically linked to a multiprotein intracellular structure known as an *exosome*, in which further breakdown of the deadenylated mRNA body can take place [6,7].

Presence of Instability Elements in Vertebrate mRNAs

It has been known for many years that instability of certain vertebrate mRNAs is conferred by AU-rich elements (ARE) within the 3′ untranslated region (UTR) of mRNAs [8]. Removal or mutation of these elements results in a more stabilized mRNA, whereas insertion of such elements

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into otherwise stable mRNAs can destabilize them. These instability-inducing AREs were originally classified into three major groups by Xu *et al.* [9]; this classification has been broadened recently by Willusz *et al.* [2]. A recurring element in these classifications is the AUUUA pentamer, which is linked in five tandem copies in examples of class IIA element-containing mRNAs such as those encoded by granulocyte macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor α (TNFα). Progressively fewer of these pentameric elements are found in mRNAs containing class IIB, C, D, and E AREs, whereas class I mRNAs contain a single pentamer plus a U-rich region, and class III mRNAs are U-rich but do not contain the pentameric motif [2].

Possibly because many of the mRNAs containing class II AREs are of intense clinical interest (e.g., GM-CSF, TNFα, cyclooxygenase 2, interleukin 2, interleukin 3, vascular endothelial growth factor, interferon α), the mechanism by which these elements confer instability on an mRNA has received intense scrutiny. From the work of Chen and Shyu [10], a consensus has emerged that these mRNAs are first subjected to processive poly(A) exonuclease action, resulting in the formation and possible accumulation of deadenylated mRNA bodies; the bodies are then further degraded by other cellular nucleases, perhaps in the exosomal compartment as described earlier. How this process is stimulated by the presence of the class II AREs, and how it is otherwise regulated, are not currently known, but are the subject of much current research.

Effects of ARE Binding Proteins on mRNA Turnover

Given the apparent importance of the class II AREs in the enhanced turnover of the mRNAs that contain them, much research has been focused on the identification and functional analysis of ARE binding proteins. A number of such proteins has been identified, and most seem to exert a protective or inhibitory effect on ARE-containing mRNA destruction. Others appear to have minimal effect on mRNA turnover rate, but instead modulate the translation of the target mRNAs. In most cases, it has not been possible to identify precise mechanisms by which the protective mRNA binding proteins exert their effects, but an obvious possibility is the inhibition of "pro-turnover" binding proteins by physical interference.

Our group has identified one such family of pro-turnover ARE binding proteins, the tristetraprolin (TTP) family of CCCH tandem zinc finger proteins. The prototype of this group, TTP, was originally identified by virtue of its immediate early response gene characteristics in a screen for genes whose transcription was rapidly stimulated by insulin [11]. It was identified independently by groups studying genes rapidly turned on by phorbol esters [12,13] and serum [14]. Its role in the turnover of ARE-containing mRNAs was discovered after TTP knock-out mice exhibited physical characteristics of TNF excess, and macrophages derived from them secreted excess TNF, due in part to increased stability of the TNF mRNA [15–17]. TTP was then identified as a class II ARE binding protein, able to stimulate the destruction of this mRNA by unknown means [17]. Later studies showed a similar effect on the turnover of GM-CSF mRNA, and it was suggested that increased secretion of this factor in the TTP knock-out mice might account for some of the myeloid hyperplasia characteristic of the TTP deficiency syndrome [18,19]. The GM-CSF experiments also demonstrated that the primary effect of TTP deficiency was to inhibit deadenylation of this mRNA, leading to the hypothesis that the primary effect of TTP, after ARE binding, was in some way to promote the deadenylation of ARE-containing mRNAs.

The two other known members of the TTP protein family, now known as Zfp36L1 and Zfp36L2 in mice, are able to exert similar effects to TTP in cell transfection studies [20], but their physiological mRNA targets are still not known. A fourth family member, C3H-4, has been identified as an apparently purely maternal gene product in *Xenopus* and fish oocytes and eggs [21], and it is interesting to speculate that it plays a role in mRNA deadenylation during early development.

Regulation of TTP Activity in Cells

It was apparent from the very earliest studies of TTP induction in response to insulin, growth factors, cytokines, and so on, that cellular levels of this mRNA, and presumably protein, could be regulated rapidly and dramatically through stimulated transcription. The very transient nature of the TTP mRNA induction curve indicates a rapid shut-off of transcription within an hour or so of its stimulation, coupled with a very unstable mRNA. Although the protein levels in cells appear to be rather low, making antibody detection difficult, it has been possible to confirm in several studies that the changes in protein levels largely parallel those of the mRNA. This rapid and dramatic transcriptional response to mitogens and growth factors represents one major mode of regulation of TTP's cellular activity, that is, regulation of its steady-state protein concentrations.

A second mode of regulation was suggested by early experiments in which TTP's subcellular compartmentalization was regulated by the same mitogens and growth factors that stimulate its transcription [22]. These studies indicated that serum, growth factors, and phorbol esters could rapidly (within 5 min) stimulate TTP's translocation from nucleus to cytosol. More recently, we have shown that the nuclear export of TTP and its two mammalian relatives is mediated through the exportin CRM1, using nuclear export sequences on the amino terminus of TTP and on the carboxyl termini of Zfp36L1 and Zfp36L2 [23]. We also demonstrated that the return to the nucleus was mediated through the tandem zinc finger domain itself, although this intracellular movement did not require that the protein be competent to bind mRNA. Thus, a second mode of regulation of TTP's (and related proteins') cellular activity is change in nuclear to cytosol localization. Very little is known about how these

processes are regulated, but the rapid nature of the nuclear export in response to growth factors suggests the involvement of phosphorylation or dephosphorylation.

A third mode of regulation was suggested by the early recognition that TTP is phosphorylated in cells in response to many of the same agonists that stimulate its rapid transcription on nuclear to cytoplasmic translocation [24]. We found that recombinant TTP could be phosphorylated in a cell-fee assay by MAP kinase, and that at least one of the serines phosphorylated by MAP kinase in the cell-free assay was also phosphorylated in intact cells in response to agonists that activate MAP kinase; however, replacement of this serine by alanine did not affect the ability of the stimulatory agonists to promote TTP's nuclear to cytoplasmic translocation. It is now clear that many other phosphorylated residues exist on TTP in intact cells, and we are attempting to catalog these and determine which are modified in response to stimulation of cells, and how these modifications might affect TTP's translocation and RNA binding activity.

We recently analyzed the last point as part of a study evaluating the possibility that TTP might be modified in response to another MAP kinase family member, the p38 kinase [25]. Although previous data on the relationship between p38 kinase activation and TNF secretion are somewhat contradictory, most authors have found that the use of relatively specific p38 inhibitors inhibits lipopolysaccharide (LPS)-stimulated TNF secretion from macrophages. A recent example of this demonstrated that, in freshly isolated human mononuclear cells, the inhibitor SB202190 at $3\,\mu M$ could inhibit LPS-stimulated TNF secretion by approximately 90%, and that this was associated with dramatic destabilization of TNF mRNA [26]. These and other data raised the interesting possibility that the p38 effect on TNF mRNA stability might be mediated in part by changes in the phosphorylation status of TTP. We first demonstrated that TTP-deficient macrophages were relatively refractory to the same p38 inhibitors that were capable of inhibiting TNF secretion from wild-type littermate macrophages. In addition, recombinant TTP could be phosphorylated by p38 in a cell-free system, and its phosphorylation in response to LPS in macrophages could also be inhibited by the p38 inhibitors. These data led us to propose a scheme in which phosphorylation of TTP by p38 kinase would result in a decrease in its affinity for the TNF mRNA, thus allowing for increased TNF mRNA stability and cellular concentrations and for commensurate increases in TNF secretion. This would be reversed by the p38 inhibitors, which would have little influence on the TTP-deficient cells because of loss of this critical target. We attempted to validate this scheme by performing "global" dephosphorylation of TTP expressed in 293 cells by alkaline phosphatase, and then comparing the binding affinities of phosphorylated and dephosphorylated TTP in an RNA gel shift assay. In keeping with our proposed model, the TTP phosphorylated in 293 cells had considerably less affinity for its ARE target than the dephosphorylated form. Thus, although the phosphorylation sites responsible for this change in RNA binding affinity are not known, it appears that TTP activity in cells is likely to be regulated on a minute-to-minute basis by its phosphorylation status. This third mode of TTP regulation is also likely to affect the subcellular localization of the protein and, thus, control its cytosolic concentrations and likely activity indirectly.

Conclusion

It has become clear from numerous studies that mRNA turnover should not be considered a "default" pathway reversing the more regulated process of transcription, but instead a complex set of cellular processes regulated independently. Because many of the ARE-containing mRNAs encode clinically important cytokines, it seems likely that the current intensive study of the regulated turnover of these mRNAs will lead to new insights into the cellular processes of deadenylation, exosomal decay of the mRNA body, relationship of the mRNA cap to its turnover, importance of other *cis*- and *trans*-acting elements, and so on. The molecular details of these processes should be understood more completely in the coming years. In addition, it is possible that increased understanding of these processes may lead to the development of new therapies for diseases in which the goal is modulation of circulating cytokine levels.

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CHAPTER 318

CPEB-Mediated Translation in Early Vertebrate Development

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Introduction

How a single fertilized egg gives rise to the billions of specialized cells of the adult is the most basic question of developmental biology. While the transcriptional activity of certain master control genes sends cells along certain lineages that culminate in adult tissue differentiation, at very early times of development, especially during germ cell formation, translational control is particularly influential. In female germ cells, several mRNAs that are synthesized and stored during the long period of oogenesis are "masked" or repressed. These mRNAs are subsequently translated in response to exogenous cues such as hormonal stimulation during meiosis (see later discussion) or fertilization. The proteins encoded by masked mRNA are often regulatory, and they dictate the timing of cell division, establish the germ layers, and specify the body axis. Because the translation of maternal mRNAs in vertebrates has been analyzed almost exclusively in the frog *Xenopus laevis* and the mouse, it is worthwhile considering certain key steps of oogenesis in these species.

Soon after their arrival at the genital ridge, the mitotically dividing oocytes enter meiosis and progress through the end of prophase I. During this period, they form the synaptonemal complexes that are required for genetic recombination and synthesize and store mRNA, tRNA, ribosomes, and presumably all the other factors that are necessary for protein synthesis. During their growth phase, the oocytes are arrested in diplotene, the last stage of prophase I, which in many respects resembles G_2 of the mitotic cell cycle. They contain pre-MPF (M-phase promoting factor), an inactive form of the kinase cdc2/cyclin B heterodimer, which when subsequently activated by the MAP kinase cascade, stimulates the oocytes to enter M phase (metaphase I). At the peak of M phase, cyclin B is destroyed, an event that must precede the oocyte's transition into anaphase (AI), which is followed by a second round of MPF activation and entry into metaphase II (MII) [1]. At this point, the cytostatic factor (CSF) is activated, which prevents the oocytes from progressing through the cell cycle until they are fertilized [2–4].

Although many of these events take place in all vertebrate oocytes, some key differences are seen among the animal groups. For example, in *Xenopus*, progesterone, acting through a surface-associated receptor, stimulates reentry into the meiotic divisions, commonly referred to as oocyte maturation. In the mouse, it is the oocyte's liberation from the follicular environment that stimulates maturation. Another important difference in oocyte maturation between these two animals is that only in *Xenopus* is protein synthesis necessary to stimulate maturation. In this species, the MAP kinase cascade that culminates in MPF activation is initiated by Mos, a MAP kinase kinase kinase. Oocytes have no Mos protein, but instead contain dormant mos mRNA that must undergo translational activation for maturation to occur. In the mouse as in *Xenopus*, *de novo* Mos synthesis is required for the MAP kinase cascade [5] and mos mRNA is also under translational control [6]. However, the function of Mos in the mouse is not to stimulate maturation, but to arrest the cell cycle at the end of MII, an activity that is also conserved in *Xenopus* (i.e., CSF, *vida supra*). Indeed, the preponderance of ovarian cysts and tumors in female mos knock-out mice is due to the fact that unovulated oocytes undergo parthenogenetic activation and subsequently exhibit unrestricted cell division [7,8].

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Mechanism of Translational Control

In *Xenopus* and mouse oocytes, mos and several others mRNAs such as those encoding the cyclins A1, A2, and B2, are under translational control by cytoplasmic polyadenylation. That is, these mRNAs are dormant in oocytes and are appended with short poly(A) tails, usually ~20–40 nucleotides. On the induction of oocyte maturation, the tails on these messages are elongated, up to ~100–150 nucleotides, and translation ensues. During the past few years, progress has been significant in delineating the steps of cytoplasmic polyadenylation and how this process leads to translational activation. Several dormant mRNAs contain cytoplasmic polyadenylation elements (CPEs) within their 3′ untranslated regions (UTRs). These CPEs, which have the general sequence of UUUUUAU, usually reside within about 100 nucleotides 5′ of the nuclear pre-mRNA cleavage and polyadenylation hexanucleotide AAUAAA. Both of these elements are necessary for cytoplasmic polyadenylation. The CPE is bound by CPEB, an RNA recognition motif (RRM) and zinc finger type of RNA-binding protein [9–11], and the AAUAAA is bound by a cytoplasmic version of cleavage and polyadenylation specificity factor (CPSF) [12,13], a complex of at least three proteins (see later discussion). The initiation of polyadenylation takes place when CPEB serine 174 is phosphorylated by the kinase Aurora (also known as Eg2 or IAK1; for nomenclature, see [14]) [15], which itself is activated soon after the maturation process is initiated [16]. The function of this phosphorylation event is to induce CPEB to bind, with enhanced avidity, CPSF, and possibly stabilize the latter factor's interaction with the AAUAAA [17]. However, note that Dickson *et al.* [18], while confirming the CPEB–CPSF interaction observed no enhanced interaction between these factors when CPEB was phosphorylated (see [19] for review of cytoplasmic polyadenylation). Although the precise function of CPSF in cytoplasmic polyadenylation has not been demonstrated, by analogy with nuclear pre-mRNA polyadenylation, it is to bring poly(A) polymerase (PAP) to the end of the mRNA, where it catalyzes poly(A) addition [18].

Although the basic cytoplasmic polyadenylation reaction involves Aurora, CPEB, CPSF, and PAP, a number of important questions remain regarding this process. First, nuclear CPSF consists of four subunits with molecular sizes of 160, 100, 70, and 30 kDa. At least in *Xenopus* oocytes, the 70 species is missing from the cytoplasm [18]. Whether the activity of this protein is dispensable for CPSF activity or whether it is functionally replaced by a cytoplasmic form of the protein is not known. Second, it has been reported that with some CPE-containing mRNAs, mammalian nuclear CPSF can recapitulate cytoplasmic polyadenylation *in vitro* without a requirement for CPEB [12,18]. However, given that the components of cytoplasmic oocyte CPSF are not known in their entirety, an extrapolation of the *in vitro* activities of nuclear mammalian CPSF to cytoplasmic *Xenopus* CPSF may be open to argument. Clearly, it is important to isolate and characterize cytoplasmic CPSF. Third, although PAP appears to have catalytic activity both before and after maturation [18], the observation that cdc2-mediated phosphorylation inactivates the enzyme during M phase seems paradoxical. That is, as cells enter mitosis (maturation in oocytes), cdc2 (MPF) becomes active and phosphorylates PAP at multiple sites, particularly in the carboxy terminus, which, at least in somatic cells, inactivates the enzyme [20–22]. Given that cytoplasmic polyadenylation is most robust as oocytes mature [23,24], it seems plausible, if not likely, that another PAP that is not inactivated at this time would also be present in cells. Indeed, oocytes do contain a PAP that lacks a large portion of the carboxy terminus and, hence, lacks the major cdc2 phosphorylation sites [25]. In addition, another PAP that is overexpressed in certain mammalian tumors apparently is not phosphorylated M phase and, thus, also has the potential of catalyzing polyadenylation at M phase [26].

CPEB and Early Development

In the four metazoans in which it has been examined, CPEB has been shown to be essential for early development. In *Drosophila*, various mutations in the CPEB (Orb) gene lead to arrested oogenesis (the most severe allele) or defects in embryogenesis (weaker alleles) [27,28]. While Orb has clearly been demonstrated to mediate mRNA localization, it also controls cytoplasmic polyadenylation of several mRNAs, including its own [29–31]. The nematode *Caenorhabditis elegans* has four CPEB genes, and RNAi injection experiments have demonstrated that one of them is involved in spermatogenesis [32]. However, no target mRNAs have been identified, and the molecular functions of the CPEB proteins in worms are not known.

In vertebrates, the essential nature of CPEB and cytoplasmic polyadenylation for development was first demonstrated in *Xenopus*, where the injection of CPEB antibody into oocytes was shown to abrogate meiotic maturation [33]. This block occurred because mos mRNA was not polyadenylated or translated, thus preventing the MAP kinase cascade and the activation of MPF [33,34]. CPEB is also important for cell cycle progression in *Xenopus* embryos. In embryonic cells, as in all cells, progression into M phase requires the activation of MPF, whereas the subsequent destruction of cyclin B and the inactivation of MPF is necessary to transition into interphase [35]. However, at least in embryonic cells, not only is regulated cyclin B mRNA translation important for cell cycle progression, so too is regulated cyclin B mRNA localization. That is, in the blastomeres of the developing embryo, CPEB and maskin, as well as cyclin mRNA and protein are all associated with mitotic spindles and centrosomes [36]. The injection of neutralizing antibody for both CPEB and maskin blocks cell division, as does cordycepin (3′-deoxyadenosine), an inhibitor of polyadenylation. *In vitro* binding experiments demonstrated that CPEB is a microtubule-binding protein, which may explain its association with spindles. Perhaps most importantly, the injection of embryos with a deletion mutant CPEB protein that cannot interact

with microtubules, although having little effect on cyclin synthesis, caused cyclin mRNA to dissociate from spines. The result of this dissociation was the cessation of cell division.

The first interpretation of these results is that the dominant negative CPEB protein, because it retained the capacity to bind RNA, outcompeted endogenous CPEB for binding to cyclin mRNA, but because it could not also bind microtubules, it diffused away from the spindles. The second interpretation is that cyclin mRNA translation was required locally, on or near spindles, where it mediates functions related to cell division [36]. These results would also suggest that the polyadenylation of cyclin mRNA is regulated during the cell cycle, and that this regulation is necessary for cell division. Such a hypothesis has recently been tested by Groisman *et al.* [37], who have prepared cell extracts capable of undergoing cell cycle progression. These investigators found that cyclin mRNA polyadenylation increased in the S-phase to M-phase transition and that if polyadenylation was blocked, cyclin was not synthesized nor did the extract enter M phase. Thus, it appears that at least in embryonic cells, cyclin mRNA translation is essential for cell cycle progression.

Finally, a CPEB knock-out (KO) mouse has been generated, and the results firmly demonstrate the necessity of this protein for meiotic progression [38]. Adult CPEB KO females were sterile and devoid of ovaries. Female KO embryos at 18.5 days of development (E18.5) had ovaries, but for the most part these did not contain oocytes. E16.5 embryos did have ovaries with oocytes, but the oocyte nuclei stained aberrantly with hematoxylin and with antibody directed against GCNA1, a germ cell nuclear antigen of unknown function. The staining showed the chromatin to be diffuse and/or fragmented. At 16.5 days of development, the oocytes are normally in pachytene of meiosis I a time of synaptonemal complex (SC) formation. The SC, which facilitates recombination, is comprised of several proteins, but two of them, SCP1 and SCP3, are encoded by CPE-containing mRNAs. In wild-type animals, CPEB was found to be associated with the mRNAs *in vivo*. In the CPEB KO animals, SCP1 and SCP3 mRNAs were neither polyadenylated nor translated, and thus the SC was not formed. Without the SC, there is no recombination, and the oocytes and subsequently the ovary degenerate. CPEB is therefore necessary for two stages of meiosis, at pachytene and during the maturation.

Conclusions

While this brief review has focused on CPEB-induced polyadenylation as one mechanism of translational control in early development, it is by no means the only mechanism. For example, in *Xenopus* oocytes, histone mRNAs, unlike their somatic counterparts, are polyadenylated and mostly dormant. Following the oocytes' reentry into the meiotic divisions, the core histone mRNAs are activated in a poly(A)-independent manner. A stem-loop in the 3′UTRs of histone mRNAs is bound by the stem-loop binding protein (SLBP), which, in a manner yet to be deciphered, facilitates translational repression and activation [39]. In addition, during spermatogenesis in the mouse, the protamine mRNAs are also translationally regulated in a poly(A)-independent manner by other 3′UTR binding proteins [40]. Finally, it is worth noting that the mechanisms of translational control in early development could have a number of other important consequences. Consider that CPEB-mediated polyadenylation also appears to control translation in the mammalian brain, where it could influence synaptic plasticity and long-term memory storage [41,42]. Thus, nature reuses translational control mechanisms in multiple tissues, although this should come as no surprise to the 21st century investigator.

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CHAPTER 319

Translational Control in Invertebrate Development

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Introduction

Invertebrate genetic model organisms, most notably *Drosophila*, have been instrumental in efforts to understand the role of translational control in regulating gene expression that underlies development. I will summarize some of the mechanisms of translational control that are emerging from this work. Space constraints do not permit this review to be an exhaustive one, yet this article should provide the reader with the means to gain an overview of the field, and to enter the relevant literature in a focused way.

Translational control is a critical mechanism for establishing pattern in the *Drosophila* oocyte, and the axes that are set down in the developing egg define the positional information of the embryo and of the adult fly [1–2]. Whereas the anterior determinant, Bicoid, is deployed mostly through asymmetric localization of its mRNA, many of the proteins required for posterior and germ cell specification are deployed both through localization of their mRNAs and position-dependent translational regulation. These mRNAs accumulate preferentially in a specialized cytoplasm, called *pole plasm*, at the posterior pole of the egg. Their translation is repressed throughout most of the oocyte, but is active within the pole plasm.

Translational Control Targets Oskar to the Pole Plasm

One gene under elaborate translational control is *oskar* (*osk*). The *osk* gene is required for specification of posterior and of the germ line, and *osk* mRNA accumulates in the pole plasm [3]. Unlocalized *osk* is translationally silent, but localized *osk* is translated from two start sites: constitutively at a low level from the more 5′ site to produce an isoform called Long Osk, and from an internal initiation codon to produce a second isoform called Short Osk. Short Osk is sufficient to fully rescue the phenotype of an *osk* mutant, and studies of the translational control of *osk* have mostly concentrated on Short Osk [4].

Translational repression of unlocalized *osk* depends on sequences within its 3′ untranslated region (UTR) and on several proteins and gene activities (Fig. 1). Bruno (Bru), an RRM-type RNA-binding protein, is a translational repressor that binds to three regions of the *osk* 3′UTR called Bru response elements (BREs). Flies bearing an *osk* transgene mutated for the BREs (*oskBRE*) translate *osk* precociously, and BREs are able to confer translational repression on a heterologous mRNA [5]. Bru also represses *osk* translation in extracts in a BRE-dependent manner [6,7]. A second protein, Apontic (Apt), interacts with Bru both in the two-hybrid system and biochemically and is believed to be an essential cofactor [8]. Another protein, p50, cross-links to both the 5′ and 3′ ends of the *osk* mRNA [9]. Transgenes carrying a mutation that specifically reduces p50 binding show precocious *osk* translation, suggesting that p50 is also required for translational repression of *osk*. Similar genetic evidence implicates Bicaudal-C (Bic-C), a KH domain protein, as another translational repressor of *osk* [10].

Activation of *osk* translation also involves several factors. A dsRBD-type RNA-binding protein, Staufen (Stau), is required for translational activation of *osk*, and *oskBRE*$^{-}$, indicating that Stau does not operate simply as a derepressor [5]. Stau is also required for *osk* localization, and both localization and translational regulation of *osk* depend on the region of Stau containing the dsRBDs. However, the precise domains

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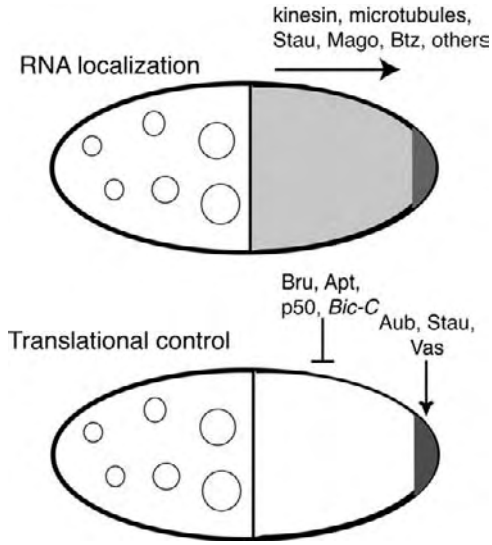


Figure 1 RNA localization and translational control work in concert to restrict the distribution of Oskar (Osk) protein to the pole plasm. *osk* RNA is transcribed in the nurse cells and transported through the ring canals into the early oocyte. Later, *osk* RNA is localized at the posterior pole in a manner that depends on microtubules and several factors, but this process does not result in an absolute restriction of the RNA to the pole plasm. Light gray indicates a low concentration of *osk* RNA, while dark gray represents the high concentration of *osk* RNA in the pole plasm. Translational regulation ensures that protein is not produced from the unlocalized component of *osk* RNA, while the *osk* RNA that is present in the pole plasm is translationally active. Osk protein is depicted in brown.

necessary for localization and translational regulation are distinct [11]. Localization of *osk* mRNA to the pole plasm is probably brought about through kinesin I-mediated microtubule-directed transport, and also requires at least two other proteins, Mago Nashi and Barentsz, which are conserved in evolution but have no previously identified functional domains [12,13].

A key activator of *osk* translation is the Aubergine (Aub) protein, which is related to, but is not the same as, translation initiation factor eIF2C [14]. Aub is a component of polar granules, but, perhaps surprisingly, its ability to activate *osk* translation does not depend on its localization to the pole plasm. Aub cannot work simply by alleviating Bru-mediated repression, because translation of *oskBRE*$^{-}$ remains sensitive to *aub* function. It has been suggested [14] that tenacious binding of localization factors to specific RNAs might itself repress translation. This idea is supported by recent work on ME31B, a DEAD-box helicase that colocalizes in RNPs with several RNAs that localize to the oocyte, and which represses their translation in nurse cells [15]. Aub may then function to alleviate this sort of translational repression.

The RNA helicase Vasa (Vas), which is related to translation initiation factor eIF4A, has also been implicated as regulating *osk* translation [4], but a careful analysis of the temporal sequence of events at the posterior pole of the oocyte indicates that the requirement for Vas is at a later stage of oogenesis than the requirement for Aub [14]. Vas interacts with the essential translation factor eIF5B, originally called dIF2, suggesting that it may activate translation at the step of ribosomal subunit joining [16,17].

Translational Control Targets Nanos to the Pole Plasm

Translational control targets Nanos to the pole plasm, and Nanos establishes the Hunchback gradient by translational control. *nos* mRNA accumulates in the pole plasm but is also present throughout the embryo; only 4% is posteriorly localized and only that localized fraction is translationally active [18]. A region of 90 nucleotides of the *nos* 3′UTR, termed the translational control element (TCE), is predicted to form two extended stem-loops, and mutations abolishing these structures render the element inactive. The loop portion of stem-loop II corresponds to the binding site for the translational repressor Smaug (Smg) [23,24], a protein lacking any previously identified RNA-binding motifs. Smg appears to be the key translational repressor of unlocalized *nos* RNA. Analysis of *nos* in polyribosomal profiles suggests that translational repression of unlocalized *nos* occurs after initiation [25].

Vas has been implicated in activating *nos* translation at the posterior pole, because *nos* transgenes containing either the TCE or the entire 3′UTR of *nos* are not translated in *vas* mutants [20]. When the TCE is deleted, translation no longer depends on *vas*, implying that the role of Vas in activating *nos* translation involves overcoming Smg-mediated repression [19]. Because Smg and Osk interact directly [23], as do Vas and Osk [26], a complex of pole plasm components including Osk and Vas may deactivate Smg on *nos* transcripts that enter the posterior region, leading to derepression of translation.

Nos generates an anterior-to-posterior gradient of the Hb transcription factor by repressing translation of the maternal *hb* transcript in the posterior part of the embryo. To do this, Nos forms a complex with Pumilio (Pum) and the Nos-response element (NRE) sequences within the *hb* 3′UTR [27]. Translational repression of *hb* involves deadenylation promoted by Nos and Pum [28], but can occur in the absence of a poly(A) tail [29]. *hb* repression also requires an association of Nos, Pum, and the NRE with the NHL protein Brain Tumor (Brat) [30]. Nos and Pum have also been implicated in repression of *cyclin B* translation in pole cells [31], and Pum binds to NRE sequences in the maternal *cyclin B* 3′UTR [30]. This function of Nos and Pum, however, is not mediated through Brat, suggesting that the ternary complex of Nos, Pum, and NRE sequences can interact with different cofactors. Proteins related to Pum are very widely conserved in evolution and may have a fundamental role in promoting proliferation of stem cells [32].

Translational Control in the *Drosophila* Nervous System

Although the developing oocyte and preblastoderm embryo are the developmental stages during which translational

control is believed to have its most important roles in regulating gene expression, a number of recent studies have implicated translational control mechanisms in later development as well. This is most notable in both the developing and the mature nervous system. For example, mechanosensory bristle development in *Drosophila* requires four successive asymmetric cell divisions, and in the first asymmetric division, one daughter cell (called IIa) gives rise to non-neuronal progeny and the other (called IIb) gives rise to neuronal progeny [33]. A transcriptional repressor, Tramtrack69 (Ttk69), is present only in IIa and is required for specification of the non-neuronal cells, yet Ttk69 mRNA is present at similar levels in both the IIa and IIb cells. This asymmetry of Ttk69 protein distribution is brought about through translational repression of *Ttk69* RNA in IIb by an interaction of the Musashi (Msi) protein with specific elements in its 3′UTR [34]. Msi itself is present in both the IIa and IIb cells, but its activity is inhibited in IIa by Notch activity, indicating that this important intercellular communication pathway is capable of influencing gene expression at the level of translation.

Translational control has also been implicated in synaptic plasticity in the mature nervous system. Aggregates containing essential translation components such as eukaryotic initiation factor 4E (eIF4E), poly(A) binding protein (PABP), and the glutamate receptor subunit II mRNA (GluR-II) accumulate in subsynaptic compartments of the larval neuromuscular junctions of *Drosophila* [35]. Alteration of the dosage of genes encoding these translation components is accompanied by changes in the number of these aggregates and the size of junctions, and with changes in the levels of synaptic proteins such as GluR-II. These results were interpreted as suggesting that localized regulation of translation at the synapse might underlie long-term alternations of neuronal function and connectivity.

Role for Translational Control in Regulating Growth

A critical signaling pathway for regulating growth is that which is activated by insulin and mediated through phosphoinositol 3-kinase (PI3K) and the kinase target of rapamycin (TOR) [36,37]. Downstream targets of insulin include factors that regulate translational activity, including ribosomal protein S6 kinase (S6K) [38] and the eIF4E inhibitor 4E-BP [39]. Genetic manipulations in *Drosophila* that alter the activities of S6K and 4E-BP result in alterations to cell and organismal size [38,39], as does a mutation that blocks phosphorylation of the cap binding protein eIF4E [40]. Thus, the general translation factors may be important targets for growth control signaling pathways.

Translational Repression through MicroRNAs

Two *C. elegans* genes that were discovered on the basis of mutations, *lin-4* and *let-7*, were found to encode very short RNAs of approximately 22 nucleotides that have an antisense sequence to a particular target RNA (lin-14 and let-41, respectively) [41–43]. A recent systematic study [44] has shown that at least 17, and perhaps hundreds, of similar genes encoding microRNAs exist in the *C. elegans* genome, and that numerous such genes are predicted in the *Drosophila* and human genomes as well. Thus microRNA-mediated translational repression could prove to be a widespread mechanism of regulating gene expression.

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CHAPTER 320

Role of Alternative Splicing During the Cell Cycle and Programmed Cell Death

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Introduction

Alternative splicing plays an important role in gene expression in metazoan organisms. By the mid-1980s, it was estimated that 5% of genes in higher eukaryotes might be subject to alternative splicing [1]. However, recent genome-wide analyses involving computational identification of alternative splicing frequencies indicate that 35–60% of human genes give rise to at least one alternatively spliced form [2–6]. As an extreme example, the *Drosophila* DSCAM gene has the potential of generating more than 38,000 isoforms by alternative splicing [7]. These findings suggest a significant role for alternative splicing in both the generation of protein diversity and also the control of gene expression.

Pre-mRNA splicing occurs in a macromolecular complex, the spliceosome, which consists of small nuclear ribonucleoprotein particles (snRNPs) and a large number of non-snRNP protein factors [8]. Regulation of pre-mRNA splicing provides the main posttranscriptional control of gene expression in higher eukaryotic cells [9]. A combination of genetic and biochemical approaches has allowed progress in identifying both *cis*-acting elements on the pre-mRNA and *trans*-acting protein factors. Among the *cis*-acting elements, exon splicing enhancers stimulate splicing of adjacent upstream introns. SR proteins, a group of *trans*-acting protein factors, play key roles in the activation of splicing enhancers [10,11]. Negative regulatory sequences also exist, and seem to interact with hnRNP proteins as well as SR proteins, and also participate in splicing regulation. Therefore, splicing control can be achieved by coordination between positive and negative regulatory elements in the pre-mRNA and the variation or activity of individual SR proteins in cells [12]. Phosphorylation of splicing factors has been demonstrated to influence pre-mRNA splicing and thus likely also contributes to splicing regulation [13]. In this chapter, we summarize some of the progress made in the past years on pre-mRNA splicing regulation, focusing on genes involved in programmed cell death (apoptosis) and cell cycle control.

Apoptosis and Splicing

A remarkable feature of genes encoding proteins that function in the apoptotic, or programmed cell death (PCD), pathway is that many of them are transcribed into mRNA precursors that can be alternatively spliced to produce proteins with opposite effects on programmed cell death, either facilitating or preventing apoptosis (see Table I for a partial list of such genes; also [14]). The fact that such splicing patterns are commonplace strongly suggests that splicing control plays a significant, physiologically relevant role in affecting programmed cell death, at least in some cases. Although conclusive evidence establishing this is not yet available, a number of studies point in this direction, and they suggest that changes in the activity of splicing factors, such as SR proteins, may be involved.

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Table I Alternatively Spliced Products with Opposite Effects on Programmed Cell Death

Receptors	Fas (Apo-1, CD95)
Bcl-2	Bcl-2
	Bcl-x
	Bcl-w
	Bax
	Bim
	Mcl-1
Ced-4	Ced-4(Apaf)
Caspases	Caspase-1 (ICE)
	Caspase-2 (Ich-1)
	Caspase-3
	Caspase-4
	Caspase-6 (Mch-2)
	Caspase-7 (Mch-3, ICE-LAP3, CMH-1)
	Caspase-10 (Mch-4)

Evidence that inactivation of an SR protein, ASF/SF2, can lead to apoptosis has recently been described. Specifically, a genetically engineered chicken DT40 B-cell line was constructed such that expression of ASF/SF2 could be strongly repressed by tetracycline [15]. Following depletion of ASF/SF2, the cells stop growing and undergo cell death via an apoptotic pathway ([16]; J. Wang, X. Li, and J. L. Manley, in preparation). Although the exact mechanism by which ASF/SF2 depletion leads to PCD is unknown, it may involve alteration of the splicing pattern of at least one transcript encoding an apoptotic regulator: Bcl-X. Production of mRNA encoding the anti-apoptotic Bcl-X_L is reduced while accumulation of the pro-apoptotic Bcl-X_S isoform increases. Although the changes in splicing are modest, and Bcl-X_L remains the predominant isoform, this change may be sufficient to at least contribute to, or facilitate, PCD in ASF/SF2-depleted cells. Consistent with this, overexpression of a Bcl-X_L cDNA significantly delays onset of cell death following ASF/SF2 depletion (J. Wang, X. Li, and J. L. Manley, in preparation).

The above-described experiments argue that changes in the concentration of an SR protein can alter splicing of apoptotic regulators and contribute to PCD. But do similar situations occur naturally in response to apoptotic stimuli? Several studies indicate that significant changes in SR protein accumulation and/or phosphorylation can occur during apoptosis, for example, induced by Fas ligand, γ-irradiation or UV light [17–19]. These experiments provided evidence that SR proteins become hyperphosphorylation or hypo-hyperphosphorylated in response to these various stimuli and can then be found in complexes, with U1 snRNPs or even snoRNPs, that did not exist prior to apoptosis induction. These changes could have significant affects on SR protein activity. For example, hyperphosphorylation and hypophosphorylation are both known to reduce SR protein activity in *in vitro* splicing assays [20] and hyperphosphorylation can also enhance or stabilize interactions with U1 snRNP [21]. Thus either changes in hyperphosphorylation *per se* or the formation of stable complexes could in effect reduce the concentration of available, active SR proteins, leading to changes in alternative splicing, such as those described earlier for Bcl-X, thereby inducing or enhancing apoptosis.

Another example of slicing regulation involves splicing of the pre-mRNA encoding the Fas receptor and the RNA dividing protein TIA-1 [22]. Alternative splicing of the Fas receptor pre-mRNA results in a membrane-bound form, which promotes PCD, or a soluble form, which inhibits apoptosis. TIA-1 appears to promote apoptosis by activating production of membrane-bound isoform of the Fas receptor. TIA-1 was found to bind to uridine-rich sequences adjacent to the 5′ splice site of exon 5 of the Fas pre-mRNA and recruit U1snRNP to the 5′ splice site, activate exon 6 inclusion, and thereby promote accumulation of the membrane-bound Fas receptor. Depletion of TIA-1 in a fibroblast cell line or deletion of the poly(U) tract adjacent to the 5′ splice site leads to skipping of exon 6, and TIA-1 overexpression increases exon 6 inclusion. Although the physiological relevance of these observations has not been confirmed yet, TIA-1 might promote apoptosis by activating a weak 5′ splice site in the Fas pre-mRNA. This mechanism for regulating alternative splicing by TIA-1 has been shown in other cases, such as the fibroblast growth factor receptor-2 pre-mRNA [23], male-specific lethal-2 pre-mRNA in *Drosophila* [24], and the meiosis-specific MER2 pre-mRNA gene in *Saccharomyces cerevisiae*, by Nam8p, the yeast homolog of TIA-1 [25].

Cell Cycle and Splicing Regulation

Transitions through the cell cycle are highly regulated by the activities of cyclin-dependent kinases (CDKs). Cyclins, which associate with and regulate the CDKs, accumulate only at specific times during the cell cycle. In addition to this temporal control, which involves ubiquitin-mediated proteolysis of the cyclin, CDK activity can be regulated by phosphorylation, and physical interaction with CDK inhibitor proteins (reviewed in [26]).

Links between pre-mRNA splicing and the cell cycle are likely, but have not been fully investigated. In yeast, both *Schizosaccharomyces pombe* and *S. cerevisiae*, several genes initially isolated by virtue of cell cycle phenotypes have been found to encode proteins involved in splicing, and mutations in some known splicing factor genes display cell cycle phenotypes (reviewed in [27]). But little is known about the functional significance of these genetic interactions.

One aspect of cell cycle control is the silencing of gene expression that occurs during mitosis in metazoan cells. Recent years have provided insights into how this occurs and into the underlying mechanisms involved, which, for example, involve phosphorylation of key components of the transcription [28–30] and polyadenylation [31] machineries, and dephosphorylation of a translation initiation factor required for cap-dependent translation [32]. In contrast to all these processes, it is unclear what happens to the splicing machinery during M phase. Our recent studies [32a]

have provided insights into this issue. We first addressed the question of whether splicing is inhibited in M-phase cells, and found that it was indeed blocked in whole-cell extracts prepared from nocadazol arrested cells. Intriguingly, though, significant inhibition was only detected in high-salt extracts, suggesting that a mitosis-specific factor associated with insoluble cellular structures is required for inhibition. A candidate is a novel SR protein, SRp38, that we have recently characterized. SRp38 is unlike other SR proteins in that it is unable to activate splicing in *in vitro* assays (reviewed in [10]). Rather, SRp38 seems to be a dedicated splicing repressor and, importantly, its activity is strongly activated by dephosphorylation. Remarkably, SRp38 becomes significantly dephosphorylated during M phase, and is extracted from cell extracts only at the same high-salt concentrations required to detect M-phase-specific repression. These studies have shown that splicing, like other steps in gene expression, is repressed during mitosis. Analogous to the mechanism involved in translational inhibition [32], splicing inhibition appears to involve dephosphorylation, specifically of the SR protein SRp38.

Recently, two cdk-related kinases have been described that may function in splicing. Ko *et al.* [33] described a novel protein kinase as *C*dc2-*r*elated *k*inase with an arginine/serine rich (*RS*) domain (CrkRS). CrkRS has extensive proline-rich regions that match the consensus for SH3 and WW domain binding sites and, most significantly, an N-terminal RS domain. RS domains, as exemplified in SR proteins, are protein–protein interactions characteristic of splicing factors. CrkRS colocalizes in nuclear speckles with other splicing factors, and immunoprecipitates of CrkRS were found to phosphorylate CrkRS itself, exogenously added ASF/SF2, and the GST-tagged C-terminal domain of the RNA polymerase II largest subunit (CTD). CrkRS is hyperphosphorylated in mitosis and hypophosphorylated in interphase. The function of CrkRS is currently unknown, but it has the potential to function in linking transcription and splicing with the cell cycle.

In keeping with the existence of an RS domain-containing cdk, a recent human database search has identified an RS domain-containing cyclin, called CYCLIN L [34]. Recently, the mouse homolog of CYCLIN L, called ania-6, has been described. Intriguingly, ania-6 is rapidly induced in the adult striatum by cocaine or direct dopamine stimulation [35]. Different types of neurotransmitter stimulation cause selective induction of distinct ania-6 isoforms (Ania-6a^{60} and Ania-6a^{25}), through alternative splicing. The longer protein, Ania-6a^{60}, contains a C-terminal RS domain, whereas the shorter isoform, Ania-6a^{25}, does not. Ania-6a^{60} is localized to nuclear speckles, whereas Ania-6a^{25} is not localized to the nucleus. Perhaps significantly, ania-6 sequences are more similar to invertebrate homologs of cyclins than to other mammalian cyclins, and closer to cyclins associated with RNA pol II (such as cyclin K or T) than to cyclins directly involved in cell cycle control (such as cyclin A1). Immunoprecipitation studies with FLAG-Ania-6a^{60} indicate that the protein is associated specifically with the hyperphosphorylated form of RNA polymerase (RNAP IIO), the splicing factor SC35, and the p110 PITSLRE cdk. The idea that RNAP II, and specifically the CTD, participates in mRNA processing is now well accepted (reviewed in [36]) and RNAP IIO has been shown to stimulate pre-mRNA splicing *in vitro* [37]. It seems possible that Ania-6a^{60}, or CYCLIN L, as well as crKRS, play roles in coupling transcription with splicing. How, and if, this coupling functions in regulating expression of specific genes, and whether such controls might be cell cycle related, are interesting questions for the future.

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CHAPTER 321

NF90 Family of Double-Stranded RNA-Binding Proteins: Regulators of Viral and Cellular Function

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Summary

Double-stranded RNA-binding proteins in the nuclear factor 90 (NF90) family participate in the regulation of gene expression at both transcriptional and translational levels. Well-known members of the family are NF90 itself and NF110, which differ from one another at their C termini and are encoded by the ILF3 gene. We propose a uniform nomenclature for these proteins, many of which have been given alternative names. Related proteins, SPNR/p74 and ZFR, are encoded by distinct genes. The NF90 family proteins contain two tandem copies of the double-stranded RNA-binding motif, flanked by regulatory regions. Present in humans as well as other mammals and *Xenopus*, NF90 family members associate with nucleic acids, both DNA and RNA, and with several proteins including nuclear factor 45 (NF45) with which they copurify. Complexes have been detected containing NF45 as well as other proteins involved in signal transduction, DNA repair, transcription, splicing, and translation. NF110 is more effective than NF90 in activating gene expression at the transcriptional level, and NF45 exerts a modulatory function. In its translational role, NF90 interacts directly with mRNAs as well as with the protein kinase PKR, which phosphorylates initiation factor eIF2. Members of the NF90 family appear to coordinate gene expression by serving as flexible adaptors that integrate cellular signals via recognition of both proteins and structured nucleic acids.

Introduction

Highly structured and double-stranded RNAs (dsRNAs) transmit a multiplicity of signals within cells. In many cases, the signal is transduced by dsRNA-binding proteins containing a motif known as the dsRNA-binding motif (dsRBM, [1]). Although more than 26 dsRBM-containing proteins have been identified in the human genome, the cellular functions of many of these proteins have not yet been fully deciphered [2]. One such group of dsRBM proteins is the NF90 family. NF90 was originally isolated with its heteromeric partner NF45 as a complex that binds to the antigen recognition response element (ARRE-2) present in the interleukin 2 (IL-2) promoter [3,4]. Independently, partial cDNAs were cloned as M-phase phosphorylated proteins [5] and homologs named 4F.1 and 4F.2 were characterized as dsRNA-binding factors present in *Xenopus* extracts [6]. Over the years, several NF90-related proteins have been found in different species, using various techniques, and were named according to the screen through which they were identified. Table I lists the NF90 homologs reported, indicating how they were isolated, the names assigned, and the functions putatively ascribed. An underlying theme that has emerged implicates the proteins as regulators of both viral and cellular gene expression pathways, particularly at the transcriptional and translational levels.

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Table I Isolation of NF90 Homologues

Name	Species	Source	Identification	Putative function	Reference
NF90	Human	Jurkat cells	ARRE-2 affinity chromatography	Regulator of activated transcription in T-cells	Kao, P.N., *et al.*, 1994
NF90	Human	293 cells	Adenovirus VA RNA_{II}-specific interacting protein	None assigned	Liao, H.-J., *et al.*, 1998
NF90	Human	Placenta	Component of DNA-PK complex	Facilitated interaction of DNA-PK/Ku complex with dsDNA	Ting, N.S.Y., *et al.*, 1998
NF90	Human	K562 cells	Autoantibodies from pristane treated mice	None assigned	Satoh, M., *et al.*, 1999
NF90	Human	HeLa cells	Interacts with encapsidation signal ε of hepatitis B virus (HBV) RNA	Facilitates binding of HBV polymerase to RNA	Shin, H.J., *et al.*, 2002
NF90	Bovine	Brain	DHS-22 binding protein	Repressor of HLA-DRα gene expression	Sakamoto, S., *et al.*, 1999
MPP4	Human	HeLa and MOLT4	MPM2 reactive protein	None assigned	Matsumoto-Taniura, N., *et al.*, 1996
DRBP76	Human	HeLa cells	Poly (I:C) chromatography and PKR-interacting protein in yeast two hybrid	Substrate of PKR	Patel, R.C., *et al.*, 1999
DRBP76 DRBP76α DRBP76δ ILF3	Human	SK-MeL-28 melanoma cells	Degenerate primers to Rel similarity domain (RSD) of NFAT	Regulator of melanotransferrin gene	Duchange, N., *et al.*, 2000
NFAR1/2	Human	Jurkat cells	PKR-interacting protein in yeast two hybrid	Enhancer of SV40 transcription in mammalian cells	Saunders, L. R., *et al.*, 2001
TCP80	Human	Liver	Interacts with GCase mRNA	Repressor of translation of GCase and other cellular RNAs	Xu, Y.-H. and Grabowski, G.A., 1999
ILF3	Mouse	Male germ cells	Library screen using Prml 3′UTR	RNA metabolism	Buaas, F.W., *et al.*, 1999
ILF3	Mouse	10d embryo	Exportin-5-interacting protein in yeast two-hybrid	None assigned	Brownawell, A.M. and Macara, I.G., 2002
ILF3	Rat	FAO cells	PRMT1-interacting protein in yeast two-hybrid	Substrate and activator of PRMT1	Tang, J., *et al.,* 2000
4F.1/4F.2	Xenopus	Ovary	Library screen using dsRNA	None assigned	Bass, B.A., *et al.,* 1994
CBTF122/ CBTF98	Xenopus	Oocyte extract	Component of CCAAT box binding factor (CBTF)	Regulator of GATA-2 transcription	Orford, R.L., *et al.,* 1998
SPNR	Mouse	Male germ cells	Library screen using Prml 3′UTR	Spermatid RNA metabolism	Schumacher, J. M., *et al.,* 1995
p74	Rat	Smooth muscle	RNA probe	None assigned	Coolidge, C.J., and Patton, J.G., 2000
ZFR	Mouse	Male germ cells	Library screen using Prml 3′UTR	None assigned	Meagher, M. J., *et al.,* 1999

ARRE-2 = Antigen Recognition Response Element-2
CBTF = CCAAT Box Transcription Factor
DRBP76 = Double-stranded RNA Binding Protein 76 kDa
GCase = Acid β-Glucosidase
ILF3 = Interleukin Enhancer Binding Factor 3
MPM2 = Monoclonal antibody reactive to a set of related M-phase phosphorylation sites
MPP4 = M-Phase Phosphoprotein 4
NFAR = Nuclear Factor Associated with dsRNA
SPNR = Spermatid Perinuclear RNA-binding Protein
TCP80 = Translational Control Protein 80 kDa

Members of the NF90 Protein Family

Homologs have been identified in *Xenopus* and three mammals, including humans, and their relationships are summarized in Table II. NF90 is the prototype of a class of human dsRNA-binding proteins generated by the alternate splicing of transcripts from the interleukin enhancer binding factor 3 (ILF3) gene locus located at 19p13 [7,8]. Two regions of the gene undergo alternate splicing events, resulting in the production of at least six unique predicted protein products. Two major species with apparent molecular weights of 90 and 110 kDa are detected by Western blotting. An alternate splicing event with major consequences occurs at the C terminus of the protein coding sequence following exon 17. The 90-kDa species, NF90 (also known as DRBP76 and NFAR1), results from the splicing of exon 17 to exon 18. Two additional cDNAs, DRBP76α and DRBP76δ, are generated via the use of alternate 3′ acceptor sites. These three species have been identified at the mRNA level. Although the corresponding protein products have yet to be conclusively identified *in vivo*, the DRBP76, DRBP76α, and DRBP76δ proteins are predicted to have 702, 694, and 690 amino acids, respectively, and masses of 75–76 kDa [8–10].

Nuclear factor 110 (NF110, also called ILF3 and NFAR2) is generated by the splicing of exon 17 to exons 19, 20, and 21. The resulting slower migrating protein (~110 kDa) is predicted to have 894 amino acids and a mass of 96 kDa [8,9]. Peptides from the unique C terminus of NF110 have been detected [11]. Two mRNA species encoding the NF110 protein were identified in melanoma cells, differing in the length of the 3′ untranslated regions (UTRs) possibly because of alternative polyadenylation site usage [8]. The consequence of the presence or absence of the extended 3′UTR has not been described.

A second alternate splicing event occurs at the exon 13–14 junction and stems from the usage of two different 3′ acceptor sites. Exon 14a contains an additional 12 bp, which leads to the addition of four amino acids (NVKQ) between the two dsRBMs, giving rise to NF90b and NF110b. The corresponding isoforms from which the insert is absent are termed NF90a and NF110a. The 4-amino-acid insert has been identified in both NF90 and NF110 at the mRNA level and in NF90 at the protein level [8,11]. The presence of the insert increases the ability of NF110 to stimulate gene expression in a transfection assay [11,12].

The originally isolated cDNA encoding NF90, referred to as NF90c, appears to contain an additional two nucleotides (TC) after position 1798 of the NF110 mRNA, leading to a frame shift that results in premature termination of the protein. The origin of this variant cDNA, whether it is an artifact of cloning or a bona fide naturally occurring mutant or variant form, remains to be determined. TCP80 is another unique NF90-related cDNA, isolated from a human liver cDNA library [13]. The precise origin of TCP80 by alternative splicing is unknown but it appears to result from the usage of a unique 5′ acceptor site in exon 17 and a novel 3′ acceptor site in exon 21 (Genbank entry AF202445). The expression of this protein isoform in mammalian cells also remains to be verified.

Two additional homologs of NF90, SPNR/p74 and ZFR, are encoded by distinct gene loci and are not alternative splice products of the ILF3 gene. SPNR/p74 was picked up in biochemical screens for RNA-interacting proteins [14,15]. Unlike NF90, which is predominantly nuclear, SPNR binds to microtubules and localizes to the manchette, a spermatid-specific microtubular array [15,16]. SPNR and NF90 do not serve redundant functions in mice, since $SPNR^{-/-}$ mice have neurological, spermatogenic, and sperm morphological abnormalities [17]. ZFR contains three C_2H_2 zinc fingers in its N terminus, followed by a C terminus that is highly homologous to the N terminus of NF90 (amino acids 1–316). The cDNA encoding this protein was identified in an expression library by screening with the protamine 1 (Prm1) 3′UTR [18]. Although the function of ZFR is unknown, it is required for the embryonic development of mice [19].

Table II Comparison of NF90 Homologues from Four Different Species

Species	NF90a/b	NF110a/b	Unique cDNAs
Homo sapiens	DRBP76a DRBP76b NFAR1	ILF3a ILF3b NFAR2	NF90c TCP80 DRBP76α DRBP76δ
Mus musculus	*NI*	ILF3	
Rattus norvegicus	*NI*	ILF3	
Xenopus laevis	4F.1 $CBTF^{98}$	4F.2 $CBTF^{122}$	

NI = cDNA not identified
a or b suffix indicates absence or presence of the NVKQ insertion
For references, see text.

Domain Structure of NF90 Family Proteins

Inspection of the primary sequence of NF90 reveals homology with other proteins and the existence of several known motifs (Fig. 1). Prominent are the two type-A dsRBMs present in the center of the protein between amino acids 404–465 and 526–592. An additional short nucleic acid binding motif, the RGG motif, lies between residues 640 and 659 in NF90, NF110 and their homologs except for NF90c ZFR, and p74/SPNR. This motif was previously identified in tandem with another nucleic acid binding domain in nucleolin, the RNA recognition motif (RRM), with which it worked in concert to enhance nucleic acid binding activity [20]. When expressed in isolation, the RGG domain displays avid affinity for single-stranded (ss)RNA and RNA structures such as G-quartets [21,22]. This region also includes several asymmetric arginine dimethylation consensus sites (RxR and RGG), known targets for protein arginine methyltransferases [23]. A functional bipartite nuclear localization signal is located from amino acids 370 to 394, just upstream of the dsRBMs, and is required for strong nuclear localization of NF90 [24].

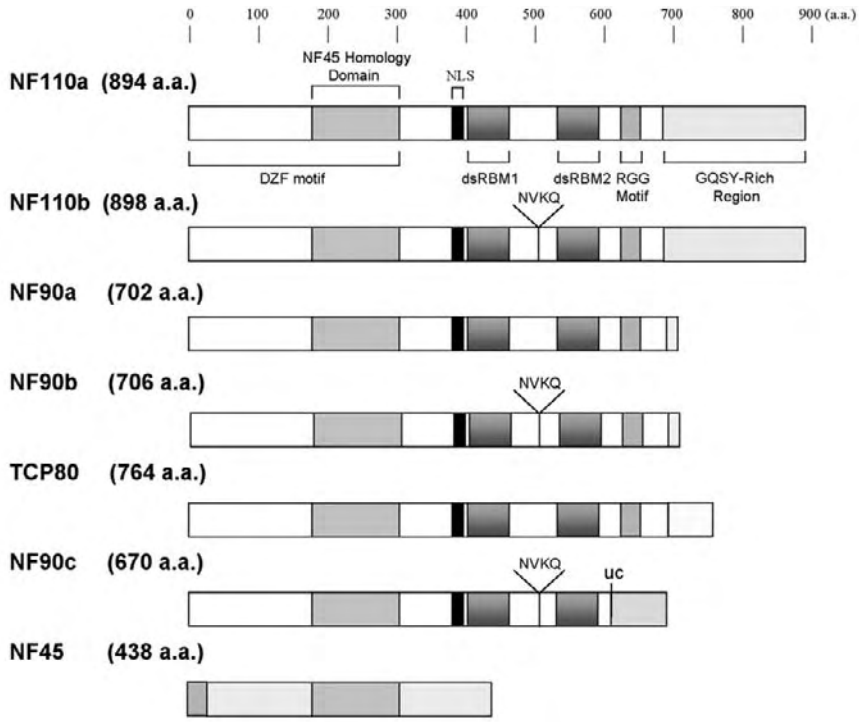


Figure 1 Domain structure of the NF90 homologs. Schematic representation of the proteins encoded by the human ILF3 gene compared with NF45, the heteromeric partner of NF90. NVKQ is the tetrapeptide insertion present between the dsRBMs in the b isoforms, and uc indicates the two bases inserted in the NF110b RNA, which encodes the NF90c protein. DRBP76α and DRBP76δ are not depicted but are identical to NF90 except for the remote C-terminal region. a.a. = amino acid.

NF90 shares extensive homology with its heterodimeric partner NF45. This motif, putatively titled the NF90/NF45 homology region, is located from amino acids 136 to 337. A similar but more extended motif, the DZF motif (acronym for domain in DSRM or ZnF C2H2 domain containing proteins), encompasses the entire N terminus of NF90. This motif is also found in the NF90-related protein, ZFR [18]. In NF90, the NF90/NF45 homology region is the binding site for NF45 and is thought to play a regulatory role by inhibiting the transcriptional activation function of NF90's C terminus. Binding of NF45 to NF90 relieves the inhibitory effect of this domain on the C terminus of the protein, an effect that can be mimicked by deletion of the N terminus from NF90 [24]. It is tempting to speculate that the DZF motif plays a similar regulatory role in ZFR, and that NF45 acts as a general regulator of proteins that contain this motif.

The NF110 isoforms have an additional motif encoded by exons 19, 20, and 21. Their alternatively spliced C-terminal region contains a motif rich in glycine, glutamine, serine, and tyrosine, which we have provisionally titled the GQSY domain. This domain shares limited homology with regions found in other RNA-binding proteins including RNA helicase A (RHA), hnRNP A, and the EWS (Ewing sarcoma) family of proteins that includes EWS, TLS/FUS, and the TATA box protein (TBP)-associated factor $TAF_{II}68$. The GQSY domains of other proteins have been reported to have both nucleic acid binding [25] and protein–protein interaction properties [26]. In NF110, this domain modulates dsRNA binding and increases the protein's ability to enhance gene expression in transient expression assays [9,12]. As discussed later, NF110's GQSY region was found to interact with TLS/FUS and SMN in a yeast two-hybrid assay [9].

Proteins That Interact with NF90

Association of NF90 family members with several different proteins and protein complexes has been reported, suggestive of participation in various cellular processes (Fig. 2). The *Xenopus* orthologs of NF90 and NF110, $CBTF^{98}$ and $CBTF^{122}$ (also known as 4F.1 and 4F.2), were isolated as part of a CCAAT box transcription factor complex that binds to the CCAAT box element present in the GATA-2 promoter. In the early stages of *Xenopus* development, this protein complex is localized in the cytoplasm, anchored to a large pool of untranslated maternal mRNAs via the dsRBMs of CBTF. Prior to the midblastula transition (MBT), the cytoplasmic RNA is degraded and the complex translocates to the nucleus, where it presumably activates transcription of its target genes [27,28].

NF90, NF110, and p74 all interact with and act as substrates for PKR, a critical regulator of translation in mammalian cells [9,10,14,29,30]. PKR's translational function is exerted via the phosphorylation of translation initiation factor eIF2,

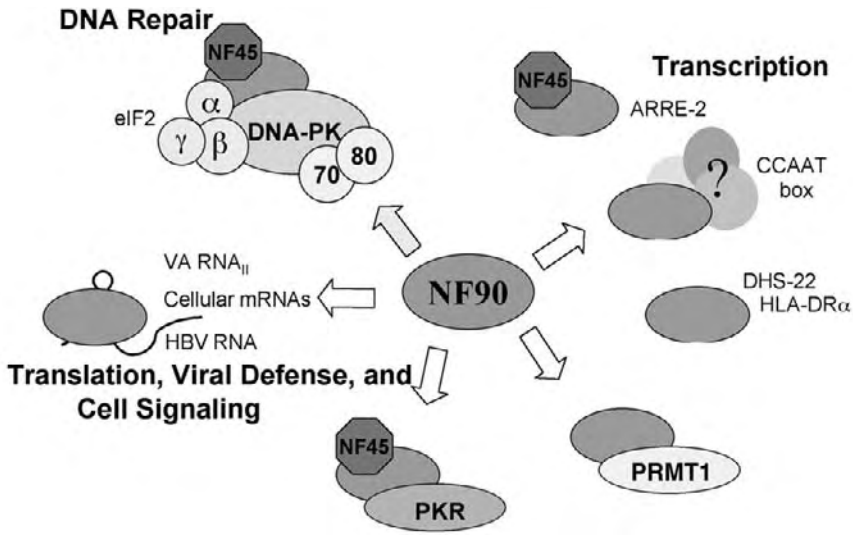


Figure 2 NF90 family proteins: functions and complexes. For details, see text.

resulting in the inhibition of protein synthesis. In an *in vitro* assay, NF90c blocked the activity of PKR, presumably by sequestering dsRNA [30]. NF110 also interacts with and is a substrate for the protein arginine methyltransferase 1 PRMT1. Furthermore, NF110 stimulated the activity of PRMT1 in an *in vitro* methylation assay [23]. NF110 was also isolated as an exportin 5-interacting protein using a yeast two-hybrid screen. Exportin 5 is a karyopherin that functions to transport dsRBM proteins from the nucleus to cytoplasm. Interestingly, the NF110:exportin-5 interaction is inhibited by dsRNA [31].

Two NF110-specific interacting proteins, SMN and TLS, were isolated in a yeast two-hybrid screen conducted with the unique C terminus of NF110 [9]. SMN is an essential component of a complex required for the biogenesis and function of several ribonucleoprotein (RNP) complexes [32–38]. The N terminus of TLS was originally identified as part of a fusion protein resulting from chromosomal translocations in several cancers [39]. Subsequently, TLS itself was found to function in splicing and transcription as well as other cellular processes [40–42]. The interaction of NF110 with TLS and the immunoprecipitation of a spliceosome complex with NF90-specific antibodies suggest a possible role for NF110 in mRNA splicing [9].

NF90 and NF45 copurified in a large complex from placenta that contains the translation factor eIF2, the double-stranded DNA-dependent protein kinase DNA-PK, and the DNA-binding proteins Ku70 and Ku80 [43]. In this complex, NF90/NF45 stabilized the association of Ku/DNA-PK with dsDNA, possibly implying a role for these proteins in DNA repair and perhaps suggesting that they form a link between DNA repair and translation. NF90 and NF45 were also recently identified as components of a complex that binds to the encapsidation signal ε of hepatitis B virus (HBV) RNA and facilitates binding of HBV polymerase to the RNA [44]. This finding extends the range of activities with which NF90 family members are associated to include viral replication, but the functional significance of these diverse interactions remains to be determined.

Nucleic Acid Binding Properties of NF90

Not surprisingly, the nucleic acid binding properties of NF90 and its homologs have been studied extensively. The proteins bind to both DNA and RNA although some of the interactions, especially with DNA, may be indirect. As mentioned earlier, NF90 and its *Xenopus* homologs CBTF^{122} and CBTF^{98} were originally isolated by virtue of their affinity for the enhancer elements ARRE-2 and CCAAT, respectively [3,28]. Furthermore, the CCAAT box DNA-binding factor present in the complex was identified by an expression library screen and determined to be CBTF^{122} [28]. NF90 and NF110 were also identified as factors binding to a unique sequence (DHS-22) present in the DNase I hypersensitive site II in HLA-DRα [45]. Complexes containing NF90, NF110, and NF45 were affinity purified from mammalian cell extracts using ssDNA cellulose and dsDNA cellulose [46]. In many cases, however, the ability of individual purified recombinant proteins to bind directly to dsDNA has yet to be demonstrated, leading to the suggestion that post-transcriptional modification may be required and/or that NF90/NF110 binds DNA via cooperation or interaction with other dsDNA-binding proteins.

In contrast to the absence of a characteristic dsDNA-binding domain, the two dsRBMs of NF90 and its homologs interact tightly with dsRNA and the synthetic dsRNA-like polymer poly(I:C) [6,9,10,14,29,30]. In addition, NF90 binds with somewhat lesser affinity to highly structured ssRNAs such as VA RNA$_{II}$, RNA-DNA hybrids, and ssRNA at high concentrations [6,30,47]. The rat homolog p74 also interacts with additional nucleic acid substrates, having high affinity for poly(dI:dC) and poly(G) and weaker affinity for

poly(U) and poly(C). A p74 deletion mutant lacking its two dsRBMs bound almost equally as well to poly(dI:dC), suggesting that the dsDNA-binding domain resides in distinct sequences outside the dsRBM region [14]. It has been inferred from transient expression and RNA-binding assays that the C-terminal RGG and GQSY domains modulate the affinity and specificity of the dsRBMs for dsRNA[12].

Functions of NF90 Homologs

A broad spectrum of functions has been imputed to members of the NF90 family (Table I, Fig. 2) based on their interactions with nucleic acids and other proteins and on their presence in complexes, but the most direct experimental evidence relates to roles in transcription and translation.

NF90 and NF45 were first identified as regulators of the IL-2 promoter. Using polyclonal antibodies specific for NF90 or NF45, this complex was found to be essential for activated transcription from the ARRE-2 enhancer element in an *in vitro* transcription assay using an extract from activated Jurkat T cells [3]. In a transient expression system, NF90 and NF110 function as both positive and negative regulators of gene expression of several cellular and viral promoters in mammalian cells, including the proliferating cell nuclear antigen (PCNA) and the HIV-LTR promoters [9,11,12]. NF90 and NF110 also bind to a specific regulatory sequence in the HLA-DRα gene where they are hypothesized to exert a negative effect on gene expression. Interestingly, NF45 did not copurify as a cofactor in this case, implying that it regulates the activation but not the inhibitory function of NF90 [24,45].

The TCP80 isoform of NF90 acts as a negative regulator of translation in cell-free extracts. Originally isolated as a cytoplasmic protein that binds to the acid β-glucosidase (GCase) mRNA, TCP80 was further characterized as a repressor of translation *in vitro* of several mRNAs including aldolase B, complement protein 8 γ-subunit, and fibronectin receptor β1. This function is dependent on the presence of the N terminus of TCP80 and certain structured regions present in the inhibited mRNAs [13]. TCP80 inhibited the association of GCase mRNA with polysomes with little effect on mRNA translation following initiation [13,48].

Consistent with its role as a regulator of translation, NF90 inhibits the activity of PKR *in vitro* [30]. In *Saccharomyces cerevisiae*, however, NF90 appears to function like PACT, another dsRNA-binding protein [49], causing a slow growth phenotype in the presence of PKR. NF90 also rescued the growth of yeast under conditions of amino acid starvation, an effect that is dependent on PKR activation [50]. Furthermore, NF90 inhibited the translation of both globin mRNA and luciferase mRNA in a cell-free translation system [50]. Using a similar yeast assay, NF45 inhibited the function of PKR and rescued yeast from a slow-growth phenotype. Evidently NF90 and NF45 can function independently of each other and can exert different functions depending on the circumstances.

Cellular Regulation of NF90 and NF45

Despite extensive structural and functional analysis of both NF90 and NF45, little is known about how these proteins are regulated. To date, both NF90 and NF110 have been detected at varying levels via Western blot in every cell line tested, and the two isoforms are always coexpressed. A survey of human tissues revealed that the proteins are expressed at high levels in the testes, ovaries, thymus, and kidney [9]. Similar results were obtained in mice except that no expression was detected in the kidney, but weak expression was detected in the spleen [51]. The association of NF90 with cell growth is further confirmed by the overexpression of NF90 mRNA in nasopharyngeal carcinoma cells [52] and NF90 protein levels in transformed as compared to untransformed fibroblasts [50]. In contrast, THP cells induced to arrest and differentiate using 12-*O*-tetradecanophorbol-13-acetate (TPA) had decreased levels of NF90 mRNA levels as compared to the controls [45]. Examination of the protein levels of NF90 through the cell cycle revealed subtle changes in protein expression with NF90 expression peaking at the G_2/M-phase boundary concomitant with phosphorylation of the protein [5,50].

Even less is known about the expression of the putative regulatory factor of NF90, NF45. The tissue distribution of NF45 has not been reported, but like NF90, NF45 has been found in every cell line examined [4,50,53]. NF45 mRNA is overexpressed in cervical cancers when compared to normal tissue, further supporting a role for NF90/NF45 in regulating genes required for cell growth and proliferation [54]. In contrast, exposure of HL60 cells to X-rays (5 Gy) strongly down-regulated NF45 [55]. Similarly, treatment of bronchial epithelial cells with triptolide, a potent immunosuppressant and anti-inflammatory drug, inhibited TPA-induced expression of NF45 [56]. Examination of the NF90 and NF45 protein levels in various cell types revealed that the levels of the two proteins and the ratios between them vary considerably [50]. These data suggest that the ratio of expression between the two proteins could act to regulate their function; in addition, as suggested by the yeast data discussed earlier [50], these proteins can not only function together in a complex, but may also function separately.

It is likely that NF90 function is regulated by posttranslational modification. As mentioned, NF90 can be phosphorylated and methylated *in vitro* by two known protein modifiers, PKR and PRMT1, respectively [9,23,30]. The PKR phosphorylation site of NF90 maps within the C-terminal portion of the protein containing its two dsRBMs [30]. A close NF90 homolog, MPP4, was identified by virtue of its interaction with MPM2, a monoclonal antibody that recognizes a set of M-phase phosphorylation sites. NF90/ NF110 is phosphorylated specifically during the M-phase of the cell cycle, and homology searches identified five potential MPM2 phosphorylation sites [5]. Seven putative CKII and 10 putative PKC phosphorylation sites have been identified in TCP80 [13]. The consequences of these modifications have yet to be determined, but their existence

supports a role for NF90 and its homologs in regulating cell cycle and proliferation.

Conclusions

The NF90 family is a group of dsRNA-binding proteins shown to regulate both transcription and translation in vertebrate cells. They are also implicated, but less firmly, in a diverse set of viral and cellular functions ranging from replication and DNA repair to splicing. Members of this family have been identified in several organisms using various techniques. They are found in a variety of protein complexes, most notably with NF45, and bind to nucleic acids including dsRNA, dsDNA, and structured RNAs including adenovirus VA RNA_{II}, HBV RNA, and some mRNAs. Conceivably they serve as flexible adaptors that recognize classes of both nucleic acids and proteins and modulate macromolecular synthesis at several levels. Further identification and characterization of novel RNA and protein partners should create a more detailed blueprint of the cellular and viral processes regulated by the NF90 family and will provide insight into their roles in normal and abnormal cellular processes.

Acknowledgments

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CHAPTER 322

Signaling Pathways That Mediate Translational Control of Ribosome Recruitment to mRNA

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Introduction

Alterations in mRNA translation rates in eukaryotes accompany cell proliferation, differentiation, and growth. Translation initiation is the major target of translational control [1,2]. Specifically, the step of recruitment of ribosomes to the mRNA is tightly regulated. This control is exerted primarily by phosphorylation of initiation factors. Some of the signaling pathways that mediate phosphorylation of initiation factors have been extensively studied and are described in this chapter.

Nuclear transcribed eukaryotic mRNAs possess at their 5′ termini the structure m^7GpppX (where X is any nucleotide), termed the *cap* [3,4]. The cap structure strongly facilitates the translation of a large majority of mRNAs [4]. However, some viral mRNAs (such as those of picornaviruses) [5] and a subset of cellular mRNAs translate by a cap-independent mechanism [6]. Cap-dependent mRNA translation initiation is dependent on eIF4E, the cap binding protein, and requires the participation of a set of initiation factors (the eIF4 family), which facilitate the recruitment of the ribosome to the 5′ end of the mRNA [1,4,7]. Cap-dependent translation is counteracted by members of a family of translational inhibitors (the eIF4E binding proteins; 4E-BPs). Many of the initiation factors that promote ribosome recruitment to the mRNA and the repressors of this process are phosphoproteins. The phosphorylation status of these proteins is modulated by extracellular stimuli and environmental stress [1,4]. We describe the current model for cap-dependent, initiation factor-assisted assembly of a ribosome–mRNA complex and how this activity is regulated by translational repressors via specific signaling pathways in mammals. Phosphorylation of initiation factors is also important in regulation of mRNA recruitment in plants, but the signaling pathways have not been elucidated [8]. It is also unclear if and how phosphorylation of initiation factors plays a role in mRNA recruitment to ribosomes in yeast [9].

eIF4F Complex Formation

Affinity chromatography using m^7GDP-agarose was used to identify and purify eIF4E, the cap-binding protein [10,11]. Subsequently, it was discovered that eIF4E functions as a complex, termed eIF4F, with two other polypeptides: eIF4A and eIF4G [12]. eIF4A is an RNA helicase, which in combination with two RNA-binding proteins, eIF4B and eIF4H, and accompanied by the hydrolysis of ATP, is believed to unwind the mRNA 5′UTR secondary structure [1,13]. eIF4G serves as a modular scaffolding protein for several proteins, including eIF4A, eIF4E, and eIF3 (a ribosome-associated initiation factor). The interaction between eIF4G and eIF3 is required for the recruitment of the 40S ribosomal subunit with its associated factors (Fig. 1). eIF4G also binds to PABP, an interaction that brings about the circularization of the mRNA [14]. eIF4G also possesses a C-terminal docking site for Mnk kinase, which phosphorylates eIF4E at Ser209 [15].

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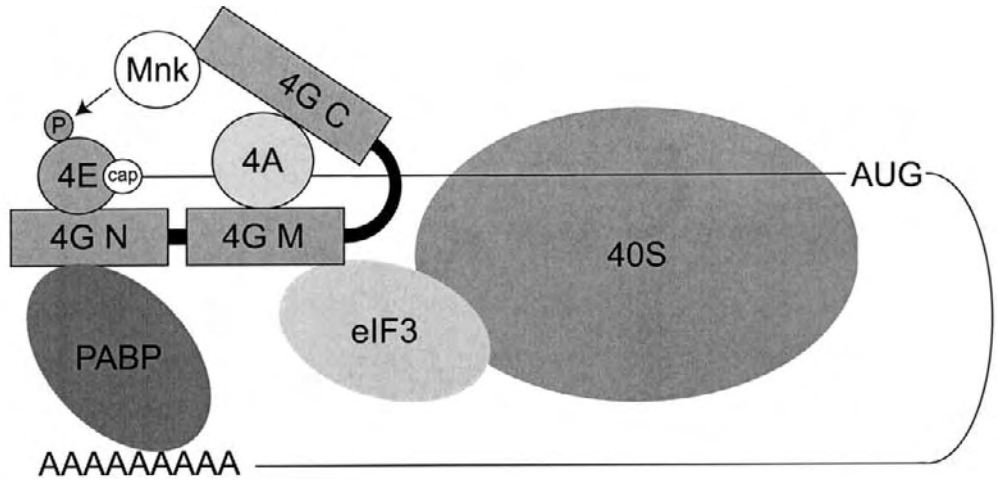


Figure 1 Recruitment of the 40S ribosome to the 5′ end of mRNAs by initiation factors. The eIF4F complex (comprised of eIF4E, eIF4G, and eIF4A) coordinates the recruitment of the 40S ribosomal subunit to the 5′end of eukaryotic mRNAs. The three modular domains of eIF4G (N-terminal domain, 4G N; middle domain, 4G M; C-terminal domain, 4G C) are docking sites for PABP, eIF4E, eIF4A, eIF3, and Mnk1. eIF3 functions to bridge between eIF4G and the 40S ribosomal subunit. eIF4E (indicated as 4E) binds the 5′ cap (m^7G) structure of mRNA. Poly(A)-binding protein (PABP) interacts with the N-terminal domain of eIF4G [62], to mediate circularization of the mRNAs [14]. (Adapted from [61].)

eIF4E Phosphorylation occurs in response to a variety of extracellular stimuli including hormones (such as insulin), growth factors (such as epidermal growth factor), cytokines (tumor necrosis factor α), and mitogens (phorbol ester), and in general positively correlates with cell growth and enhanced translation rates [4]. However, stress induced by arsenite or anisomycin, which inhibit translation, also results in phosphorylation of eIF4E [16]. Both extracellular stimuli and stress activate the Ser/Thr kinase Mnk1 (Mnk2, a homolog of Mnk1 is constitutively active [17]). Mnk-mediated phosphorylation of eIF4E is effected by two pathways: the PD98059-sensitive Ras/Raf/MEK/ERK kinase cascade and the SB203580-sensitive stress-activated p38MAPK signaling pathway (Fig. 2) [16,18]. There is no consensus as to whether phosphorylation of eIF4E stimulates translation, and the mechanism of such stimulation is also controversial (for a review, see [19]). Although some evidence suggests that phosphorylation of eIF4E is not required for general translation *in vitro* [20] or *in vivo* [21], it is noteworthy that flies, which express a mutant eIF4E, Ser209Ala, develop slowly and are smaller than their wild-type counterparts, demonstrating that phosphorylation of Ser209 is biologically significant [22]. In addition to Mnk, PKC (α, β, γ) also phosphorylates eIF4E on Ser209 *in vitro* [23], and in certain circumstances Ser209 is phosphorylated in a PKC-dependent manner *in vivo* [24,25]. Thus, PKC may also play a role in the phosphorylation of Ser209.

Based on the X-ray crystal structure of eIF4E, it was suggested that phosphorylation of Ser209 results in the formation of a salt bridge with Lys159, thus forming a clamp over the mRNA to stabilize its interaction with eIF4E [26]. However, two recent papers [27,28], reported that phosphorylation of Ser209 causes a two to fourfold reduction in the affinity of eIF4E for the cap. Thus, the molecular mechanism by which eIF4E phosphorylation affects its function is unclear.

Repressors of Cap-Dependent Translation

The formation of the eIF4F complex is inhibited by a family of proteins, termed eIF4E binding proteins (4E-BPs) [1]. The 4E-BP family is comprised of three members, 4E-BP1, 4E-BP2, and 4E-BP3, all of which inhibit specifically cap-dependent translation [29,30]. 4E-BPs are also known as PHAS (*p*hosphorylated *h*eat and *a*cid *s*table proteins) [31]. 4E-BPs specifically inhibit cap-dependent translation by binding to eIF4E to impede the eIF4E–eIF4G interaction and, consequently, the assembly of the eIF4F complex [26]. The consensus sequence shared among the 4E-BPs and eIF4Gs, YXXXXLφ (where X is any amino acid and φ is a hydrophobic residue), directly interacts with eIF4E. Biochemical and structural studies demonstrated that the 4E-BPs inhibit the eIF4E–eIF4G interaction by a molecular mimicry mechanism [26]. Indeed, peptides harboring the eIF4E binding motif inhibit cap-dependent translation *in vitro* [26,32].

Modulation of 4E-BP Phosphorylation by FRAP/mTOR

Binding of the 4E-BPs to eIF4E is regulated by phosphorylation (Fig. 2). Hypophosphorylated 4E-BPs bind tightly to eIF4E, resulting in translational inhibition, whereas hyperphosphorylation of 4E-BPs engenders their release from eIF4E [1,29]. Increased phosphorylation of 4E-BPs occurs in response to treatments with hormones, growth factors, mitogens, cytokines, and G-protein-coupled receptor agonists [1].

The PI3K-Akt-FRAP/mTOR signaling pathway plays a critical role in the phosphorylation of 4E-BP1 [33]. PI3Ks regulate a large number of cellular processes including

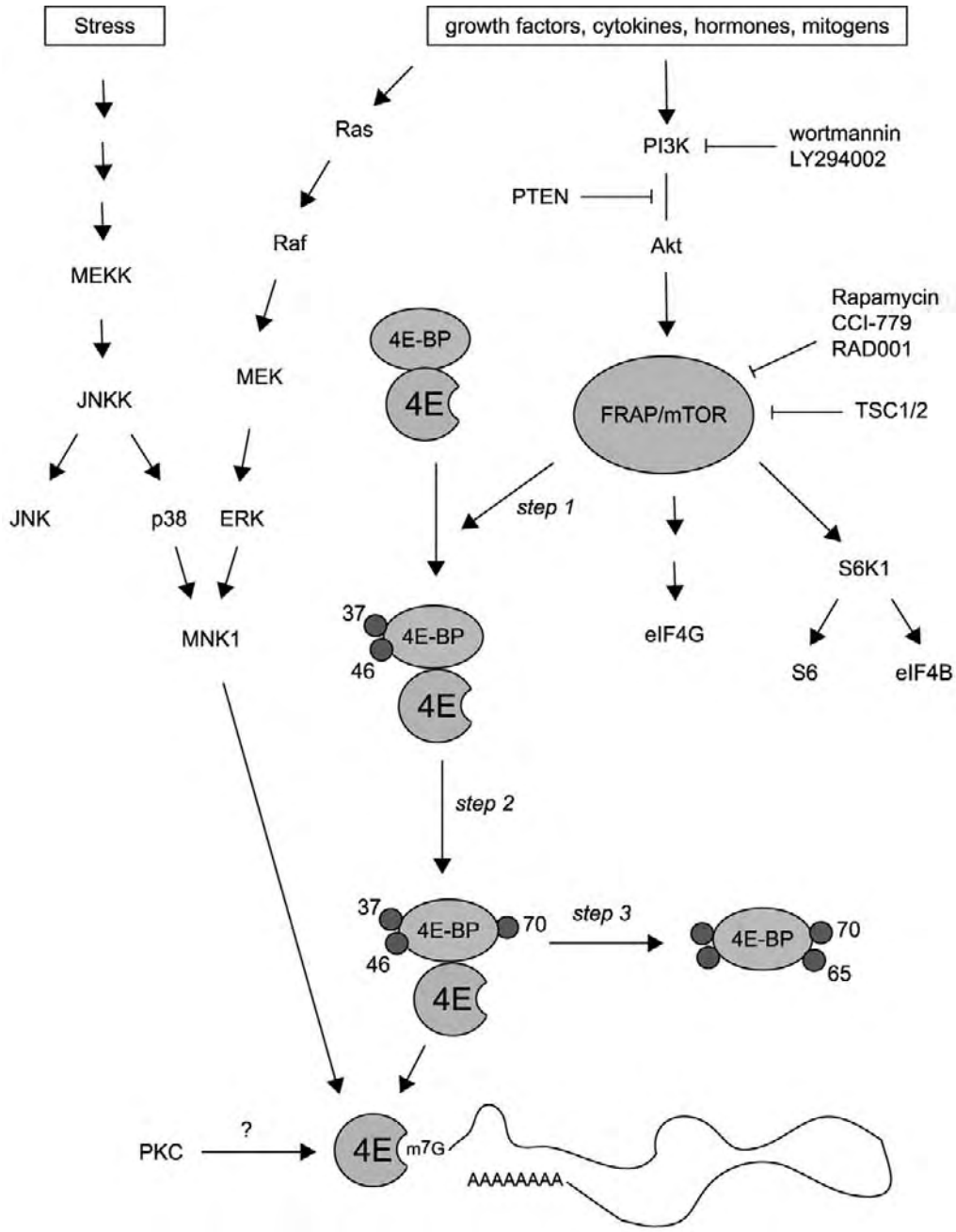


Figure 2 Signaling pathways leading to eIF4E and 4E-BP phosphorylation. Stimuli (i.e., growth factors) activate the ras-signaling and the PI3K-Akt-FRAP/mTOR signaling pathways. 4E-BP1 is phosphorylated through a multistep mechanism. FRAP/mTOR signaling mediates phosphorylation at Thr37 and Thr46 prior to phosphorylation of Ser65 and Thr70. Hyperphosphorylated 4E-BP1 causes its release from eIF4E, allowing for eIF4E to bind to cap (m^7G)-bearing mRNAs. Mutations in PTEN and TSC1/2 contribute to elevated FRAP/mTOR activity, increased levels of unbound eIF4F, and enhanced translation of a subset of mRNAs that harbor extensive secondary structure. FRAP/mTOR activity is inhibited by rapamycin and its derivatives (CCI-779, RAD-001) [63]. The stress-activated and the ras-signaling pathways converge on Mnk1, which phosphorylates eIF4E. PKC may also phosphorylate Ser209.

proliferation, apoptosis, and protein synthesis [34]. PI3K phosphorylates phosphatidylinositol-(4,5)-biphosphate to generate phosphatidylinositol-(3,4,5)-triphosphate. This reaction is negatively regulated by PTEN, a lipid phosphatase, which exhibits tumor activity and is mutated in many cancers [35]. PI3K-generated lipid products bind the pleckstrin homology (PH) domain of the Akt/PKB Ser/Thr protein kinases and target them to the plasma membrane [36]. Overexpression of the p110α PI3K catalytic subunit or Akt/PKB enhances 4E-BP1 phosphorylation [37], providing evidence that 4E-BP1 is a target of PI3K signaling.

FRAP/mTOR (*F*K506 *r*apamycin-*a*ssociated *p*rotein/*m*ammalian target *o*f *r*apamycin) is the mammalian homolog of the yeast TOR proteins, TOR1 and TOR2 [38] (also see

chapters by Schmelzle *et al.* and Radimerski and Thomas in this volume). FRAP/mTOR functions as a major integrator of a variety of physiological states, such as nutrient availability (i.e., amino acids, glucose), extracellular stimuli (growth factors, hormones and cytokines), and energy levels (ATP), to effect cell cycle progression, cell growth, cell size, translation, and protein turnover. Importantly, FRAP/mTOR activity is inhibited by a highly specific drug, rapamycin, a bacterial macrolide that forms a gain-of-function complex with FKBP12 (FK506-binding protein). This complex binds with high affinity to FRAP/mTOR to inhibit its kinase activity toward 4E-BPs and p70 S6 kinase [39].

FRAP/mTOR directly phosphorylates 4E-BP1, because a rapamycin-resistant form of FRAP/mTOR confers rapamycin resistance to 4E-BP1 phosphorylation [40–42]. The phosphorylation sites in human 4E-BP1 are Thr37, Thr46, Ser65, Thr70, Ser83, and Ser112. The phosphorylation of these sites except for Ser83 (equivalent to Ser82 in rat 4E-BP1) [43] and Ser112 is dependent on FRAP/mTOR [33,41]. Ser112, which is present only in 4E-BP1, is phosphorylated by ataxia-telangiectasia mutated (ATM) protein kinase [44] and was reported to be a major insulin-dependent phosphorylation site in adipocytes (equivalent to Ser111 in rat 4E-BP1) [45]. Phosphorylation of 4E-BP1 occurs in a hierarchical, multistep process [33,46]. Phosphorylation of Thr37 and Thr46 is necessary for subsequent phosphorylation at Ser65 and Thr70 [46], because substitution of Thr37 and/or Thr46 for alanine residue(s) prevents phosphorylation of Ser65 and Ser70 [46]. In contrast, substitution of serine for glutamic acid at amino acid residues Thr37 and Thr46 partially restores phosphorylation at Ser65 and Ser70. Thus, Thr37 and Thr46 are phosphorylated first. This primes the phosphorylation of Ser70, followed by the phosphorylation of Ser65 [46]. Thr37 and Thr46 are phosphorylated to a considerable extent even in the absence of serum stimulation and in the presence of rapamycin [33]. In comparison, Ser65 and Thr70 are rapidly dephosphorylated in serum-deprived conditions or in the presence of rapamycin [33]. It was proposed that a protein phosphatase 2A (PP2A) is activated by rapamycin treatment and dephosphorylates Ser65 and Thr70 [47]. The proposed model suggests that FRAP/mTOR also phosphorylates PP2A in order to inhibit dephosphorylation of 4E-BP1 (in addition to p70 S6 kinase) [47].

Phosphorylation of eIF4G and eIF4B

Phosphorylation of eIF4G and eIF4B is also rapamycin sensitive, implicating FRAP/mTOR in their phosphorylation. eIF4G becomes phosphorylated on several residues in response to a diverse array of extracellular stimuli. Phosphorylation of eIF4G positively correlates with translational efficiency, and similar to 4E-BP1, a two-step mechanism for eIF4G phosphorylation has been suggested [48]. Two of the phosphorylation sites were mapped to the linker region that separates the middle and the C-terminal eIF4A binding domains (see Fig. 1) [49]. It was suggested that a single eIF4A molecule contacts both the middle domain and the C-terminal domains [48,49]. The linker region could then serve as a hinge, and its phosphorylation might affect the interaction of eIF4A with eIF4G and, in turn, the activity of eIF4F. eIF4B is phosphorylated on two sites (Ser406 and Ser422) by p70 S6 kinase *in vitro*. These sites are highly likely to be phosphorylated *in vivo* by p70 S6 kinase, because their phosphorylation is inhibited by rapamycin. Strong evidence that phosphorylation of eIF4B on these sites is downstream of S6K1 was obtained by expression of a rapamycin-resistant form of S6K1 (J. W. B. Hershey, personal communication). Under these conditions, serum-stimulated phosphorylation is rendered rapamycin resistant. As is the case for eIF4G, phosphorylation of eIF4B positively correlates with enhanced translation [50]. However, it is not known how the phosphorylation of either eIF4G or eIF4B affects their biochemical activities.

Control of Cell Growth and Proliferation by eIF4E: Link to Cancer

eIF4E is a rate-limiting factor for mRNA translation. Overexpression of eIF4E induces malignant transformation of NIH 3T3 and primary rodent cells [51,52] through mechanisms that are thought to selectively enhance the translation of a subset of mRNAs [51,53]. Poorly translated mRNAs generally harbor long and structured (G/C rich) 5′UTRs. In eIF4E-transformed cells, the translation of such mRNAs is enhanced [53], probably as a result of the increased formation of the eIF4F complex that effects mRNA unwinding activity. Consistent with the transformation activity of eIF4E, poorly translated mRNAs usually encode proteins that regulate cell growth, differentiation, and development [54]. Also consistent with the role of eIF4E in cellular transformation, overexpression of 4E-BP1 or 4E-BP2 in eIF4E-transformed cells, or ras- or src-transformed cells, partially reverted the transformed phenotype [55]. eIF4E is overexpressed in many tumors and transformed cell lines [56,57]. It is thus paramount that rapamycin, which inhibits eIF4E activity, is showing promising results as an anticancer drug [58]. It is a tantalizing possibility that rapamycin acts as an anticancer drug by inhibiting eIF4E activity. Consistent with this scenario, it is noteworthy that mutations in the tumor suppressor PTEN result in elevated Akt inputs to FRAP/mTOR signaling [59]. Also, mutations in the tuberous sclerosis complex (TSC1/2), which is a tumor suppressor and inhibits FRAP/mTOR, lead to benign tumors (hamartomas) [60]. Such mutations cause an increase in 4E-BP phosphorylation [60].

Conclusions

FRAP/mTOR plays an important role in the phosphorylation of the 4E-BPs and consequently in regulation of mRNA recruitment to ribosomes. The phosphorylation status of the 4E-BPs is an important readout of FRAP/mTOR activity that directly correlates with eIF4F assembly and activity.

FRAP/mTOR integrates inputs from many diverse extracellular stimuli, intracellular physiological cues, and environmental stress to affect either an anabolic or catabolic output. Understanding the mechanisms that control 4E-BP phosphorylation should shed light on the involvement of 4E-BPs in physiological processes and in disease.

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PART IV

Events in Intracellular Compartments

Marilyn Farquhar, Editor

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PART IV

Introduction

Marilyn Farquhar

Overview of Signaling from Intracellular Compartments

Over the last 10 years research in signaling has shifted from an exclusive consideration of events that occur at the cell membrane to focus on those events that occur inside the cell. Among the startling revelations of current research in this field has been the realization that signaling can actually be initiated from intracellular compartments. Classically signaling was assumed to initiated only by cell surface receptors and transduced across the cell membrane to reach intracellular compartments. Many of the other Parts of this book deal with the intracellular consequences of signaling initiated from the outside. Part IV focuses on events initiated or taking place exclusively from the inside of the cell.

The articles in Part IV describe what is known at present about many of the best-studied signaling pathways that are initiated or propagated from intracellular compartments. The information summarized also reveals the interplay between signaling and protein trafficking in that often trafficking of signaling molecules is necessary to effect signaling, and protein targeting and trafficking are typically regulated by classical signaling molecules such as GTPases and phosphoinositides.

The best understood signaling pathways that are initiated from intracellular compartments are those from the endoplasmic reticulum and mitochondria. Two fascinating and well-worked out pathways for signaling from the endoplasmic reticulum are described in the chapters by Espenshade, Goldstein and Brown and by David Ron. In the former the authors summarize their pioneering work demonstrating that the ER contains a sterol sensor SCAP in complex with SREBP, a membrane bound transcription factor. When cells are depleted of sterols, SCAP escorts SREBP to the Golgi where SREBP undergoes proteolytic cleavage liberating a fragment of SREBP that acts as a transcription factor and activates genes involved in cholesterol and fatty acid biosynthesis. This work represents a novel paradigm and provides an intriguing example of how interacting components of intracellular signaling networks located in different intracellular compartments are brought together by regulated trafficking. The chapter by David Ron describes the ER stress response or unfolded protein response—i.e., the pathway initiated by the presence of unfolded proteins in the ER. When cells are stressed a pattern of response similar to that for sterols is initiated involving a putative sensor, proteolysis and generation of a transcriptional regulator that also modifies gene expression and up-regulates stress genes and down-regulates others.

Signals can also be initiated from mitochondria as described in the chapters by Liu and Butow and by Pagliari, Pinkoski and Green. The former reviews how the changes in the functional state of mitochondria elicit specific responses mediated via for example, by increased cytosolic Ca2+ and NAD+, leading to effects on transcription and proliferation of mitochondria. The Chapter by Pagliari *et al.* describes signals for apoptosis or cell death that can be initiated either from mitochondria in response to stress or from the cell membrane. Both involve activation of specific enzymes, the executioner caspases that cleave specific proteins leading to DNA fragmentation and cell death.

Many signaling events, particularly stimulation of growth factor receptors, lead to mitosis and cell proliferation. The article by Clare McGowen provides a comprehensive description of signaling events that initiate the cell cycle, which is controlled by cyclin dependent kinases that act in series to coordinate duplication and segregation of cell components during mitosis.

Another basic tenet that has come to light in the last 10 years has been the realization that signaling and trafficking of cell surface receptors are irrevocably intertwined. Most if not all cell surface receptors must traffic to the cell interior for many of the consequences of their activation to be signaled. Moreover, receptor fate is determined within endosomes where the decision is made that they be either recycled to

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act again or degraded (down-regulated). The chapter by Benovic and Keen summarizes current information indicating that growth factor and G protein coupled receptors (GPCR) must be taken up and transported to endosomes for effective signaling to take place. The complementary chapter by Difiore and Scita clarifies the interplay between endocytosis and the cytoskeletal system—e.g., actin, myosin VI and dynamin, and the importance of small GTPases of the rho superfamily in the regulation of endocytosis. This chapter brings out the widespread importance of signaling through small GTPases in regulation of the cytoskeleton as well as endocytic trafficking.

The chapter by Eiden provides a more classical description stimulus-secretion coupling which is outside to inside signaling where the stimulus is provided by a ligand or secretagogue and the response is release of secretion granules from endocrine or exocrine organs or synaptic vesicles from neurons or neurosecretory cells. The emphasis is on the role of Ca2+ and cAMP in stimulating exocytosis and the role of snare complexes in recognition and fusion. The chapter by Prudovsky *et al.* describes the generation and release of signaling molecules such as FGF1 that lack classical signal sequences and appear to be transported independent of the ER-Golgi pathway by mechanisms that remain unclear.

Many signaling events affect the expression of specific genes, which requires trafficking of signaling regulators such as transcription factors and cyclins between the nucleus and cytoplsm. The chapter by Cingolari and Gerace describes current knowledge of the molecular mechanisms involved in trafficking of molecules into and out of the nucleus which involves targeting signals, receptors and signaling molecules, as in the case of other protein targeting events. Here, once again, another small GTPase—ran–plays a key role.

Finally, it should be stated, that it seems likely that what we know at present about signaling from intracellular compartments represents only the tip of the iceberg, and it is probable that cells can respond to changes in the environment of many if not all cell organelles. It seems intuitive that the cell must be able to respond and adapt to changes in key metabolites as it responds to changes in cholesterol composition. Although not dealt with here, it is clear that signals are generated via peroxisomes in response to changes in lipid metabolism that lead to peroxisomal proliferation. It seems likely that signaling events can also be initiated from the Golgi apparatus, given its role in trafficking, processing and packaging of macromolecules for transport along the exocytic pathway, and the fact that abundant signaling molecules such as small GTPases, heterotrimeric G proteins, protein kinases, and phosphoinositides, among others, are found there. It follows that many other signaling pathways initiated from inside the cell remain to be discovered, and many gaps in our knowledge remain to be filled in.

CHAPTER 323

SREBPs: Gene Regulation through Controlled Protein Trafficking

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Introduction

Cholesterol is an essential component of mammalian cell membranes. Sufficient levels of cellular cholesterol are required for the integrity and impermeability of the plasma membrane, for the proper assembly of cell surface lipid rafts and caveolae, and for the posttranslational modification of at least one protein, the morphogen Hedgehog [1,2]. However, too much unesterified cholesterol is toxic to cells. Thus, levels of intracellular cholesterol must be tightly regulated [3]. Here, the cell faces a fundamental problem: How does a cell measure the concentration of insoluble, membrane-embedded cholesterol and then appropriately adjust its levels? This chapter outlines the molecular mechanism that cells utilize to maintain proper cholesterol homeostasis.

Mammalian cells obtain cholesterol from two sources. Cholesterol can be synthesized *de novo* from acetyl-CoA or taken up in the form of lipoprotein particles by the low density lipoprotein (LDL) receptor [3]. Cells maintain cholesterol homeostasis through a feedback regulatory mechanism that acts at the level of transcription to coordinately control the input of sterols from these two sources [4]. When cells are depleted of sterols, transcription of genes required for the uptake and synthesis of cholesterol, such as the LDL receptor and 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, increases. Conversely, when intracellular cholesterol levels are high, transcription of these target genes decreases, and sterol levels fall. Analysis of the LDL receptor gene identified a *cis*-acting, positive regulatory sequence in the promoter that mediates transcription in the absence of sterols and is silenced by sterols [5]. This sterol-regulatory element (SRE) was used as bait in the purification and subsequent cDNA cloning of a family of membrane-bound transcription factors, designated sterol regulatory element-binding proteins (SREBPs) [5,6].

SREBPs: Membrane-Bound Transcription Factors

SREBPs transmit information to the nucleus about the sterol content of membranes [4]. This information is generated through the process of regulated intramembrane proteolysis (Rip) [7]. Newly synthesized SREBPs are inserted into the membranes of the ER and nuclear envelope in a hairpin orientation such that the NH_2 and COOH termini project into the cytosol (Fig. 1). These termini are separated by two transmembrane segments that surround a short ~30-amino-acid lumenal loop. The NH_2-terminal domain of ~480 amino acids is a transcription factor of the basic helix–loop–helix leucine zipper family, whereas the COOH-terminal domain of ~580 residues performs a regulatory function. Three SREBP proteins, encoded by two genes, are present in humans, hamsters, and mice [8]. *SREBP1* encodes two isoforms (SREBP-1a and SREBP-1c) through the use of alternate promoters that generate different first exons that are spliced to a common second exon. *SREBP-2* encodes a single protein. SREBP-2 preferentially activates genes involved in cholesterol biosynthesis, whereas SREBP-1 activates genes required for fatty acid synthesis. To date, SREBPs have been shown to directly activate more than

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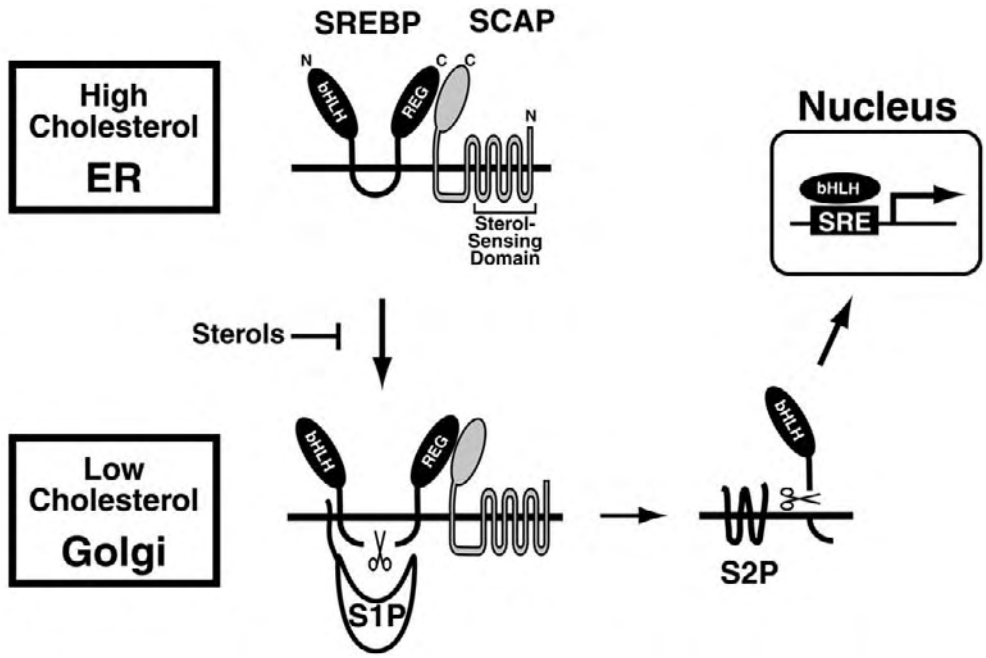


Figure 1 Sterol-regulated transport of SCAP/SREBP complex from ER to Golgi. SCAP/SREBP complexes localize to the ER in the presence of sterols. When cells are depleted of sterols, SCAP escorts SREBP from the ER to the Golgi where SREBP is cleaved at Site-1 by S1P. Then, S2P cuts at a site within the first transmembrane segment. This cleavage by S2P releases the transcription factor from the membrane and allows it to enter the nucleus where it activates transcription of target genes.

30 genes involved in the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids (see [9] for a detailed review).

When cells are depleted of sterols, SREBPs are activated by two sequential proteolytic cleavage events that release the NH_2-terminal transcription factor from the membrane, allowing it to enter the nucleus and activate transcription of target genes (Fig. 1). The first cleavage occurs in the luminal loop of SREBP at Site-1 and is catalyzed by a transmembrane protease, called Site-1 protease (S1P) [10]. Cleavage by this 1052-amino-acid, subtilisin-related protease occurs after the consensus sequence RXXL and separates the molecule into two halves [11]. S1P is not restricted to proteins involved in cholesterol homeostasis. S1P cleaves and activates ATF6, a transcription factor involved in the endoplasmic reticulum (ER) stress response [12], and it also participates in the processing of Lassa virus glycoprotein precursor GP-C, a step required for production of infectious virus [13].

Following cleavage by S1P, the NH_2-terminal domain of SREBP remains bound to the membrane until a 519-amino-acid, membrane-bound zinc metalloprotease, the Site-2 protease (S2P), cleaves SREBP within the first transmembrane segment [14,15]. The liberated transcription factor now enters the nucleus and activates target gene transcription. Sterols control the activation of SREBPs by regulating cleavage at Site-1. When cholesterol accumulates, cleavage of SREBP by S1P is blocked. Cleavage by S2P is not regulated by sterols, but requires the prior cleavage by S1P [16].

SCAP: Sterol Sensor and Escorter of SREBP from ER to Golgi

Cleavage of SREBP at Site-1 requires a 1276-amino-acid, polytopic membrane protein called SREBP cleavage-activating protein (SCAP) [17]. SCAP is divided into two domains. The NH_2-terminal's 730 amino acids contain eight transmembrane segments that attach SCAP to membranes of the ER and nuclear envelope [18]. The COOH-terminal's 546 amino acids contain five copies of the WD-40 motif, which form β-propeller structures that mediate protein–protein interactions [19]. This domain of SCAP forms a tight complex with the COOH-terminal regulatory domain of SREBP, and this interaction is essential for cleavage of SREBP by S1P (Fig. 1) [20,21]. Chinese hamster ovary (CHO) cells lacking SCAP fail to process SREBP at Site-1 and show reduced levels of SREBP precursor protein, indicating that SCAP is also required for stability of SREBP [22].

SCAP functions as the sterol sensor in the SREBP pathway. The sterol-sensing activity localizes to transmembrane segments 2–6 (~170 amino acids) in the NH_2 terminus of the protein (Fig. 1). Mutant CHO cells that contain single amino acid substitutions in this sterol-sensing domain of SCAP (Y298C and D443N) fail to sense sterols and continue to process SREBPs even in the presence of high levels of sterols [17,23].

Genetic, biochemical, and live-cell microscopy studies have established the following mechanism for sterol-mediated regulation of the processing of SREBP (Fig. 1). In the absence

of sterols, SCAP escorts SREBP from the ER to the Golgi where S1P cleaves SREBP at Site-1 and initiates release of the NH_2-terminal transcription factor. Following cleavage of SREBP, SCAP recycles to the ER [24]. In the presence of sterols, the SREBP/SCAP complex remains in the ER and is compartmentally separated from active S1P. The initial evidence for this model emerged from studies of the glycosylation state of SCAP. In wild-type CHO cells cultured in the presence of sterols, the *N*-linked carbohydrate chains of SCAP are sensitive to digestion with endoglycosidase H, indicating that the protein resides in the ER. However, when cells are depleted of sterols, *N*-linked carbohydrates on SCAP become resistant to endoglycosidase H treatment, suggesting that SCAP travels to the Golgi, but only in the absence of sterols [23].

The cloning and characterization of S1P provided further support for this model (Fig. 1). S1P is synthesized as an inactive zymogen (S1P-A). Autocatalytic processing of S1P at two sites in the NH_2 terminus of the protein leads to the removal of an inhibitory prosegment and production of the active form of the enzyme (S1P-C) [25]. Glycosylation and immunolocalization studies demonstrated that S1P-C resides in the Golgi. Therefore, in order to be processed at Site-1, SREBP must move from the ER to the Golgi. SCAP is the key to this movement.

Additional evidence for the role of SCAP in the trafficking of SREBP comes from experiments using SCAP-deficient mutant CHO cells (SRD-13A cells) that possess two copies of a nonfunctional SCAP gene [22]. These cells fail to process SREBPs, and they are therefore unable to synthesize cholesterol. Relocalization of active S1P from the Golgi to the ER bypasses the requirement of SCAP for processing of SREBP in SCAP-deficient SRD-13A cells [26]. This relocalization can be accomplished experimentally in two ways: (1) by treatment of SRD-13A cells with the fungal metabolite, brefeldin A, which results in fusion of the Golgi with the ER; and (2) by transfection of SRD-13A cells with a cDNA encoding a fusion protein of S1P (lacking its membrane-spanning segment) attached to the ER retrieval sequence, KDEL. In both of these situations, cleavage of SREBP occurs constitutively and is not inhibited by sterols [26]. Considered together, these results demonstrate that SCAP functions to escort SREBP from the ER to the Golgi and that sterols act on SCAP to block this transport.

The regulated transport model for SREBP processing is supported by light and electron microscopy studies of CHO/GFP-SCAP cells, a line of CHO cells that stably expresses a fusion protein consisting of the green fluorescent protein (GFP) covalently attached to the NH_2 terminus of SCAP [27]. In the presence of sterols, the GFP-SCAP/SREBP complex localizes to the ER. When CHO/GFP-SCAP cells are depleted of sterols, GFP-SCAP/SREBP moves from the ER to the Golgi by way of the ER–Golgi intermediate compartment (ERGIC) [27,28]. Time-lapse imaging of living CHO/GFP-SCAP cells reveals that on acute depletion of sterols, GFP-SCAP initially accumulates in the ERGIC and then the Golgi [28]. A movie of the ER to Golgi transport of GFP-SCAP can be found at http://www.cell.com/cgi/content/full/102/3/315/DC1. These data suggest that sterols block an early step in the ER to Golgi transport of SCAP.

Sterols Control Sorting of SCAP/SREBP into ER Vesicles

Dissection of the sterol-mediated regulation of ER-to-Golgi transport of SCAP/SREBP necessitated the development of assays that reconstitute this event *in vitro*. Studies in the yeast *Saccharomyces cerevisiae* by Wuestehube and Schekman [29] and in mammalian cells by Rowe and colleagues [30] led to the development of an *in vitro* assay that reconstitutes vesicle formation from the ER using isolated ER membranes and cytosol. These assays were used as a starting point for the development of an *in vitro* reaction that reconstitutes the sterol-regulated formation of SCAP-containing vesicles [28]. For these experiments, Nohturfft *et al.* [27] utilized CHO/VSVG-T7 cells, a cloned line of CHO-K1 cells that stably expresses a temperature-sensitive mutant (tsO45) of vesicular stomatitis virus G protein (VSVG) containing a cytoplasmic, COOH-terminal T7 epitope tag (VSVG-T7). VSVG, a type I transmembrane protein, is a classic marker of the mammalian secretory pathway. Incubation of CHO/VSVG-T7 cells at the nonpermissive temperature (40°C) results in accumulation of VSVG-T7 in the ER [31]. VSVG-T7 subsequently moves to the Golgi when the temperature is lowered to 32°C *in vivo*. When ER membranes are prepared from CHO/ VSVG-T7 cells grown in the absence of sterols, SCAP enters ER vesicles in a reaction that requires ER membranes, rat liver cytosol, ATP, and GTP. When ER membranes are prepared from cells grown in the presence of sterols, vesicles continue to form, as indicated by their content of VSVG-T7. However, SCAP is no longer incorporated into these vesicles. Immunoisolation experiments demonstrate that sterols act by regulating sorting of SCAP into a common class of ER vesicles that contain VSVG, rather than regulating the formation of a unique class of vesicle, specific for SCAP [28].

Recent data indicate that SCAP leaves the ER in COPII-coated vesicles, the same vesicles that carry VSVG and other secretory and membrane-bound proteins destined for the Golgi [28]. Five cytosolic proteins (Sar1, Sec23/24, and Sec13/31, collectively called COPII) can substitute for cytosol in the *in vitro* vesicle formation reaction. Detailed biochemical studies in yeast and mammalian cells have elucidated the mechanism of ER vesicle formation [32]. COPII vesicle formation is initiated by the recruitment of Sar1, a small GTP-binding protein, to the ER membrane through an interaction with a transmembrane, guanine nucleotide exchange protein called Sec12. GTP-bound Sar1 then recruits two heterodimeric complexes, Sec23/24 and Sec13/31, to the membrane. Binding of COPII to the ER membrane forms a protein coat and drives vesicle budding. Hydrolysis of GTP by Sar1 destabilizes the vesicle coat and permits fusion with distal compartments. The Sec23/24 heterodimer is believed to function in cargo selection, while Sec13/31 stimulates the

GTPase activating protein (GAP) activity of the Sec23/24 complex [32].

Characterization of SCAP vesicles produced in the aforementioned *in vitro* vesicle formation assay revealed that SCAP exits the ER in COPII-coated vesicles [28]. But how do sterols control the packaging of SCAP into COPII vesicles? Complexes of Sar1, Sec23/24, and cargo molecules, such as VSVG, can be isolated from ER microsomes that have been incubated with purified COPII proteins and then solubilized with digitonin [33,34]. Using a similar protocol, Espenshade *et al.* [28] showed that SCAP forms a ternary complex with mammalian Sar1 and Sec23/24 and that the formation of this complex is regulated by sterols. Complex formation occurs when ER membranes are prepared from cells cultured in the absence, but not the presence, of sterols [28]. These data indicate that sterols control the packaging of SREBP/SCAP into ER vesicles by regulating the ability of SCAP to interact with COPII vesicle coat proteins (Fig. 2). In the absence of sterols, SCAP/SREBP binds to COPII, enters vesicles, and then moves to the Golgi for proteolytic processing of SREBPs. In the presence of sterols, SCAP/SREBP is unable to interact with the COPII proteins and remains in the ER.

ER Retention of SCAP/SREBP

Clues to the mechanism by which SCAP/SREBP is retained in the ER come from experiments that examine the effects of overexpressing the sterol-sensing domain of SCAP [35]. In the presence of sterols, SCAP/SREBP is retained in the ER. However, when a truncated form of SCAP containing the sterol-sensing domain, transmembrane domains 1–6 (TM1–6), is overexpressed, SCAP/SREBP exits the ER, and SREBP is cleaved even in the presence of sterols. Importantly, overexpression of SCAP(TM1–6) carrying the Y298C mutation that confers sterol resistance has no effect on cleavage of SREBP [35]. These data suggest that in the presence of sterols SCAP binds to a putative retention protein. Overexpression of SCAP(TM1–6) competes for binding of full-length SCAP to this putative retention protein and allows SCAP/SREBP to exit the ER. It is unclear whether the Y298C mutation disrupts the ability of SCAP to sense sterols directly, or whether it impairs its interaction with the putative retention protein (or both).

Current data support the following model, shown schematically in Fig. 2. In the presence of sterols, SCAP/SREBP binds to a retention protein in the ER (protein X in Fig. 2), which prevents its interaction with COPII proteins. This failure to interact with COPII could result from either a conformational change in SCAP itself or from the sequestration of the SCAP/SREBP complex in a domain of the ER that has low vesicle formation activity. In the absence of sterols, SCAP/SREBP no longer interacts with the retention protein and is available to bind to COPII, enters vesicles; it is also transported to the Golgi where SREBP is cleaved sequentially by S1P and S2P.

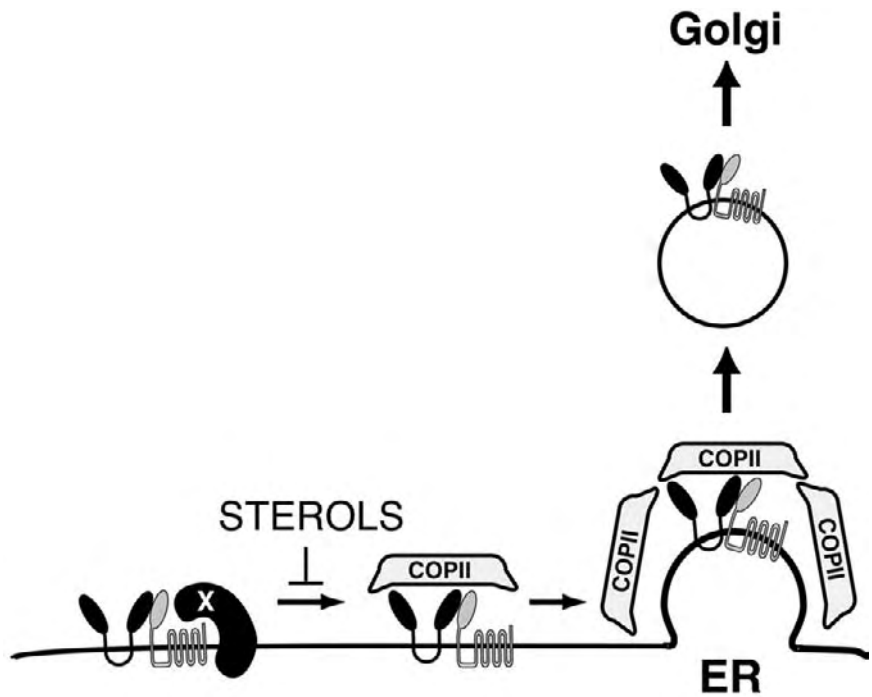


Figure 2 Model for ER retention of SREBP/SCAP complex. In the presence of sterols, SCAP binds to a putative ER retention protein (labeled X). Binding of SCAP to protein X prevents interaction of SREBP/SCAP with COPII proteins. When cells are depleted of sterols, SREBP/SCAP binds to one of the components of COPII, allowing the SREBP/SCAP complex to be packaged into COPII-coated vesicles. SREBP/SCAP is then transported to the Golgi where SREBP is cleaved sequentially by S1P and S2P.

Conclusions

A complete understanding of the mechanism by which sterols regulate the sorting of SCAP/SREBP into ER vesicles awaits the identification of the putative ER retention protein. Detailed structural studies of SCAP will also be required to learn how SCAP senses sterols. Does SCAP directly recognize cholesterol (or an hydroxylated derivative of cholesterol)? Or, does SCAP monitor a more general property of membranes when the local environment surrounding SCAP is perturbed by sterols [36]? Several other proteins that function in various aspects of cholesterol homeostasis contain sterol-sensing domains, including the rate-controlling enzyme in cholesterol synthesis; HMG CoA reductase; the Hedgehog receptor, Patched; and the Niemann-Pick Type C disease gene, NPC1 [4,37]. It remains to be determined whether these other sterol-sensing domains function in a manner similar to the sterol-sensing domain of SCAP. Much has been learned, but more remains to be discovered about this fundamental control mechanism for cellular cholesterol homeostasis.

Acknowledgments

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CHAPTER 324

Endoplasmic Reticulum Stress Responses

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Introduction

Eukaryotic cells must adapt to wide variation in the load of client proteins that their endoplasmic reticulum (ER) must process. They do so by modulating the synthesis of client proteins and the expression of genes whose products determine the folding capacity of the organelle. The signaling pathways activated to effect these adaptations are part of the unfolded protein response (UPR), which is similar in many ways to the cytoplasmic heat-shock response. In mammalian cells, signaling is initiated by three known ER resident transmembrane proteins (IRE1, ATF6, and PERK) that can sense the stress caused by imbalance between capacity and demand in the ER. IRE1 and PERK are transmembrane protein kinases whose activity is repressed by free chaperones in the ER lumen.

During ER stress, as free chaperones are recruited to serve client proteins, these transducers are activated, resulting in downstream signaling. IRE1 effector functions include endonucleolytic processing of a substrate mRNA (HAC1 in yeast and XBP-1 in metazoans) and activation of JUN N-terminal kinase, resulting in activated gene expression. PERK phosphorylates the translation initiation factor eIF2 and mediates both translational repression of client protein biosynthesis and activation of specific mRNA translation. The latter culminates in increased expression of the transcription factor ATF4 and activates a gene expression program that is also accessed by other forms of stress and is referred to as the *integrated stress response.* ATF6 activation is mediated by proteolytic processing and liberation of an active N-terminal protein fragment that serves as a transcription factor for activating gene expression in the UPR.

ER Stress Defined

Proteins destined for secretion and membrane insertion are synthesized on ribosomes associated with the endoplasmic reticulum. Such client proteins of the ER are translocated into the organelle's lumen where they undergo posttranslational modifications specific to that environment. Chaperone proteins specific to the ER assist in the folding and posttranslational processing of the client proteins, and quality-control mechanisms operating within the organelle clear properly folded client proteins for transport to the Golgi apparatus or earmark others for degradation by the ER associated degradation apparatus (ERAD).

The ER is thus a dynamic cellular compartment whose activity level varies among different cell types and between physiological states. In complex metazoans it is most developed in cells devoted to secretion, such as those of the exocrine and endocrine glands, immunoglobulin-secreting plasma cells, and cells with extensive membranous processes such as neurons and myelin-producing glial cells. In other words, the quantity and intrinsic properties of client proteins synthesized in response to developmental programs and physiological states set the level of *demand* made of the ER. The ability of the ER to *supply* this demand is impacted by the size of the organelle, by the complement of chaperones and other intrinsic protein and lipid components at its disposal, and by the physiological state of the cell. The latter is particularly important in pathological states associated with nutrient limitation, hypoxia, or toxins that degrade the ER's ability to process client proteins (reviewed in [1–3]).

Specific signaling pathways are activated by an imbalance between the demand on the ER and the ability of the organelle

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to meet the demand, so-called "ER stress." Activation of these pathways occurs under both pathological conditions and in response to normal physiological demands. However, much of what we have learned about them is derived from experiments in which cells were treated with toxins, for example, tunicamycin, an inhibitor of N-linked glycosylation. Because such toxins cause ER stress by interfering with protein folding, the signaling pathways that they activate came to be known as the *unfolded protein response* (UPR) [4,5]. This is a somewhat misleading term; because the pathways in question appear to be activated by the threat of malfolded proteins and do not necessarily require the accumulation of measurable amounts of malfolded proteins.

The UPR in Yeast

The UPR was first studied in yeast where treatment with tunicamycin or mutations that affect protein folding in the ER were noted to activate transcription of the *KAR2* gene encoding the yeast homolog of the mammalian ER chaperone BiP [6,7]. Genetic analysis of signaling from the yeast ER to the *KAR2* promoter unveiled a truly novel pathway. Signaling in response to ER stress is initiated by a type 1 ER resident transmembrane protein encoded by the *IRE1* gene [8,9]. The N-terminal lumenal domain of Ire1p responds to the ER stress signal and the signal is transmitted to the C-terminal, cytoplasmic effector domain in which two distinct activities reside: a protein kinase activity for which the only known substrate is Ire1p itself [8,9] and, remarkably, an endonuclease activity that removes an intron from the *HAC1* mRNA [10–12]. Ire1p activation thus entails transautophosphorylation that unmasks the endonucleolytic activity [13]. The cleaved ends of the *HAC1* mRNA are joined by tRNA ligase to generate an activated, spliced form of the *HAC1* mRNA [14]. The unspliced *HAC1* mRNA is poorly translated, whereas the protein encoded by the spliced form is well expressed. Thus, noncanonical splicing controls expression of Hac1p, a transcription factor that activates downstream target genes of the yeast UPR [15,11].

In yeast the IRE1>HAC1 pathway is a simple linear one that is required for activation of all known target genes of the UPR. These have been the subjects of detailed analysis by expression microarrays. Surprisingly, the genes induced by the UPR in yeast include not only the expected ER chaperones but also components of the apparatus for degrading ER proteins, the machinery required for membrane biosynthesis and non-ER components of the secretory apparatus [16,17]. The yeast UPR, therefore, appears to up-regulate the capacity of the entire endomembrane system to deal with client proteins. In this context the UPR should be viewed as a pathway concerned both with adapting to stress in the short term and with growth and anabolism [18]. It is also clear, however, that the ability to up-regulate target genes of the UPR is an important adaptation, because yeast defective in this function are hypersensitive to ER stress [16,17,19].

IRE1 proteins are conserved in higher eukaryotes [20,21] and their mechanism of action appears to be conserved too (see later discussion), however their preeminent role in regulating all aspects of the UPR has been superseded by new components that are lacking in yeast. One important difference between higher eukaryotes and yeast is that the former rapidly and reversibly attenuate protein biosynthesis in response to ER stress. This adaptation decreases the quantity of client proteins that are delivered to the stressed ER and reduces the demand on the organelle.

The UPR is Metazoans

Translation control by ER stress is mediated by PERK (also known as PEK or EIF2AK3), a type 1 transmembrane ER resident protein kinase related to IRE1 in its stress-sensing lumenal domain. The C-terminal, cytoplasmic effector domain of PERK is a protein kinase that phosphorylates the alpha subunit of translation initiation factor 2 [22]. Phosphorylation of eIF2α inhibits nucleotide exchange on the eIF2 complex and blocks the initiation step of translation. An important component of the regulation of PERK and IRE1 activity depends on an interaction between their related N-terminal lumenal domains and the ER chaperone BiP. In unstressed cells, dispensable BiP protein is available to bind the lumenal domains of PERK and IRE1. BiP binding maintains these proteins in a monomeric, inactive state. When the folding reserve of the ER is challenged, either by increased demand or reduced function, levels of dispensable BiP decrease and PERK-BiP and IRE1-BiP complexes dissociate. Freed from the inhibitory effects of BiP binding, PERK and IRE1 oligomerize, transautophosphorylate, and initiate downstream signaling [23,24].

Cells lacking PERK are unable to reduce translation when experiencing ER stress and consequently are exquisitely sensitive to agents and conditions that cause such stress [25]. Humans and mice with *PERK* mutations develop early onset diabetes mellitus caused by destruction of the insulin-producing pancreatic β cells [26,27]. Protein synthesis in β cells is strongly activated by plasma glucose; PERK modulates this increase, ensuring that the quantity of proteins translocated into the ER does not exceed the capacity of the organelle to fold them. In $PERK^{-/-}$ islets one finds cells with distended ER, and the presence of these correlates with high rates of programmed cell death in the mutant islets. It is clear, therefore, that PERK signaling is activated under normal physiological circumstances, notably in professional secretory cells.

The hypothesis whereby loss of translational control leads to stuffing of the ER with client proteins goes a long way toward explaining the phenotype of the *PERK* mutation. However, this simple hypothesis does not take into account the role of PERK in regulating gene expression in the UPR. eIF2α phosphorylation decreases the efficiency of translation of most mRNAs; however, some mRNAs are translated more efficiently in such circumstances. The mechanism for

this has been worked out in great detail in the case of the yeast *GCN4* mRNA, which encodes a transcription factor that activates genes involved in amino acid biosynthesis [28]. Translation of the mammalian *ATF4* mRNA is similarly up-regulated by eIF2α phosphorylation [29]. Thus, ATF4 translation during ER stress and up-regulation of its downstream target genes require PERK. Because this signaling pathway is activated both by ER stress and other conditions associated with eIF2α phosphorylation (e.g., amino acid starvation, arsenite treatment), we propose referring to it as the *integrated stress response* (ISR). Preliminary results suggest that the ISR may contribute substantially to cell survival during ER stress and to developmental programs [30].

GADD34 is an interesting target gene of PERK (and the ISR). The encoded protein binds the catalytic subunit of protein phosphatase 1 and recruits it to dephosphorylate eIF2α. Thus, the integrated stress response induces a negative feedback loop that serves to terminate the response [31]. It seems likely that eIF2α dephosphorylation plays a role in the recovery of protein biosynthesis, which, in turn, is required for the translation of mRNAs that are up-regulated as part of the gene expression program activated during the UPR (and other stresses associated with an ISR). According to this model, the first line of defense against ER stress in complex metazoans is translational repression. It is followed, soon thereafter, by remodeling of the internal milieu; the latter process requires new protein synthesis that is favored by GADD34.

Higher eukaryotes possess a third signaling pathway from the ER to the nucleus (Fig. 1), which is mediated in mammals by two ATF6 genes, α and β, encoding very similar type 2 transmembrane ER resident proteins. The N-terminal cytoplasmic portion of these proteins is a bZIP transcription factor that binds and activates ER stress response elements (ERSEs) in the promoter of such classic UPR target genes as BiP and CHOP [32–34]. In unstressed cells ATF6 is maintained in an inactive state by being tethered to the ER membrane. In response to ER stress, the protein is proteolytically cleaved, generating a free N-terminal fragment that migrates to the nucleus and activates transcription through ERSEs [35,33]. This mode of activation resembles that of another ER-tethered bZIP protein, the sterol-regulated transcription factor SREBP. This is more than a superficial resemblance, because the proteases that process and activate SREBP were found to be essential also for the activation of ATF6 [36]. These proteases are found in the Golgi apparatus. It is likely, therefore, that ATF6 activity is controlled by ER retention, and migration of ATF6 to the Golgi apparatus during ER stress likely leads to processing and liberation of the active transcription factor.

The comprehensive analysis of the yeast UPR, carried out by expression microarrays, has not yet been extended to mammalian cells. Consequently, we do not have detailed information on how the different signaling components of the UPR interact to control gene expression in higher eukaryotes. However, based on even the limited information available, it is clear that the mammalian UPR has diverged considerably from that of the yeast. The ATF6 pathway appears to have assumed a major role in controlling chaperone gene expression. This is revealed by the severe defect in *BiP*

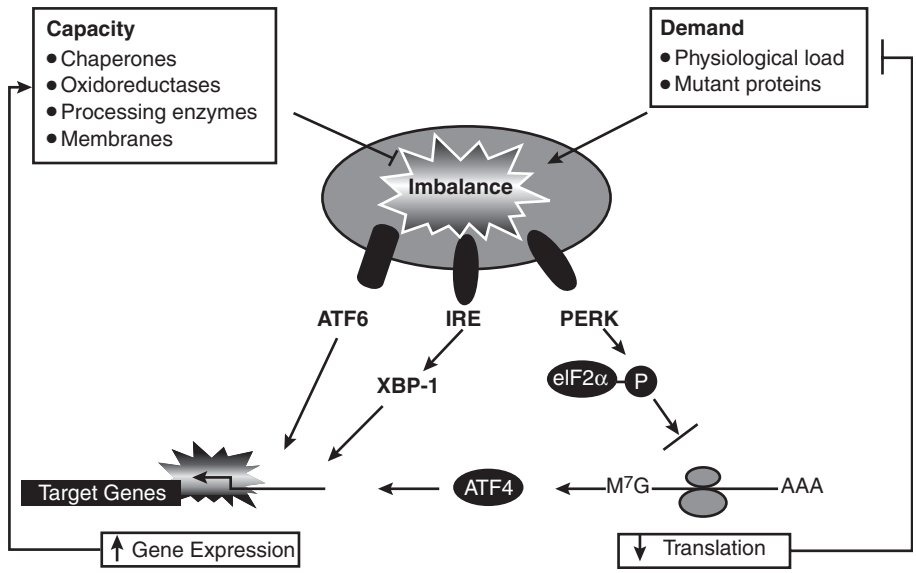


Figure 1 Depiction of the major aspects of the endoplasmic reticulum stress response and its mediators in mammalian cells. An imbalance between the load of client proteins imposed on the ER (demand) and capacity of the ER to fold client proteins is sensed by the three ER stress transducers: ATF6, IRE1, and PERK. These coordinate two functional responses: activation of the expression of genes whose products increase ER folding capacity and inhibition of protein synthesis that attenuated demand on the ER. IRE1 activity is transduced though XBP-1 to activate gene expression, whereas ATF6 directly mediates ER to nuclear signaling. PERK signaling is mediated by eIF2α phosphorylation modifying translation initiation rates. The latter results in a general decrease in demand on the ER and at the same time activates the translation of transcription factors such as ATF4 that contribute to activated gene expression.

expression manifested by cells that lack ATF6-processing protease activity [36]. PERK and the integrated stress response also contribute significantly to gene expression in the UPR [29,30]. Some target genes of the UPR, such as *GADD34* and *CHOP*, are highly dependent on the ISR, whereas other genes, such as *BiP,* are less dependent on PERK signaling and the ISR. IRE1 genes, of which mammals have two, appear to have been diverted from exclusive control of a broad set of UPR genes (as is the case in yeast) toward certain specific cellular functions.

We have found that mouse cells lacking *IRE1* are virtually unimpaired in activating most target genes of the UPR. Furthermore, such mutant cells are not manifestly hypersensitive to pharmacological manipulations that cause ER stress. One measure of the divergence of IRE1 function from yeast to higher eukaryotes is the finding that mammalian IRE1 activates stress-activated protein kinases during ER stress. It does so by recruiting TRAF2 to the ER membrane [37]. The physiological significance of this signaling pathway is not fully understood, but it may link ER stress and programmed cell death. The association between ER stress and programmed cell death is a poorly understood but potentially important subject, particularly in light of recent findings suggesting that ER stress may accompany common pathological conditions [38]. Several signaling pathways leading to CHOP [39], JNK [37], CASPASE-12 [40], and cABL [41] activation may be involved, but the manner in which these are integrated into the UPR remains to be studied in greater detail.

Further clues to the role of mammalian IRE1 have been provided by the identification of a direct downstream target mRNA (analogous to yeast *HAC1*). The *XBP-1* mRNA is processed by mammalian IRE1 in an ER-stress-dependent manner and IRE1 thus controls the expression of this important transcription factor [42–44]. The target genes of XBP-1 have not been identified, but analysis of *XBP-1* knock-out mice reveals its essential role in development of B cells to plasma cells [45]. XBP-1 mediated signaling presumably up-regulates the capacity of the endomembrane system to deal with the load of immunoglobulins that must be secreted by the developing B cell. The finding that XBP-1 is controlled by IRE1 suggests that in mammalian cells the IRE1 genes have specialized to link the load on the ER to certain long-term "developmental" adaptations of the ER. This hypothesis is further supported by the observation that both XBP-1 and IRE1 are enriched in cells that specialize in secretion.

Conclusion

In summary, the mammalian UPR consists of rapid and transient translational repression, which is completely PERK dependent. This short-term adaptation is supplanted by up-regulation of genes that enhance the capacity of ER to deal with increased load. All three known signaling components of the UPR (PERK, IRE1, and ATF6) participate in this latter adaptation. Additional signaling downstream of these and other ER stress responders effects important and poorly understood life and death decisions.

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CHAPTER 325

Signaling Pathways from Mitochondria to the Nucleus

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Introduction

Mitochondrial research has entered a new era, which recognizes that mitochondria are key players in intracellular signaling. Cells monitor and respond to changes in the functional state of their mitochondria, resulting in responses as severe as apoptosis and as subtle as changes in nuclear gene expression. The general term for this mitochondria-to-nucleus signaling is *retrograde regulation*. In animal cells, compromises in mitochondrial function often lead to increases in mitochondrial biogenesis, with attendant increases in mitochondrial DNA levels and mitochondrial gene expression. In many instances, changes also occur in the expression of nonmitochondrial components associated with mitochondrial dysfunctions. In animal cells, many of these changes can be attributed to increased cytosolic Ca^{2+}, derived in part from mitochondrial stores. Mitochondria-derived NAD^{+} may also be important in signaling the retrograde response. In yeast, both positive and negative regulatory factors, particularly the *RTG* genes, have been identified that control retrograde regulation. Transcriptome analyses reveal that the retrograde regulation is both a stress response and a mechanism to overcome metabolic defects imposed by dysfunctional mitochondria. Many of these effects are signaled by intra- and extracellular glutamate levels. In both yeast and filamentous fungi, the retrograde response is intimately tied to aging and cellular senescence. Altogether, retrograde regulation adapts cells to increased energy demands, and links mitochondrial function to fundamental processes of nutrient sensing, carbohydrate and nitrogen metabolism, stress, and aging.

Milestones in Mitochondrial Research

The history of mitochondrial research can be divided conveniently into three areas of accomplishment, each revealing unique and fascinating intricacies of this eukaryotic organelle. Spanning a period of roughly 50 years, these studies broadly include (1) structure–function, (2) biogenesis, and (3) intracellular signaling. First was the elucidation of the primary function of mitochondria—the synthesis of ATP via oxidative phosphorylation—culminating with the recognition that the structure of mitochondria, the ATP synthase complex, and the organization of components of the electron transport chain within the inner mitochondrial membrane were integral features of the coupling of electron transport to ATP synthesis through an electrochemical gradient.

The second area followed the discovery of mitochondrial DNA (mtDNA) and its role in mitochondrial biogenesis. This led immediately to the general concept of nuclear-mitochondrial cross-talk and the idea that there is coordinate regulation between the nuclear and mitochondrial genomes. These findings also provided a basis for understanding mitochondrial diseases and offered a new paradigm for studies in molecular evolution using mtDNA as an evolutionary clock. Control of the replication, transmission, and expression of the mitochondrial genome is determined largely by nuclear gene products that function either directly within mitochondria, or indirectly through regulation of the expression, targeting, stability, and so on of mitochondrial proteins. These studies culminated with the detailed dissection of the mechanisms of targeting and import of proteins into mitochondria.

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The third and most current area of mitochondrial research relates to the unexpected finding that mitochondria are players in intracellular signaling. Changes in the functional state of mitochondria, often subtle, are now known to elicit a wide range of cellular responses: apoptosis, aging and cellular senescence, tumor progression, changes in gene expression leading to metabolic reorganization, and patterns of differentiation, to name a few. Here we review some examples of mitochondrial signaling in animal cells and in fungi, and we also highlight the molecular events that trigger and transduce mitochondrial signals to effect changes in nuclear gene expression. Some aspects of these signaling pathways have been discussed recently [1]. Collectively, signaling from mitochondria to the nucleus is called *retrograde regulation* to distinguish it from nuclear control over mitochondrial biogenesis and mitochondrial activities.

Mitochondrial Signaling

Responses in Animals

Stimulation of Mitochondrial Biogenesis

Different cellular and environmental cues can lead to various tissue-specific increases in mitochondrial biogenesis. Stimulation of skeletal muscle contractility, for example, leads to a dramatic muscle-specific proliferation of mitochondria because of an increased demand for energy [2]. Heat production in brown fat tissue in adaptive thermogenesis is the result of both an increase in mitochondrial mass as well as an increase in uncoupled respiratory activity due to specific expression of uncoupler proteins [3,4]. The regulatory factors that control these responses include the transcription factors NRF-1 and NRF-2, which regulate the expression of a number of nuclear genes encoding mitochondrial proteins [5], and additional activities of the transcription factor PPARγ and its coactivator, PGC-1 [3,4]. Because these proteins also regulate the expression of nuclear genes that control the copy number and expression of mtDNA, they are key components of the interorganelle cross-talk pathway that coordinately regulates expression of the nuclear and mitochondrial genomes.

In cells with dysfunctional mitochondria, increased expression of one or more components of the mitochondrial bioenergetic apparatus, or increased mitochondrial biogenesis, is easily interpretable as an attempt by the cell to overcome the mitochondrial defects. Responses resulting in down-regulation of gene expression, however, are sometimes more difficult to understand. One such example is the decreased steady-state levels of the ADP/ATP translocator mRNA observed in HeLa cells treated with the mitochondrial protein synthesis inhibitor, chloramphenicol, or with the uncoupler, dinitrophenol [6]. This finding contrasts with an earlier observation by the same group that, in ρ° derivatives of human 143B cells (cells that lack mtDNA), expression of an isoform of the ADP/ATP translocator increased [7]. The difference might possibly be related to acute versus chronic failure of mitochondrial function.

In tissues of patients with pathogenic mtDNA mutations, increased expression is seen of nuclear genes encoding components of the oxidative phosphorylation apparatus, including the heart-muscle isoform of adenine nucleotide translocator (ANT1), the ATP synthase β-subunit, and ancillary genes including hexokinase I and glycogen phosphorylase [8]. Additionally, an increase is seen in the expression of mtDNA transcripts, although the amount of mtDNA was unchanged. This contrasts with findings in *Drosophila* that mtDNA copy numbers increase in cells with mtDNA deletion mutations as a mechanism to compensate for reduced expression of genes within the deleted mtDNA [9,10].

In cells with intact, wild-type levels of mtDNA, a retrograde response can often be initiated by perturbation of a specific component or pathway within mitochondria. For example, extensive proliferation of mitochondria is seen in skeletal muscle of mice lacking the heart-muscle ANT1 [11]. Limited transcriptional profiling of skeletal muscle of such ANT1-deficient mice revealed some 17 genes that were up-regulated, including mitochondrial tRNA and rRNA genes, and mitochondrial and nuclear genes encoding components of the oxidative phosphorylation apparatus [12].

An important feature of retrograde regulation is that the response is not restricted to changes in expression of nuclear genes encoding mitochondrial components (see later section). For example, in ρ° chicken cells, an increase is seen in expression of genes encoding elongation factor 1β [13], β-actin, and v-Myc [14] and a decrease in expression of *MEK2* (encoding a dual specificity kinase), the latter having been observed both in ρ° cells and in ρ^+ cells treated with drugs that block the expression of mtDNA genes [15].

Signals

Ca^{2+} What are the signals that originate from dysfunctional mitochondria in retrograde response pathways, and how are those signals transduced to effect such dramatic changes in nuclear gene expression? It is likely that, depending on the cell type and the kind of mitochondrial lesion, the retrograde response will be initiated by different, but not necessarily exclusive, signals. In the case of the apoptotic response, the release of cytochrome c and other pro-apoptotic factors from mitochondria of many different cell types represents one pathway for the initiation of cell death (see [16] for a recent review).

For less severe responses, specific signals or effectors arise as a consequence of changes in the functional state of mitochondria. One of these effectors is Ca^{2+}. Mitochondria of animal cells transport Ca^{2+} very efficiently, and are thus likely to be important in regulating the distribution of intracellular Ca^{2+} [17]. Given that so many pathways are regulated through Ca^{2+} signaling, it is perhaps not surprising that alterations in the mitochondrial function of animal cells can elicit such profound cellular responses.

Lowering the mtDNA copy number in mouse C2C12 myocytes, or treatment of those cells with mitochondrial inhibitors, results in changes in the expression of a number of cellular and regulatory factors [18]. There is increased

expression of the sarcoplasmic reticular ryanodine receptor-1, calcineurin, calcineurin-dependent NFATc, c-Jun N-terminal kinase (JNK)-dependent ATF2 (activated transcription factor 2), and subunit Vb of cytochrome oxidase, and decreased expression of NFκB. These changes can be attributed to increased cytosolic Ca^{2+} levels arising, in part, because of increased Ca^{2+} efflux from defective mitochondria. Treatment of rat PC12 cells with uncouplers similarly increases intracellular Ca^{2+} levels, resulting in activation of the mitogen-activated kinases, ERK1 and ERK2 [19]. These studies clearly establish that mitochondrial stress can disrupt Ca^{2+} homeostasis and either initiate changes in nuclear gene expression or affect signaling pathways directly.

Ca^{2+}-related mitochondria-to-nucleus signaling induces invasive phenotypes in otherwise noninvasive C2H12 myocytes and in human pulmonary carcinoma cells [20]. Expression of cathepsin L and TGFβ, two tumor marker genes, increases in C2H12 cells depleted of their mtDNA or when treated with an uncoupler of oxidative phosphorylation. Increased cathepsin L gene expression in mitochondria-stressed cells results partly from the activation of calcium-dependent protein kinase C. Together, these results suggest a novel connection between retrograde signaling and tumor progression and metastasis. Because of a less efficient repair system, it has long been recognized that mutations are fixed at a higher rate in mtDNA compared to the nuclear genome. This observation has led to some speculation that the accumulation of mtDNA mutations in tumor cells may underlie tumorigenesis by providing some selective advantage to cells, leading eventually to cell populations that contain only mutant mitochondrial genomes (i.e., they are homoplasmic for their mtDNA) [21]. Although it remains possible that some mtDNA mutations could influence tumor progression through a retrograde response, a recent study suggests that simple random segregation of mtDNA in a rapidly dividing cell population, as in tumors, could lead to homoplasmy without any selection [22].

Arnould *et al.* [23] used similar strategies of mtDNA depletion and treatment of cells with mitochondrial poisons to show that mitochondrial dysfunction causes the activation of the cAMP responsive element binding protein (CREB), with attendant activation of CREB target gene expression. CREB is activated by phosphorylation at serine 133 by calcium/calmodulin kinase IV (CaMKIV). The increased cytosolic Ca^{2+} due to mitochondrial dysfunction disrupts the interaction of CaMKIV with protein phosphatase 2A, leading to the activation of the kinase. Among the genes affected by CREB phosphorylation in mtDNA depleted cells is p53. Phosphorylated CREB interacts with p53, affecting p53's transcriptional activity. One consequence is an increased expression of p21, a cyclin-dependent kinase inhibitor, which results in an inhibition of the entry of cells into S phase. These findings suggest that the slower growth rate of cells with mitochondrial defects may not be due solely to compromises in energy metabolism.

Differentiating between a "true" mitochondrial signaling pathway versus secondary responses resulting from decreased ATP supplies because of mitochondrial defects can be discriminated by the specificity of the response. If, for example, cell viability is compromised, many of the molecular indicators of cellular robustness will change; such changes would thus be considered secondary to the mitochondrial dysfunction. This issue was clearly addressed by Rochard *et al.* [24], who observed that treatment of avian myoblast cells in culture with mitochondrial poisons inhibited myogenic development without affecting cell viability or ATP levels. The specificity of the response was evident from the observation that these treatments down-regulated the expression of the muscle-specific regulatory protein, myogenin. Moreover, stimulation of mitochondrial activity enhanced myoblast differentiation, suggesting that some subset of nuclear genes involved in myoblast differentiation is likely to be affected by the functional state of their mitochondria.

NAD+ Besides its role in oxidation-reduction, NAD^+ has been implicated in a variety of signaling pathways related particularly to both mono- and polyADP ribosylation of proteins and to the formation of NAD^+ derivatives, such as cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP). These derivatives are formed by NADase activities (reviewed in [25]), and both cADPR and NAADP are effective agents for the mobilization of Ca^{2+} from the endoplasmic reticulum (reviewed in [26]).

Di Lisa and Ziegler [25] proposed a model in which mitochondrial alterations could lead to the release of mitochondrial NAD^+, providing additional substrate for nuclear polyADP ribosylation catalyzed by poly(ADP-ribose) polymerase. That enzyme responds to DNA damage—with its attendant changes in nuclear gene expression—and could be linked to the functional state of mitochondria via the generation of reactive oxygen species (ROS) by mitochondrial electron transport activity. Release of mitochondrial NAD^+ would also contribute substrate for cADPR formation catalyzed by an outer mitochondrial membrane NADase activity.

Yeast

THE *RTG* PATHWAY

The most extensive mechanistic information on retrograde regulation has come from studies of *CIT2* gene expression in the budding yeast *Saccharomyces cerevisiae*. *CIT2* encodes a glyoxylate cycle isoform of citrate synthase, one of three citrate synthase genes in yeast. In respiratory competent cells with fully functional mitochondria, *CIT2* expression is low, whereas in cells with dysfunctional mitochondria, *CIT2* transcription, as well as the level of citrate synthase enzyme activity, is dramatically elevated [27–29]. As discussed in a later section, the increases in *CIT2* expression and activity reflect the activation of pathways that resupply intermediates to the TCA cycle when they become severely depleted in cells with dysfunctional mitochondria. Three positive regulatory factors, Rtg1p, Rtg2p, and Rtg3p [29,30], and four negative regulatory factors, Mks1p [31,32], Lst8p [33], and two redundant 14-3-3 proteins encoded by *BMH1*

and *BMH2* (Z. Liu, 2002) function in this retrograde pathway. As we will see, the apparent complexity of retrograde regulation in yeast reflects, in part, the intersection of mitochondria-to-nucleus signaling with nutrient sensing, carbohydrate and nitrogen metabolism, and stress.

Rtg1p and Rtg3p are basic helix–loop–helix leucine zipper (bHLH/Zip) transcription factors that bind as a heterodimer to activate transcription at a novel DNA target site, GTCAC, called an R box [30]. This site differs from the canonical E-box recognition site, CANNTG, used by most bHLH proteins. Rtg3p has both C- and N-terminal transactivation domains [34], but only the N-terminal region of the protein is critical for regulated target gene expression [35]. In addition, Rtg3p contains an AD1 (activation domain 1) region at its N terminus [36]. AD1 is a prototypical activation domain conserved from yeast to mammals and is restricted to a subset of bHLH transcription factors. The AD1 domain of Rtg3p is required for its interaction with the nuclear histone acetyltransferase complex, SAGA, to activate transcription. Although the requirement of the AD1 domain in Rtg3p for regulated *CIT2* expression has not been tested directly, mutants lacking components of the SAGA complex show about a 50% reduction in *CIT2* expression [37]. This suggests that the AD1domain in Rtg3p may not be essential for transcriptional activation of target genes. Compared with other bHLH transcription factors, Rtg1p has a truncated basic domain and lacks any transactivation domains. Rtg1p's main function may be to facilitate Rtg3p binding to R boxes.

Rtg2p is a cytoplasmic protein with an amino terminal ATP binding domain similar to the hsp70/actin/sugar kinase superfamily of ATP binding proteins [38,39]. Rtg2p acts as a proximal sensor of mitochondrial dysfunction, transducing signals to the Rtg1p–Rtg3p complex. When the retrograde response is off, Rtg1p and Rtg3p are complexed together in the cytoplasm and Rtg3p is phosphorylated at multiple sites within the protein [35]. When the retrograde response is activated, Rtg3p becomes partially dephosphorylated and accumulates in the nucleus (along with Rtg1p) by processes that require Rtg2p. In *rtg2* mutant cells, Rtg1p and Rtg3p remain in the cytoplasm and Rtg3p is hyperphosphorylated. In addition to its transcriptional function, Rtg1p is also required to sequester Rtg3p in the cytoplasm when the retrograde response is off: Inactivation of *RTG1* results in constitutive nuclear localization of Rtg3p even in the absence of Rtg2p. Rtg3p, but not Rtg1p, contains a nuclear localization signal (NLS) located within its basic domain. Whether retention of Rtg3p in the cytoplasm is due to occlusion of its NLS or to more complicated mechanisms has not been established. Most, if not all, of the phosphorylation sites of Rtg3p are in the N-terminal region of the protein, upstream of the bHLH motif. Mutation of a number of these potential phosphorylation sites leads to constitutive nuclear localization of Rtg3p, but activation of its transcriptional function nevertheless requires additional, but unknown, retrograde signals (T. Sekito, 2002).

Among the genes regulated by the Rtg proteins are *CIT1*, *ACO1*, *IDH1*, and *IDH2* [40]. Those genes encode the enzymes catalyzing the first three steps in the TCA cycle that lead to the formation of α-ketoglutarate. In cells with robust mitochondrial activity, their expression is under the control of the HAP transcription complex [41]. In cells with reduced or compromised mitochondrial function, however, expression of those TCA cycle genes becomes largely dependent on the *RTG* genes [40]. This switch to *RTG* control ensures that sufficient α-ketoglutarate, the direct precursor of glutamate, is made to maintain glutamate supplies in cells with dysfunctional mitochondria. This conclusion is consistent with the observation that (1) none of the TCA cycle genes downstream of α-ketoglutarate is regulated by the *RTG* genes, (2) glutamate is a potent repressor of *RTG*-dependent gene expression, and (3) strains harboring *rtg* mutations are glutamate auxotrophs. Thus, a key function of the retrograde response and *RTG*-dependent gene expression is glutamate homeostasis.

Lst8p is an essential 7 WD-repeat protein that has been implicated in the targeting of the general amino acid permease, Gap1p, from the late-Golgi to the plasma membrane [42]. As a class, WD-repeat proteins are involved in diverse cellular activities through their interactions with other proteins. WD-repeat proteins adopt a seven-bladed β-propeller fold, with each repeating unit consisting of a four-stranded antiparallel β-sheet [43]. WD-repeat proteins can likely use different blades of the β-propeller to interact with different proteins and, thus, regulate different pathways [44]. In this connection, analysis of different Lst8p mutants shows that the protein negatively regulates *RTG*-dependent gene expression at two sites, one downstream of Rtg2p, perhaps at the level of the Rtg1p–Rtg3p complex, and the other upstream of Rtg2p by regulating the targeting of an amino acid sensor, Ssy1p, to the plasma membrane. Ssy1p signaling is coupled to the cytoplasmic proteins Ptr3p and Ssy5p (reviewed in [45]). External glutamate represses *RTG*-dependent gene expression via the Ssy1p/Ptr3p/Ssy5 pathway [33]. This same signal transduction pathway is also responsible for the induction of amino acid permeases [45]. It remains to be determined how these pathways of glutamate sensing, retrograde regulation, and amino acid permease induction and targeting are integrated and where the inductive and repressive components of the pathway branch off.

Mks1p has been reported to be involved in multiple pathways: Ras-cAMP signaling [46], negative regulation of lysine biosynthesis [47], positive regulation of nitrogen catabolism [48], and the generation of the yeast prion [URE3] [49]. [URE3] is a non-Mendelian determinant of Ure2p, a negative regulator of the nitrogen catabolite repression (NCR) pathway, with properties fully consistent with it being an infectious prion. The NCR pathway is activated by release of the GATA transcriptional factor, Gln3p, from a cytoplasmic complex with Ure2p, allowing Gln3 to translocate to the nucleus to activate expression of genes involved in the utilization of nonpreferable nitrogen sources such as urea [50].

Mks1p is a phosphoprotein [31,32] whose phosphorylation state mirrors that of Rtg3p [31]. Negative regulation of the retrograde pathway by Mks1p helps explain the proposed roles for the protein as a negative regulator of lysine

biosynthesis and as a positive regulator of [URE3] prion formation. Inactivation of *MKS1* results in the constitutive expression of *RTG* target genes. As a consequence, the levels of α-ketoglutarate, a precursor to lysine, are high, resulting in an increase the intracellular lysine pool. The apparent requirement of Mks1p in [URE3] prion formation is also linked to the retrograde pathway. Inactivation of *rtg2* or *rtg3* results in a 20- to 30-fold increase in the frequency of cells harboring the [URE3] prion [31]. In *rtg2 mks1* double mutant cells, in which *RTG* target gene expression is high (because inactivation of *MKS1* bypasses the requirement for Rtg2p), the frequency of [URE3] formation is suppressed to a very low level. However, in *rtg3 mks1* double mutants, in which *RTG*-target gene expression is blocked because of the transcriptional requirement for Rtg3p, the frequency of [URE3] prion formation is high. In other words, [URE3] prion formation correlates with the presence or absence of *RTG*-dependent gene expression, rather than with the presence or absence of Mks1p. Is the regulation of [URE3] formation by the *RTG* pathway related to glutamate homeostasis? Probably yes. In addition to repressing *RTG*-dependent gene expression, glutamate can also completely suppress [URE3] formation [31]. The *RTG* pathway, and glutamate homeostasis may, therefore, be intimately linked to one way that cells could regulate the sensing of the quality of nitrogen sources—by the conversion of the active negative regulator of the NCR pathway, Ure2p, to its inactive prion form, [URE3].

One recent report suggests that the *RTG* genes play no role in [URE3] generation [51]. The discrepancy might lie in whether glutamate is included in the growth medium in assays of [URE3] prion formation. That assay is based on the ability of cells to take up ureidosuccinic acid (USA) when the NCR pathway is activated. Severe glutamate starvation of an *rtg2* mutant strain would activate the NCR pathway by releasing Gln3p from Ure2p. One of Gln3p's targets is *DAL5*, which encodes an allantoate permease that enables cells to take up USA. Because *rtg2* mutant cells are leaky glutamate auxotrophs, they would thus appear as being USA^+ in medium lacking glutamate. Sekito *et al.* [31] supplemented the medium with small amounts of glutamate to allow *rtg* mutant cells to grow robustly if Ure2p were inactivated. In that case, all of the cells that were USA^+ were [URE3].

The connection between *RTG*-dependent gene expression and nitrogen metabolism was clarified by the finding that the RTG pathway is also regulated by target of rapamycin (TOR) signaling [52,53]. TOR signaling is mediated by two TOR kinases, Tor1p and Tor2p [54]. Rapamycin, an immunosuppressant that binds to FKBP12, a prolyl isomerase, inactivates the kinase activity of Tor1p and Tor2p. In the presence of rapamycin TOR signaling affects multiple cellular processes including activation of the NCR pathway and the expression of stress-related genes in response to carbon limitation [50,54–56]. When rapamycin is added to yeast cells, or when cells are grown on poor nitrogen sources, *RTG*-dependent gene expression is activated by processes that are, for the most part, similar to their activation in cells with dysfunctional mitochondria [35,53].

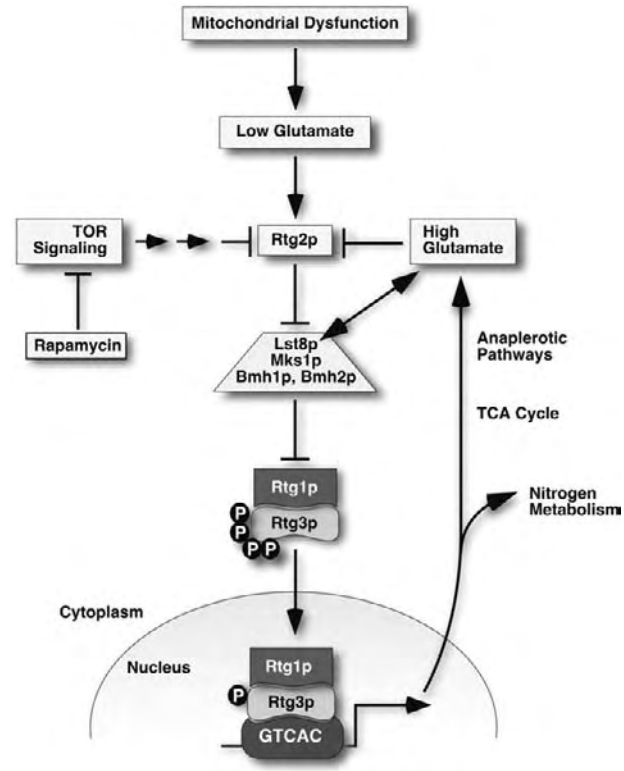


Figure 1 Regulation of *RTG*-dependent gene expression. Translocation of Rtg1p–Rtg3p from the cytoplasm to the nucleus is shown to be regulated by the levels of glutamate and by rapamycin, an inhibitor of the TOR pathway. The negative regulator of the *RTG* pathway, Lst8p, acts both downstream and upstream of Rtg2p. The latter site of action is through the Ssy1p–Ptr3p–Ssy5p signal transduction cascade that responds to external glutamate (see text for details). Inhibition of TOR signaling by rapamycin also activates the RTG pathway through Rtg2p [53], but for reasons that are not yet clear, the relation between nuclear localization of Rtg3p and Rtg3p phosphorylation is opposite to that observed for activation of the RTG pathway by mitochondrial dysfunction or limiting glutamate.

Finally, *BMH1* and *BMH2* also negatively regulate the *RTG* pathway downstream of Rtg2 (Z. Liu, 2002). This is consistent with a recent report suggesting that 14-3-3 proteins are required to keep Rtg3p in an inactive state [57]. Figure 1 summarizes the essential features of the control of *RTG*-dependent gene expression.

ADDITIONAL PATHWAYS

Pleiotropic drug resistance (PDR) is often due to the overexpression of genes encoding ATP-binding cassette (ABC) transporters. In yeast, dysfunctional mitochondria results in the induction of members of this family of PDR genes, such as *PDR5* [58]. The induction of *PDR5* appears to be routed through *RTG*-dependent and *RTG*-independent pathways. Which pathway is chosen may depend on the nature of the mitochondrial lesion [58]. Expression of the *PDR* genes is controlled by two zinc finger transcription factors, Pdr1p and Pdr3p [59]. Enhanced expression of *PDR5* in cells with dysfunctional mitochondria is the result of both increased expression of *PDR3* and posttranslational autoregulation of Pdr3p [58]. In ρ° cells, about one-half of the increased level

of *PDR* expression is dependent on the *RTG* pathway. In cells with a mutation of *OXA1*, a gene encoding an inner mitochondrial membrane protein required for cytochrome oxidase and ATPase assembly [60], the up-regulation of *PDR* genes is also observed, but in this case, induction is independent of the *RTG* genes [58]. These findings also suggest that more than one retrograde pathway can affect the same target genes in response to mitochondrial dysfunctions. The *RTG*-independent components of *PDR* expression in ρ° and *oxa1* responses can be traced to the reduction or loss of the F_0 subunit of the mitochondrial ATP synthase complex [61]. How the state of the F_0 subunit is transduced to changes in gene expression is not clear.

It has long been recognized that the functional state of mitochondria is affected by carbon source or growth conditions. In an hypoxic environment, for instance, cells repress the expression of aerobic genes, such as those encoding subunits of the cytochrome oxidase complex; at the same time, they induce the expression of hypoxic genes required for the survival of cells in a low-oxygen environment. In some, but not all cases, a functional mitochondrial electron transport chain is necessary for the induction of hypoxic genes [62]. Repression of cytochrome oxidase subunit (*COX*) genes also occurs in ρ° cells grown aerobically [63,64]; however, under hypoxic growth conditions, expression of *COX* genes in ρ° cells is only about 20% of that in hypoxic ρ^+ cells [63]. These observations suggest that, like the induction of *PDR* genes in ρ° cells, some specific mtDNA-encoded component, perhaps a subunit of the ATP synthase complex, is required to maintain a level of expression of *COX* genes independent of whether the cells have a functional electron transport chain.

As a protection against oxidative damage, cells induce the expression of genes that detoxify ROS. Cytosolic thioredoxin peroxidase I, a thiol-dependent peroxidase encoded by the *TSA1* gene, is an enzyme induced in cells that have been oxidatively stressed, for example, by exposure to high concentrations of H_2O_2 [65]. A role for retrograde signaling in this regulation was noted by the observation that in ρ° cells or in ρ^+ cells treated with the respiratory chain inhibitor, antimycin A, there was a greater induction of *TSA1* expression by H_2O_2 treatment than in ρ^+ cells alone [65]. Induction may be in response to increased ROS production in cells with blocked electron transport activity, and is consistent with the finding that under derepressed growth conditions, inactivation of *TSA1* leads to greater sensitivity to oxidative stress in cells with compromised mitochondrial function.

Genome-Wide Transcriptional Responses to Mitochondrial Dysfunction

Some insights into the scope of the retrograde response have been provided from microarray experiments in yeast by Traven *et al.* [66] and Epstein *et al.* [64] who determined the global changes in gene expression associated with mitochondrial dysfunction in ρ° cells. In addition, Epstein *et al.* [64] evaluated the role of the *RTG* genes in the ρ° responses, and the effects of treatment of respiratory competent cells with three inhibitors of oxidative phosphorylation, antimycin A, an uncoupler (carbonyl cyanide *m*-chlorophenylhydrazone [CCCP]), and an inhibitor of the ATP synthase complex (oligomycin). In comparing the data sets from these studies, it is important to note that different carbon sources were used to grow cells for transcription profiling. Traven *et al.* [66] used glucose, a repressing carbon source, whereas Epstein *et al.* [64] used raffinose, a fermentable but nonrepressing carbon source. In the experiments of Traven *et al.* [66], genes encoding mitochondrial proteins involved in respiratory function and mitochondrial biogenesis were up-regulated in ρ° cells, whereas Epstein *et al.* [64] observed that many of those genes were down-regulated in ρ° petites. One plausible explanation for these differences may be related to the onset of the diauxic shift, in which genes involved in aerobic metabolism become derepressed as glucose is exhausted from the medium [67]; these effects would not be superimposed on a comparison of cells grown on nonrepressing, but fermentable carbon sources. This interpretation is also consistent with the up-regulation of stress response genes and genes involved in the synthesis of reserve carbohydrates in ρ° petites grown on glucose medium [66]. Such responses are typical of yeast cells that begin to deplete glucose from the medium and whose growth rate slows down as they approach stationary phase. Particularly striking was the up-regulation of genes encoding many of the reactions that function to replenish supplies of acetyl-CoA and oxaloacetate to the TCA cycle (i.e., anaplerotic reactions), as well as small molecule transporters, a number of which function in the mobilization of metabolic intermediates of these anaplerotic pathways. In cells grown under derepressing conditions a major source of acetyl-CoA is from peroxisomal β-oxidation of fatty acids. Not surprisingly, respiratory deficient cells dramatically up-regulate peroxisome biogenesis—an outcome that was predicted from the expression profiling data [64].

What is the rationale for these metabolic changes in ρ° petites? A consequence of respiratory deficiency is that the TCA cycle no longer operates as a cycle because of the failure to oxidize succinate. Thus, oxaloacetate, together with its condensation partner, acetyl-CoA, must be provided by other pathways to maintain sufficient amounts of α-ketoglutarate for glutamate biosynthesis. Moreover, the regulation of expression of genes encoding the first three steps of the TCA cycle from which α-ketoglutarate is produced switches from *HAP* control to control by the *RTG*-genes in cells with reduced or compromised mitochondrial function [40]. Loss of respiratory function can also result in the up-regulation of those TCA cycle genes upstream of α-ketoglutarate [66,68].

Transcription profiling reveals that not all genes whose expression changes in respiratory-deficient cells are dependent on the *RTG* genes [64], further supporting the idea of multiple pathways in the retrograde response. Moreover, different mitochondrial lesions clearly elicit different patterns of gene expression as seen from a comparison of the expression profiles of genes up-regulated in respiratory competent cells treated with the different inhibitors of oxidative phosphorylation (Fig. 2). When compared with

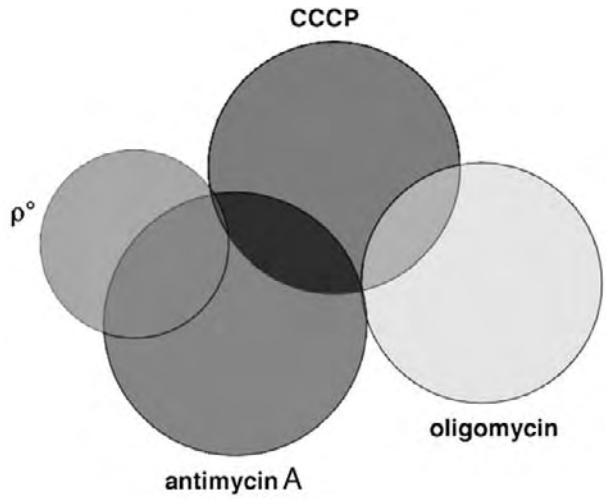


Figure 2 Different mitochondrial inhibitors elicit different genome-wide responses in gene expression. Shown is a Venn diagram of genes that are up-regulated in ρ^+ cells grown in raffinose medium in the presence of the respiratory chain inhibitor, antimycin A, the uncoupler, CCCP, or the ATP synthase inhibitor, oligomycin. These data are compared with a subset of up-regulated genes in a ρ° petite. The diameters of the circles are roughly proportional to the number of up-regulated genes, with the ρ° result set at 42 genes. (Data taken from. [64].)

the response in ρ° cells, only treatment of cells with antimycin A showed a significant overlap in the pattern of gene expression. These findings suggest that in ρ° cells, the predominant observed responses in gene expression are due to respiratory deficiency rather than to the lack of ATP synthesis from oxidative phosphorylation.

Aging and Retrograde Regulation

Aging is a complex phenomenon whose molecular basis is poorly understood. A detailed discussion of aging is well beyond the scope of this review, and so we shall only emphasize the role of mitochondrial function and the retrograde response in aging and cellular senescence. Declining mitochondrial function has often been associated with aging, but, as is the case with most aging-related events, distinguishing between cause and effect has proved difficult. In the filamentous fungi *Podospora anserina* and *Neurospora crassa*, mitochondrial respiratory function and rearrangements of mtDNA eliminating or inactivating essential respiratory genes are intimately related to senescence in these organisms. Senescence in *Podospora anserina* has been associated with the accumulation of senDNAs—circular oligomers of regions of wild-type mtDNA—in which most of the mtDNA coding sequences have been eliminated [69]. Recent studies have now established a direct link between mitochondrial electron transport function, particularly cytochrome oxidase activity, and increased longevity in *P. anserina*. Specific inactivation of nuclear or mtDNA-encoded *COX* genes leads to increased life span [70]. Similarly, life span can be extended by inactivation of the nuclear gene GRISEA [71,72]. GRISEA encodes a transcription factor involved in Cu^{2+} homeostasis. Because Cu^{2+} is an essential component of cytochrome oxidase, inactivation of GRISEA leads to cytochrome oxidase deficiency. In both of these examples, we see an induction of a cyanide-sensitive alternate oxidase (Aox) activity, which shunts electrons directly to oxygen upstream of complex III. It is believed that the presence of Aox induced by retrograde signaling depresses the production of ROS, which otherwise would be high in cytochrome oxidase-deficient mitochondria lacking Aox.

Replicative aging in *S. cerevisiae* is determined by the number of daughter cells that are produced from an individual yeast cell. A relation between the functional state of mitochondria, retrograde signaling, and longevity has become apparent from the finding that some ρ° petite strains show a life span extension that is dependent on the *RTG* genes [73]. Moreover, the correlation between these pathways of life span extension and the *RTG* retrograde pathway was strengthened by the finding that a ρ° petite strain that did not show extended life span had a weak or nonexistent retrograde response as measured by *CIT2* expression. Because of the complete absence of a mitochondrial electron transport chain, life span extension in petite cells, when observed, may be due to decreased production of ROS, to a general increase stress resistance, or to both. We have noted in an earlier section that in one study [66], several stress response genes were reported to be up-regulated in ρ° petites.

The most effective, documented strategy for environmental manipulation of life span is caloric restriction. Restricting calorie intake while maintaining adequate nutrition has been shown to prolong life span in organisms ranging from yeast to mammals. In yeast, glucose limitation results in a clear-cut life span extension, but unlike life span extension in petite cells, life span extension by caloric restriction does not appear to be dependent on the *RTG* genes [74]. On the contrary, inactivation of *RTG*-dependent gene expression in calorically restricted yeast cells has an additive effect on life span extension. These finding led Jiang *et al.* [74] to suggest that life span extension by retrograde regulation and by caloric restriction are separate and exclusive pathways. Just how caloric restriction extends longevity is an issue of some controversy, but it is likely to involve changes in metabolism and cellular redox states. Whether mitochondria play a role here is uncertain. In any case, it is clear that there are *RTG*-independent pathways of mitochondrial retrograde signaling, and it remains to be seen whether any of those pathways participate in life span extension imposed by caloric restriction.

In *Caenorhabditis elegans*, inactivation of an insulin-like signaling pathway and mitochondrial dysfunction resulting from decreased levels of coenzyme Q (CoQ, a component of the electron transport chain), imposed either by diet [75] or by a mutation in CoQ biosynthesis [76,77], result in increased life span. These pathways have been suggested to effect life span extension by an increase in ROS scavenging or production. Because mitochondrial function is affected in both cases, it is plausible that retrograde signaling may also contribute to the control of life span extension in worms.

Conclusions

Mitochondria, once acquired by cells as a free-living organisms, retained, on the one hand, a degree of semiautonomy and, on the other hand, adopted retrograde signaling to alert their "cellular host" to changes in the state of their mitochondria. The responses are usually seen as altered patterns of nuclear gene expression. In some cases, global mitochondrial biogenesis is affected; in other cases, specific changes are seen in the expression of some mitochondrial or nonmitochondrial components. In most instances, retrograde regulation can be rationalized as attempts by cells to make accommodations to their altered or defective mitochondria. Retrograde regulation is likely to play a role in diverse cellular processes, including metabolism, environmental sensing, development, aging, and disease. Some signaling molecules and regulatory factors have been identified, and others are certain to emerge as new retrograde pathways are uncovered.

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CHAPTER 326

Signaling During Exocytosis

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Introduction

Metazoan cells communicate with one another by secreting informational molecules. Regulated secretion starts, or stops, in response to an extracellular stimulus. In regulated exocytotic secretion, secretory vesicles fuse with the plasma membrane, and extrude their neurotransmitter or hormone contents, after cell stimulation by a secretagogue.

Many types of cells undergo regulated, exocytotic secretion (Table I). Secretion from other cell types is controlled by signaling that stimulates biosynthesis of secreted products, with subsequent constitutive, rather than regulated, secretion (e.g., aldosterone and cortisol secretion from the adrenal cortex, thyroid hormone secretion from thyroid follicular cells, cytokine secretion from T lymphocytes and macrophages, immunoglobulin secretion from B lymphocytes, protein secretion from hepatocytes, androgen and estrogen secretion from the gonads, mucopolysaccharide secretion from lung epithelial cells, bile salt secretion from the liver and gall bladder, and melatonin secretion from pinealocytes). The distinction between exocytotic and nonexocytotic secretion emphasizes the secretory vesicle as a "macromolecular machine" [1] that is an important locus of regulation in cell signaling. Consideration of regulated exocytotic secretion organizes a great deal of information about neuronal, endocrine, exocrine, and immune/inflammatory homeostasis. Solving the biophysical problem of passing packets of informational molecules across cell membranes in a defined period of time, in response to multiple specific extracellular signals, is the special bioengineering triumph of exocytotic secretion.

Some general statements can be made about exocytotic secretion. Endocrine and exocrine exocytosis is mainly "slow" (milliseconds to seconds) and occurs from large dense-core vesicles (LDCVs) or secretory granules (SGs). Neuronal secretion is either fast (release from small synaptic vesicles, or SSVs, within microseconds) or slow (release from LDCVs, as in endocrine cells). Neuroendocrine secretion may be acute (episodic or pulsatile, over minutes) or sustained (over hours). The rate of secretory vesicle exocytosis can be modulated at multiple steps called *docking, priming*, and *fusion.* Fast/acute secretion is calcium dependent, while slow/sustained secretion may involve both calcium and cyclic AMP (cAMP). Exocytosis of a secretory vesicle may deliver all of its contents to the exterior of the cell ("all-or-none") or only a fraction ("kiss-and-run"). Each mode of secretion has unique kinetic features and unique problems of membrane protein retrieval and reutilization. Regulated secretion can be modulated at the level of organellogenesis—the number of secretory vesicles in a particular type of secretory cell. The quality and quantity of the vesicle's content can be further regulated via vesicular transporter trafficking and function, as well as prohormone synthesis, sorting, and processing. The probability of vesicular secretion in response to a given stimulus may vary as a function of the previous stimulatory experience of the cell—this is secretory plasticity.

These factors may account for variation in regulated secretion from different cell types, secretion of different substances from the same cell, and even secretion of the same substance from the same cell in response to different secretagogues. Thus, SSV and LDCV compartments within the same cell behave differently; one secretagogue can interact with multiple receptors to stimulate different modes of regulated secretion at different times; secretion can be augmented or depressed during episodes of continuous stimulation. Differences between fast and slow neurotransmission, SSVs and LDCVs, acute and sustained secretion, and adaptation during continuous secretion have presumably evolved to allow neuronal, endocrine, exocrine, and inflammatory secretory

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Table I Some Secretagogues for Regulated Exocytosis and Their Targets

Secretagogue	Source	Target	Secreted material and function
ACh and PACAP	Coreleased from splanchnic nerve at adrenomedullary synapse	Adrenal chromaffin cells	Epinephrine and norepinephrine; hormonal cardiovascular and metabolic regulation
Gastrin and PACAP	Endocrine cells	Enterochromaffin-like cells (ECL cells)	Histamine; gastric acid secretion
Various hormones and paracrine factors	gastrointestinal epithelium	Enterochromaffin cells (EC cells) of gut	Serotonin; to supply 5-HT to platelets and mast cells of the gut
IgE	Circulation/tissue	Mast cells	Histamine; inflammation
Neurotransmitters; action potentials	Presynaptic neurons; nerve terminal depolarization	Neurons (postsynaptic)	Excitatory neurotransmitters (e.g., glutamate) Inhibitory neurotransmitters (e.g., GABA); neurotransmission
Calcium	Systemic	Parathyroid gland	PTH; control of calcium, mineral, and bone metabolism
ACh and cholecystokinin	Parasympathetic nerve terminals; paracrine secretion	Pancreatic acinar cells (exocrine)	Zymogens; amylase secretion into gut to control digestion
Glucose; epinephrine; PST; ACh; galanin and NE	Serum; adrenal medulla; adrenal medulla; islets; parasympathetic nerves; sympathetic nerves	Pancreatic islet cells (endocrine)	Insulin and glucagon; glucose uptake and utilization
Epinephrine	Circulation	Parotid gland	Amylase; regulation of salivation/digestion
Hypophysiotropic hormones (CRH; LH-RH; GH-RH; TRH; DA; somatostatin)	Hypothalamus	Pituitary, anterior	ACTH (+); LH/FSH (+); GH (+); TSH (+); prolactin (−); growth hormone (−). Endocrine regulation of reproduction, growth and metabolism
Sodium (hyperosmolarity)	Systemic	Pituitary, posterior	Oxytocin and vasopressin; salt and fluid retention
Thrombin, collagen, epinephrine, ADP	Circulation, blood vessels	Platelets (dense granules)	Serotonin, ADP & Ca^{2+}; hemostasis (platelet aggregation and vasodilatation, formed element adherence)
Calcium	Systemic	Thyroid C cells	Calcitonin; calcium metabolism

Abbreviations: ACh, acetylcholine; ACTH, adrenocorticotropin hormone; CRH, corticotropin releasing hormone; DA, dopamine; FSH, follicle-stimulating hormone; GABA, gamma-aminobutyric acid; GH, growth hormone; GH-RH, GH releasing hormone; LH, luteinizing hormone; PACAP, pituitary adenylate cyclase-activating polypeptide PST, pancreastatin; PTH, parathyroid hormone; TRH, thyrotropin releasing hormone; TSH, thyroid stimulating hormone

cells to perform homeostasis-by-secretion in response to myriad unique physiological circumstances.

Functional, Morphological, and Historical Aspects of Exocytosis and Stimulus-Secretion Coupling

Bayliss and Starling [2] discovered secretin, and articulated the general concept of regulated hormone secretion, in 1902. The modern understanding of exocytotic secretion, combining its morphological, biochemical, and cell physiological features, evolved during the latter half of the last century. In the 1950s, Hillarp [3] characterized the adrenaline-containing secretory granule, hypothesizing that it was the morphological substrate for catecholamine hormone secretion from the adrenal medulla. Adrenaline was found to be coreleased from the adrenal medulla, upon nerve stimulation, with chromogranins, large proteins also stored in secretory granules. Secretion had to occur via exocytosis to allow passage of these molecules into the circulation in the same ratios as found in the granules [4,5]. Morphological and biochemical evidence for secretory granules and exocytotic hormone release in the adrenal medulla, the exocrine pancreas, from mast cells, and from other secretory tissues followed [6].

Douglas and Rubin [7] showed that acetylcholine-stimulated catecholamine release from the perfused adrenal medulla required calcium influx into chromaffin cells. Douglas proposed the term *stimulus-secretion coupling* for this calcium-dependent process, analogous to stimulus-contraction coupling in myocytes [6]. In stimulus-contraction coupling, acetylcholine release from nerve terminals stimulates muscle cell depolarization, leading to calcium release from

the endoplasmic reticulum, triggering muscle contraction. In stimulus-secretion coupling, by contrast, acetylcholine release from nerve terminals stimulates chromaffin cell depolarization, leading to calcium influx and triggering exocytosis. Cytosolic calcium elevation driving exocytosis in most neuronal and endocrine cells is supplied primarily by influx of calcium through voltage-sensitive channels in the plasma membrane. However, the importance of calcium release into the cytosol from within the cell (calcium mobilization) in fine-tuning and in some cases primarily driving exocytotic secretion has been increasingly appreciated in the last decade or so, in neuronal, endocrine, and especially exocrine secretory cells [8,9].

Endocrine and exocrine cells contain relatively large (200 to >500 nm) secretory vesicles called *secretory granules*. Neurons and neuroendocrine cells contain LDCVs 100–200 nm in diameter that store peptides and biogenic amines, and SSVs 40–70 nm in diameter clustered near synapses in nervous tissue and at neuromuscular junctions. Small (40 nm), clear SSVs were first isolated from the electric organ of Torpedo, and shown to contain acetylcholine [10]. Katz, Miledi, and others postulated that these vesicles contained the *quanta* released on invasion of the nerve terminal by a depolarizing action potential, resulting in the miniature end-plate potential (mepp) [11]. Miledi demonstrated in 1973 that calcium entry accompanying the action potential was necessary and sufficient to trigger the release of the acetylcholine quanta at the neuromuscular junction [12,13]. The importance of amine neurotransmitter accumulation into SSVs and LDCVs for neurotransmission in the peripheral nervous system has been appreciated for at least 30 years on pharmacological grounds: The highly specific inhibitor of acetylcholine uptake into cholinergic SSVs, vesamicol, and the highly specific inhibitor of catecholamine uptake into LDCVs, reserpine, are potent inhibitors of cholinergic and adrenergic neurotransmission, respectively, *in vivo* [14,15]. The vesicle hypothesis of neurotransmission [11] has been reconfirmed by modern genetic techniques. Impairment of the gene encoding the protein that transports acetylcholine into vesicles disrupts cholinergic neurotransmission in the nematode *Caenorhabditis elegans*, even though intracellular levels of acetylcholine are increased in the absence of its vesicular accumulation [16]. Similar genetic findings in *C. elegans* support the critical role of vesicular transporters for GABAergic and biogenic amine neurotransmission [17,18].

Several technical and conceptual advances in the last two decades have greatly accelerated progress in understanding signaling during exocytosis. Development of real-time assays for exocytosis have been critical. These include measurement of capacitance changes in whole patch-clamped cells due to plasma membrane addition by vesicle fusion [19]; evanescent wave energy field changes to probe vesicle fusion at the plasma membrane [20,21]; electrophysiological monitoring of single calcium and other ion channels during addition of exogenous compounds to the inside or outside of the cell [22]; instantaneous release, and measurement of, calcium within the cell using caged calcium reagents and low-affinity calcium probes [23]; electrochemical measurement of neurotransmitter release from single vesicles [24]; and measurement of membrane fusion events using lipophilic dyes [25]. Reverse genetics in mammals and high-throughput forward genetics in *C. elegans* and *Drosophila* have also contributed to rapid progress by focusing on the proteins and phenotypes of regulated exocytosis [26]. Added to the classical availability of giant axons and their synapses and the ability to pipet cell-membrane impermeable substances into them during exocytosis [27], and further development of cell-free models for exocytosis [28,29], these new approaches have reconciled and integrated physiological, electrophysiological, biochemical, and molecular biological observations and experiments on the real-time, *in vivo* platform on which exocytosis occurs. The salient features of signaling during regulated exocytosis uncovered by these multiple approaches are summarized here.

Secretion Begins with Secretagogues

Secretagogues are molecules released by other cells into the circulation, a synapse, or the interstitial space that interact with target cell-surface receptors to initiate signaling, leading to exocytosis. In neuronal secretion, the action potential itself is a surrogate secretagogue. It invades and depolarizes the nerve terminal, triggering secretion in a frequency-encoded manner. The action potential is in turn a direct result of depolarizations caused by neurotransmitters released onto the dendrites and soma of the neuron. These neurotransmitters are the effective secretagogues whose messages are encoded by the action potential, transmitted through the axon, and decoded at the nerve terminal. Experimentally, elevated extracellular potassium mimics action potential-generated depolarizations of nerve terminals, through passive depolarization of the cell and opening of voltage-sensitive calcium channels. Depolarization by elevated extracellular potassium, however, even when tightly temporally controlled, only approximates the actual alternating amplitude- and frequency-modulated regulation of exocytosis that happens at synapse-to-axon-to-nerve terminal trios of the central and peripheral nervous systems. A brief illustrative table of physiological secretagogues, their target cells, and secreted products are listed in Table I.

Secretagogues Act at Target Cell Receptors

How do secretagogues act as first messengers to cause regulated secretion from neurons and endocrine and exocrine cells? First, the type of receptor at which the secretagogue acts determines whether secretion will be fast or slow. *Ionotropic* receptors are ion channels whose permeability is altered, or *gated*, by ligand binding. The ionotropic *N*-methyl-D-aspartate and kainic acid receptors allow increased calcium influx upon binding of the excitatory amino acid transmitter glutamate. The nicotinic receptor gates sodium

influx upon binding the neurotransmitter acetylcholine, causing subsequent cell depolarization and calcium influx via voltage-gated channels. Ionotropic receptors mediate fast neurotransmission at neuronal synapses (and rapid release of hormones from neuroendocrine and exocrine cells) because their action is immediate and does not depend on the accumulation of a second messenger via intermediary metabolic events. *Metabotropic* receptors show enhanced coupling to intracellular proteins, when liganded to their first messenger, that results in enzyme activation in the target cell. G-protein-coupled receptors (GPCRs), for example, couple to one of four types of G-proteins that mediate stimulation of adenylate cyclase, stimulation of phospholipase C, inhibition of adenylate cyclase, or stimulation of GTP binding proteins of the Ras superfamily [30]. Activation of metabotropic receptors can result in calcium influx just as stimulation of ionotropic receptors does; the difference is that ionotropic receptors mediate increased calcium influx in under a millisecond, whereas metabotropic receptors require tens to hundreds of milliseconds.

Signal propagation mediated by exocytosis through neuronal synapses can occur by either slow or fast transmission. Glutamate in the central nervous system, or acetylcholine at the neuromuscular junction, act via fast transmission. An action potential causes calcium influx, triggering the release of neurotransmitter (glutamate or ACh) from SSVs, and their interaction with ionotropic (NMDA or nicotinic) receptors will result in cation (calcium or sodium) influx into the target cell (neuron or muscle cell), generating a depolarizing signal, or excitatory postsynaptic potential (*epsp*), in less than a millisecond. Dopamine in the striatum acts via slow transmission. Action potentials cause the rapid release of dopamine from SSVs in dopaminergic nerve terminals, but the released dopamine acts on postsynaptic receptors to increase intracellular cAMP, which alters the excitability of the target cell over a period of milliseconds to minutes, or even hours, and affects additional properties of the postsynaptic cell such as cell-specific gene transcription [31]. The fact of slow or fast transmission is not a property of ligand or receptor only, but of the ligand-receptor dyad. ACh released onto cells with muscarinic rather than nicotinic receptors elicits slow responses; and glutamate released onto cells that express metabotropic (mGluR) receptors mediates slow synaptic responses. Mixed fast and slow neurotransmission occurs at the synapse between preganglionic neurons of the splanchnic nerve and chromaffin cells of the adrenal medulla. At high stimulation frequencies, the splanchnic nerve secretes acetylcholine from SSVs, which causes catecholamine release via a rapid, and rapidly desensitizing, action of acetylcholine at nicotinic receptors on chromaffin cells [32]. Acetylcholine can also act at muscarinic chromaffin cell receptors, permitting metabotropic modulation of calcium levels via stimulation of calcium release from intracellular stores mediated by inositol trisphosphate. Stimulation at low frequency causes sustained release of catecholamines from chromaffin cells via release of a peptide transmitter from the splanchnic nerve [33]. The peptide transmitter at the adrenomedullary synapse is PACAP [34]. Presumably the combination of slow and fast transmission, and ionotropic and metabotropic receptors on chromaffin cells, allows fine-tuning of secretory responses at the adrenomedullary synapse. Many central and peripheral neurons contain neuropeptides like PACAP which are released from LDCVs, and classical transmitters like ACh, which are released from SSVs [35,36].

Calcium and Cyclic AMP: The Two Main Second Messengers for Secretion

Exocytotic secretion from neuronal, endocrine, and exocrine cells is a result of elevation of cytosolic calcium or cAMP, or both, following secretagogue receptor stimulation. Calcium influx is the dominant mode of regulation in most secretory cells, intracellular calcium mobilization is more critical in others, and cAMP controls secretion in a few prominent secretory cell systems. In some cases, exocytotic secretion is regulated negatively rather than positively (see later section), and cAMP and calcium signals are attenuated or their effects antagonized.

The dependence on increased intracellular calcium for exocytotic secretion was worked out in chromaffin, mast, and posterior hypophysial cells by several laboratories using various mainly pharmacological maneuvers and observing secretion biochemically or morphologically [6], and in neurons by pipetting calcium ions into the squid giant synapse and observing secretion electrophysiologically [12]. The ability to bypass secretagogue stimulation by increasing cytosolic calcium concentration alone and to "clamp" secretion even in the presence of secretagogue (or electrical stimulation) by removal and/or chelation of calcium have been the sine qua non for calcium as the prime regulator of exocytosis [37]. Strictly speaking, calcium is often the third rather than the second messenger in exocytotic secretion. Thus, glutamate directly opens a calcium channel to allow cytosolic calcium to rise to levels sufficient to trigger exocytosis [38], but acetylcholine gates a channel for sodium, which causes cell depolarization, after which voltage-dependent calcium channels of several types are opened to allow calcium influx [39].

Metabotropic receptor stimulation is responsible for elevation of cytosolic calcium not through opening of calcium channels in the plasma membrane, but via stimulation of other second messengers, such as inositol trisphosphate (IP3), that act together with calcium on receptors on the endoplasmic reticulum (ER) to elevate cytosolic calcium levels. Secretion of insulin from the ß cells of the pancreas by the antidiabetic sulfonylureas can be triggered by calcium influx through L-type voltage-sensitive channels, via inhibition of an ATP-operated potassium channel, blockade of potassium efflux, and subsequent depolarization of the cell [40]. It is not yet clear if depolarization alone can be a second messenger in the absence of calcium entry or mobilization, for example, via sodium influx, changes in the equilibrium potential at the cell membrane, or physical reorganization of lipids and proteolipids at the cell surface. The pore-forming capabilities of

Vo ATPase subunits, found in both the plasma membrane and within the high-molecular-weight vacuolar ATPase of all secretory vesicles, suggest a potential mechanism for calcium-independent vesicular fusion [41].

In neurons and chromaffin cells, physiologically relevant secretion appears to be regulated wholly via calcium as a second messenger (as defined earlier). In other cell types, calcium and cAMP are both required, either in series, or in parallel, for full secretagogue action. Lactotrophs secrete prolactin in response to first messengers that increase intracellular cAMP and their action of forskolin is blocked by verapamil, suggesting that cAMP mediates calcium channel opening resulting in secretion in these cells [42–46]. Secretion from gonadotrophs by luteinizing hormone-releasing hormone requires calcium, supplied via intracellular mobilization following elevation of intracellular IP3 [47], and has also been suggested to require interaction of calcium mobilized from intracellular stores, with activation of PKC and elevation of cAMP [48].

In some secretory systems cAMP is the dominant signaling molecule mediating secretagogue-stimulated exocytosis. CRH-stimulated secretion of ACTH from corticotrophs shows only a partial sensitivity to blockade of calcium influx, and is mimicked by elevation of intracellular cAMP [46]. The calcium influx-dependent component of CRH-induced ACTH release is thought to be mediated by cAMP-dependent protein kinase (PKA), via inhibition of outwardly direct potassium conductance and subsequent cell depolarization [49]. CRH also mediates long-term secretion of catecholamines and enkephalin peptides from adrenal chromaffin cells, a process mimicked by the adenylate cyclase activator forskolin [50,51].

In contrast to regulation of ACTH secretion by CRH in primary corticotrophs, CRH acts to enhance ACTH secretion in AtT20 cells at multiple loci, including cAMP-dependent calcium mobilization independent of effects on potassium conductances [52]. Differences between CRH-regulated ACTH secretion in primary corticotrophs and the corticotroph-like AtT20 tumor cell line are illustrative of important differences between primary cells and tumor cell lines with respect to signaling mechanisms underlying regulated hormone secretion. The mechanisms of secretory regulation characterized in tumor cell lines may illuminate, but should not be confused with, those occurring in primary secretory cells, especially since malignant transformation is likely accompanied by profound changes in the regulation of calcium influx, efflux, and buffering that may also impact on exocytotic secretion. There is also evidence that regulated secretory behavior in isolated pituitary cells may differ from that in the perfused pituitary, in part due to the modulatory influence of factors secreted from the folliculostellate cells of the intact gland [53].

The insulin-secreting ß cell of the pancreatic islets is a prototype secretory cell in which calcium is critical for exocytosis, yet cAMP and phosphorylation via PKA play an important role in augmentation of secretion [54]. The primary insulin secretagogue is plasma glucose. When glucose levels are sufficiently high, elevation of intracellular ATP leads to the closure of an outwardly directed, ATP-regulated potassium channel, causing cell depolarization, opening of voltage-sensitive calcium channels, and exocytotic release of insulin [55–57]. Due to its importance in regulating fuel availability and consumption, insulin secretion is also modulated by a large number of peptides and neurotransmitters, including PACAP, acetylcholine, epinephrine, norepinephrine, arginine, VIP, glucose-independent insulinotropic polypeptide (GIP), and glucagon-like peptide 1 (GLP-1) [58]. Peptides can modulate both the fast and slow phases of insulin secretion. These phases describe secretion from the readily releasable pool and mobilization of the reserve pool of secretory granules (distinct from the fast and slow neurotransmission described earlier). Peptide modulation of insulin secretion may involve cAMP/PKA-dependent activation of both phosphatases and other kinases [54], modulation of calcium mobilization via stimulation of second messengers including IP3, and activation of PKC, following elevation of diacylglycerol (DAG) and IP3/Ca^{2+} [58a].

Some secretory cells are either not dependent on calcium for secretion or require it as a permissive rather than a regulatory factor. A prototype is amylase secretion from the parotid gland. Here, cAMP in the absence of extracellular calcium appears to be sufficient to trigger exocytosis [59–61], albeit calcium mobilization from intracellular stores has not been ruled out as a contributor to secretion in these cells.

A recurring theme of coregulation of exocytosis by calcium and cAMP is that the mode of regulation is highly dependent on the kinetics of secretion and, therefore, the rate-limiting step in secretion (see later section). Thus, parotid secretion, characterized by a dominant role for cAMP, is a very slow process (less than one granule per second per cell). Neuronal secretion, characterized by a greater dependence on calcium, is very fast (more than 100 vesicles per millisecond per cell) [62].

Cell permeabilization has been employed to demonstrate a role for GTP in triggering of exocytotic secretion, notably in mast cells [63] and chromaffin cells [64]. Stimulation by GTP can be mimicked by nonhydrolyzable GTP analogs, suggesting the involvement of Ras- or G-protein-type GTP-binding proteins in exocytosis. It is not yet clear, however, what GTP-binding proteins are involved, and whether they are acting in parallel or in series to calcium-mediated secretion elicited by physiological stimulators (e.g., IgG in mast cell secretion). The finding that Rab-like GTP-binding proteins are enriched on secretory vesicle membranes has not yet been developed into a coherent picture of the involvement of GTP-binding protein signaling in exocytosis, although Ras-like GTP-binding proteins are known to be important in shuttling of nonsecretory vesicles within the cell, and Rab3A knock-out mice demonstrate a neurotransmission-defective phenotype [26]. G-proteins may be important in both the priming and docking phases of secretory vesicle preparation for the final fusion event of exocytosis (see later section), and may act by coordinating the interactions of proteins and

phospholipids involved in secretion [65,66]. G-protein involvement in signaling during exocytosis awaits the identification of second and third messengers that convey secretagogue signals to them from the cell surface, and of vesicle-associated guanine nucleotide exchange factors, or GEFs, that regulate GTP hydrolysis and therefore the activities of G-proteins involved in exocytosis.

Calcium and the Regulation of Exocytosis

Calcium is important in virtually all cell functions: in exocytosis, calcium is probably involved in the formation of the secretory granule, its guidance to the plasma membrane, delay of fusion prior to a signal for secretion, and the process of exocytosis itself. Because of its primary regulatory role, calcium is extremely well buffered in secretory cells. Calcium is continually pumped out of the cell by a Ca^{2+}ATPase/transporter. It is sequestered within the endoplasmic reticulum and in mitochondria. Cytosolic calcium is normally about 100 n*M*. When it rises above this concentration (globally to about 1 μ*M* and locally to concentrations as high as 250 μ*M*) [9], it does so only briefly, due to multiple, tightly regulated mechanisms for cytoplasmic calcium clearance, calcium buffering, and inhibition of calcium influx and mobilization. These are summarized in detail in other contributions to this Handbook. The features of the regulation of calcium flux that are important in the context of signaling to exocytosis are, first, that virtually all modes of increasing cytosolic calcium, from calcium influx through voltage- and receptor-gated channels, to increased calcium release from endoplasmic reticulum through IP3, ryanodine (RY), cyclic ADP-ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP) receptors, can be regulated by first messengers/secretagogues from outside the cell; and, second, that these modes of elevating cytosolic calcium are highly interactive in the regulation of exocytotic secretion by secretagogues both singly and in physiologically relevant combinations [8,67,68].

Calcium influx is regulated by ionotropic secretagogues in two ways. The first is the direct admission of calcium to the cell through ionotropic receptors. The most prominent of these in the central nervous system are the glutamate-gated calcium channels, including the NMDA and AMPA/kainate receptors. These receptors are mainly postsynaptic, so their role in regulation of calcium flux leading to exocytosis at the nerve terminal has not been extensively explored [69,70]. Glutamate has been implicated in modulation of its own release from hippocampal synaptosomes via autoreceptors of the kainate type [71] and may inhibit secretion presynaptically by depolarizing the nerve terminal and decreasing the efficiency of action potential invasion and rapid calcium influx [72]. Acetylcholine is thought to facilitate secretion in the central nervous system by increasing calcium influx (through α_7 subunit-containing presynaptic nicotinic receptors) at presynaptic sites [73]. The chemical neuroanatomy of the striatum allows the unambiguous conclusion that ionotropic receptor regulation of exocytosis in that region is truly presynaptic and heterotypic [74]. Other examples of presynaptic regulation of neuronal exocytosis might represent heterotypic presynaptic modulation of transmission, as in the well-studied Aplysia system, or autoreceptor action, as for dopamine receptors in the striatum.

The second major mode of calcium influx regulation via secretagogue stimulation is indirect, via opening of voltage-sensitive calcium channels (VSCCs), normally closed at resting potential (−70 mV in neurons) upon cellular depolarization. In neurons, depolarization results from invasion of the nerve terminal by an action potential propagated from the cell soma via the axon. In other excitable cells, the action potential is generated directly by ligand-gated ionotropic receptors that allow sodium influx, such as the nicotinic receptor of the chromaffin cell. Generation of an action potential is capable of opening, at least transiently, all of the several types of VSCCs, and several of these are capable of admitting calcium even without complete depolarization. L-[long-lasting; dihydropyridine (DHP)-sensitive] channels are ubiquitous and are the workhorses of skeletal muscle and heart. P (Purkinje)- and N (neuronal)-type VSCCs are DHP resistant, were first identified in Purkinje cells and elsewhere in brain, and are specifically inhibited by snail venoms [75]. L-type channels are found mainly on dendrites and cell bodies of neurons, while non-L-type channels are enriched at nerve terminals [76]. Consistent with their predominant regional localizations, L-type channels are important for regulation of postsynaptic events in neuronal cells [77], whereas non-L-type channels, in various combinations, are responsible for the regulation of calcium flux in response to depolarization that mediates neurotransmitter release from nerve terminals [75]. L-type channels generally control exocytosis in endocrine tissues [78,79].

An interesting and novel third mode of calcium influx regulation involves the modulation of the voltage dependence of a voltage-sensitive general cation channel by cyclic AMP elevation, such that its probability to be open at resting potentials is significantly increased [80]. Norepinephrine and PACAP regulate pinealocyte calcium influx in this manner. It remains to be seen if secretagogues that cause elevation of cAMP in other tissues employ slow depolarization through high-conductance general cation channels to regulate calcium influx and exocytosis.

Finally, depolarization leading to calcium influx has been reported to be stimulated by at least one metabotropic receptor, a variant of the PAC1 receptor for the neuropeptide PACAP [81]. PACAP clearly stimulates calcium influx into neurons and chromaffin cells via voltage-dependent L- and non-L-type channels to stimulate exocytosis [82–85]. The effects of PACAP on calcium influx do not, as in the corticotroph, appear to be mediated by cAMP-dependent modulation of cellular potassium conductance. How this metabotropic receptor, which is not known to directly gate any plasma membrane cation or anion channel, causes the depolarization required for VSCC opening leading to exocytosis is currently a mystery. In sympathetic neurons, PACAP stimulation of

depolarization may involve a combination of sodium influx, inhibition of potassium efflux, and IP3-mediated calcium release into the cytoplasm from intracellular stores [86], although the evidence for this as a general mode of regulation of depolarization and calcium influx by PACAP is not compelling at this time.

In neurons, calcium channels are positioned close to sites at which SSVs are docked. Colocalization to this nanodomain presumably allows calcium levels above 100 μM, necessary for triggering synaptic vesicle exocytosis to be attained. By contrast, depolarization-stimulated exocytosis in neuroendocrine cells can be mimicked by intracellular calcium levels of less than 5 μM, as is also the case for secretion induced by release of caged calcium in other endocrine and in exocrine cells. Augustine [87] has succinctly summarized the concept, and proofs for, the involvement of nanodomain high calcium for fast SSV exocytosis, microdomain intermediate calcium levels for moderately fast transmission via SSV exocytosis, and low (2–20 times resting levels) "radial shell" intracellular calcium levels for neuropeptide release from LDCVs in neurons, and hormone release from the SGs of neuroendocrine cells.

As summarized earlier, ionotropic receptor activation can increase calcium influx both directly through gating of calcium influx and indirectly through cell depolarization via increased sodium conductance. Secretagogues acting via metabotropic receptors can also trigger calcium influx through voltage-sensitive channels via elevation of cyclic AMP, which in some cells, like the corticotroph, causes depolarization via inhibition of outwardly direct potassium conductance [49]. More commonly, secretagogue activation of metabotropic receptors causes an increase in cytosolic calcium via release from intracellular stores. Although influx of extracellular calcium is the most common mode of increasing cytosolic calcium for exocytosis, the role of calcium release from within the cell has become increasingly appreciated as an important one in a number of secretory cells. Douglas [88] had already noted in 1975 that calcium required for mast cell degranulation could also be supplied from intracellular stores as well as from outside the cell.

Mitochondria are the major depot for cellular calcium, and they are critical for buffering of cytosolic calcium, but are not a major source of regulated calcium release for exocytosis. Rather, calcium within the ER is released by second messenger stimulation of two or more specific ligand-operated calcium channels on the ER membrane. The generation of intracellular IP3 occurs when Gq-coupled receptors, like those for PACAP and acetylcholine, activate phospholipase C, and cleavage of phosphatidylinositol bisphosphate, causing the liberation of IP3 into the cytosol and leaving DAG within the membrane. IP3 interacts with the ryanodine receptor (RYR), so named because it contains a binding site for the alkaloid ryanodine, causing release of calcium into the cytosol. There is direct evidence that metabotropic secretagogues, such as cholecystokinin (CCK) acting on pancreatic acinar cells, cause exocytosis via IP3-mediated calcium mobilization independently of calcium influx from outside the cell [68].

It is unlikely that release from intracellular stores can supply the concentration of calcium—greater than 100 μM—needed for SSV exocytosis/fast neurotransmission. Levels of cytosolic calcium required for LDCV exocytosis are much lower [62], yet in several cases examined in detail, it appears that modulation of cytosolic calcium from intracellular stores requires the action of multiple ligands, all presumably elevated or liberated by first-messenger secretagogues, to trigger secretion. Two of these, in addition to IP3, are nicotinic acid adenine dinucleotide phosphate (NAADP) [89] and cyclic ADP-ribose (cADPR) [8,90,91]. Cancela and coworkers [68] examined this issue in pancreatic acinar cells using capacitance measurements of exocytosis and intracellular injection of second messengers cADPR, NAADP, and IP3, in conjunction with the secretagogues ACh or CCK. Local calcium spikes insufficient to cause exocytosis elicited by threshold concentrations of ACh or CCK were converted to global sustained calcium elevation in the cell sufficient to cause exocytosis by potentiation with IP3 in the case of CCK, and NAADP or cADPR in the case of ACh, respectively, indicating that CCK mobilizes calcium through NAADP and cADPR, and ACh through IP3, and suggesting a mechanism for combinatorial action of secretagogue regulation of zymogen secretion from the exocrine pancreas [68]. Yule and colleagues [92] have also suggested that sphingolipid (Sp)-activated calcium egress from the ER is involved in the control of zymogen granule exocytosis in the pancreatic acinar cell, thus implying the involvement of four types of calcium channel regulation in the mobilization of calcium from intracellular stores. How does integration of these multiple signaling mechanisms occur? Berridge *et al.* [9] have suggested that calcium initiated calcium release (CIRC) links the individual calcium spikes that result from opening of individual channels by second and third messengers to the building up of a sustained calcium transient, or *global* calcium increase, within the secretory cell. The coregulation of calcium release from intracellular stores by calcium itself, via CIRC, along with the second/third messengers IP3, cyclic ADP ribose (cADPR), NAADP, and Sp provides for instantaneous control of secretion. This type of calcium regulation of secretion is integrated over time, and based on cell sampling of the extracellular concentration of secretagogues the immediate prior history of secretagogue stimulation, and the ability to respond in an exquisitely graded fashion, to the secretory requirements of the organism as communicated by first-messenger/secretagogue signaling to the target secretory cell. In this sense, exocrine and endocrine secretion that is regulated primarily by calcium release from intracellular stores performs in slow motion the highly integrated processing of multiple inputs that is "hard wired" into neurons by their multiple synaptic contacts.

Secretory granules themselves have high concentrations of calcium, and may be involved in supplying calcium required for exocytosis [93]. Yoo and coworkers [94,95] have reported that IP3 receptors are present on bovine chromaffin granules and that IP3 can release calcium from them *in vitro*.

Gerasimenko and coworkers [96] have reported calcium release from zymogen granules of pancreatic acinar cells mediated through IP3 receptor activation, although these results have also been attributed to ER microsomes present in some zymogen granule preparations [97]. The vesicular membrane protein SV2, structurally related to the amine transporters VMAT and VAChT but without a currently assigned synaptic function, has also been implicated in calcium regulation during exocytosis by SSVs [98].

Exocytosis and SNAREs

In this section, we discuss exocytosis and SNAREs their relative importance in different secretory systems; and regulation of the function of core components of the fusion process during exocytosis.

Vesicle shuttling underlies myriad transport processes between membrane compartments within eukaryotic cells. One of the systems for membrane-bound cargo transport between various membrane compartments within the cell is the presence of receptors (SNAREs) on complementary membranes that bind to each other to form an extremely stable complex, driving fusion of the partner membranes and transfer of membrane-bound cargo from vesicles that have budded from a donor compartment, to a recipient membrane compartment. This process is followed by resegregation, mediated by dynamin and other proteins, of the donor vesicle, minus either its membrane or intravesicular cargo, from the recipient membranous compartment. The overall process of fusion and dissociation of the SNARE complex requires recruitment of soluble *N*-ethylmaleimide sensitive factor (NSF) and its attachment proteins (SNAPs), in a process dependent on ATP hydrolysis. In 1993, a landmark paper described the use of a SNAP affinity column to capture SNAREs from brain tissue extracts [99]. A triad of proteins was identified—synaptobrevin/VAMP, syntaxin, and SNAP-25 (for *Synaptosome-associated protein of Mr 25 kDa*)—that could form a 20S fusion particle with NSF and SNAPs. Synaptobrevin/VAMP had previously been identified as a component of small synaptic vesicles, and was therefore postulated to act as the *v-SNARE* or vesicle marker for fusion, and syntaxin and SNAP-25 were postulated to act as the *t-SNAREs* or target membrane receptors, the target in this case being the plasma membrane [100]. A subsequent report indicated that the SNARE complex (synaptobrevin, syntaxin, and SNAP-25) formed remarkably stably in the absence of NSF/SNAPs and could bind a fourth protein, called synaptotagmin, which competes with alpha-SNAP for binding to the SNARE complex. It was postulated that synaptotagmin might function as a "clamp," preventing NSF/SNAP-assisted secretory vesicle fusion until synaptotagmin's regulated removal from the SNARE complex, and replacement with alpha-SNAP [101]. Disruption of depolarization-induced secretion from LDCVs in PC12 cells by microinjection of synaptotagmin antibodies, or soluble fragments of synaptotagmin without a vesicle membrane attachment site, provided direct evidence of a role for synaptotagmin in regulated exocytosis [102]. The next section includes a discussion of the specific role of synaptotagmin as a calcium sensor in exocytosis.

A requirement for SNARE complex formation in regulated exocytosis from a variety of secretory cells has been demonstrated with the aid of toxins that specifically cleave each of the three individual SNAREs and impair their ability to participate in SNARE complex formation [103]. The clostridial botulinum and tetanus toxins (BoNT and TeTx, respectively) are zinc endopeptidases that disrupt neurotransmission by cleaving synaptobrevin/VAMP (BoNT-F, -G and TeTx) [104], SNAP-25 (BoNT-A, -E, and -C), and syntaxin (BoNT-C1). Cells can be permeabilized sufficiently to allow toxin entry without disrupting the exocytotic machinery, most commonly through the use of other bacterial toxins or antibiotics that form controlled-sized membrane pores [29,105], by electropermeabilization [106], or by capacitance measurements of exocytosis during cell perfusion with toxin. This has allowed facile experiments to demonstrate the involvement of the SNARE complex in a variety of secretory systems. The failure to block exocytosis with certain toxins in specialized secretory cells has pointed the way toward the identification of other SNARE-related proteins that take the place of the syntaxin 1 and 2 isoforms in the SNARE complex, to impart variant exocytotic regulatory characteristics to those cells.

Restoration of calcium-dependent secretion in permeabilized cells in which the soluble SNAPs and NSF were allowed to diffuse out, by reintroduction of these factors, has established the importance of these components of the fusion complex in exocytosis in several neuroendocrine cell types [107]. The involvement of synaptobrevin/VAMP in exocytotic release during neurotransmission has been demonstrated by microinjection of BoNT and TeTx, which hydrolyze synaptobrevin-2, into Aplysia neurons, which contain synaptobrevin-2, and rescue from blockade by co-injection of synaptobrevin-1-derived peptides resistant to toxin cleavage [104]. Similarly, BoNT-D and -C1 light chains cleaved synaptobrevin-2/VAMP-2 and blocked calcium-stimulated LDCV exocytosis in chromaffin cells [64]. Exocytosis is blocked in permeabilized pancreatic ß cells with TeTx and BoNTs, implicating both VAMP-2/cellubrevin and SNAP-25 in insulin release from secretory granules [108–110]. BoNT C1 was reported to block 95% of insulin release induced by KCl, implicating syntaxin in secretion, but only 25% of glucose-induced insulin release [111], suggesting that a related, relatively toxin-resistant protein may carry out some proportion of exocytotic secretion of insulin, and reinforcing the notion that multiple vesicle pools may make up what in aggregate is called the readily releasable pool (RRP) which comprises about 1% of the secretory granules of the normal ß cell [58]. Insulin secretion by KCl in the HIT-15, but not the RINm5F insulinoma cell line was blocked by BoNT-F [112]. The RINm5F cell line, however, is impaired in glucose-mediated insulin release and has only 1% of normal cellular levels of insulin compared to ß cells of the pancreatic islets [113], reinforcing the need for caution in applying conclusions

drawn from tumor cell lines to the physiological regulation of secretion from specific tissues *in vivo*. Calcium-dependent exocytosis from neutrophils requires VAMP-2 based on its blockade by clostridial toxin treatment [114]. Zymogen granule exocytosis from the exocrine pancreas also requires synaptobrevin-2/VAMP-2, based on clostridial toxin blockade of secretion [115]. VAMP-2 is required for amylase secretion from parotid acinar cells, despite the fact that exocytosis in these cells is controlled predominantly by cyclic AMP rather than calcium and is rather slow even by exocrine secretory standards [116]. Based in part on differential clostridial toxin sensitivity, and on the absence of syntaxin I or II expression in parotid, Fujita-Yoshigaki and colleagues [61,117] have proposed that syntaxin-4 rather than syntaxins-1 or -2, and SNAP-23 rather than SNAP-25 participate with synaptobrevin/VAMP in the SNARE complex for parotid amylase secretion, and have also put forward the intriguing speculation that this SNARE complex may exclude synaptotagmin so that other components, such as Rab3A, can participate in the fusion complex under the control of cAMP/PKA-dependent phosphorylation rather than calcium. Consistent with this hypothesis, syntaxin-4, but not the neuronal isoform syntaxin-1, can be phosphorylated by PKA, and phosphorylation affects its binding to SNAP-23 *in vitro* [118].

Cell permeabilization techniques have also been employed in "run-down" experiments, in which permeabilized cells gradually lose the ability to perform exocytosis, to add back ions, factors, and proteins that restore secretion. Experiments of this type have led to the identification of early and late phases of exocytosis, which may differ in their contributions to the overall rate of secretion in different tissues and may have distinct biochemical requirements as well. Three stages of exocytosis have been defined in secretory cells based on permeabilized cell/reconstitution experiments. They are docking, priming, and fusion [119,120]. Identification of these steps has been useful in assigning spatiotemporal "regulatory domains" not only to calcium and cyclic AMP, but to their sensors in secretory cells (see next section). Operationally, docking defines a step wherein vesicles are associated with the plasma membrane in a way that is not disrupted when cells are ruptured and undocked vesicles are washed free of the plasma membrane [121]. Docking may involve the transfer of vesicles, both SSVs and LDCVs, from a cytoskeletal matrix that either forms a barrier to free diffusion to the plasma membrane for these organelles, or to which they are tethered and must be freed prior to diffusion to the plasma membrane. The roles of kinesin, synapsin, cyclic AMP, PKA, and ATP in this step are considered in the following section.

Priming is an ATP-dependent step that, at the present time, has no morphological substrate to distinguish it from fusion, except that the exocytosis of docked vesicles requires both ATP and calcium, and that the ATP-dependent step precedes the calcium-dependent one [122]. The ATP dependence of priming suggests that NSF and SNAPs could act at this step, positioning the SNARE complex for fusion triggered by elevation of calcium in the neighborhood of the primed vesicle or granule [119], followed by addition of synaptotagmin to the SNARE complex upon elevation of calcium, which increases synaptotagmin/syntaxin affinity about 100-fold [123].

In considering the roles of docking, priming, and fusion and their regulation by calcium and cyclic AMP, it is useful to note briefly the kinetics of secretion in neuronal, endocrine, and exocrine systems, and in immune/inflammatory exocytotic secretion as well. Secretion of amylase from the rather large (>1000 nm) granules of the parotid gland acinar cells is relatively slow—it occurs only by about 10 sec after application of a cAMP-dependent secretagogue such as epinephrine or isoproterenol. Insulin exocytosis from the >200 nm-diameter ß-cell secretory granules also requires a few seconds, whereas secretion from neuronal or neuroendocrine LDCVs, 100–200 nm in size, takes between 1.3 [124] and 40 msec [125]. Exocytosis from neuronal SSVs, about 40 nm in diameter, occurs a few hundred microseconds after elevation of nerve terminal calcium levels above ~25 μM [126]. While it is axiomatic that exocytotic fusion, even in parotid cells, requires or is augmented by calcium, that requirement may be progressively less dominant from a regulatory point of view, as secretion moves along the continuum from (neuronal) very fast, all-or-none secretion of vesicles poised or accumulated in a docked and primed state followed by a refractory state during which replenishment of the releasable pool occurs, to very slow secretion during which vesicles docking, priming, and fusing populate more equivalent proportions of the overall vesicle complement of the cell.

Calcium and cAMP Sensors for Exocytosis

Synaptotagmin has already been alluded to as the primary calcium sensor in neurons, and likely in neuroendocrine cells as well. Recently, analysis of neurotransmission in synaptotagmin-1 knock-out mice, flies, and worms has confirmed its importance in neurotransmission, albeit the full analysis of the role of each of the 12 synaptotagmin isoforms in neuronal and non-neuronal SSV, LDCV, and SG exocytosis remains to be completed in conditional knock-out animals [26,127]. Synaptotagmin-1 was identified in the central nervous system as the major low-affinity sensor for synchronous SSV release based on electrophysiological criteria; and, in fact, asynchronous secretion, elicited by hypertonic medium or alpha latrotoxin, was unaffected. Leaving aside the issue of whether synaptotagmin-1 is a "clamp" whose inhibition of synchronous release is released by calcium, or a calcium-stimulated effector of secretion, its relatively low affinity for calcium accords with estimates, based on caged-calcium release, of a requirement for greater than 20 μM calcium, presumably attainable only in "nanodomains" near voltage-sensitive calcium channels following action potential invasion of the nerve terminal, for neurotransmission via SSV exocytosis in neurons, and consistent with the observation that synaptotagmin/syntaxin affinity is sharply increased only at calcium concentrations above 100 μM [123]. What of calcium-dependent secretion that occurs from LDCVs

in neurons, or LDCVs and SGs of neuroendocrine and exocrine cells, that can be triggered by calcium concentrations considerably lower (300–1000 n*M*?), or even SSV secretion from certain nerve terminals that can be triggered by calcium concentrations below the low-affinity calcium binding constant for synaptotagmin-1 [87]? Several possibilities exist, and include (1) altered calcium binding affinity for synaptotagmin due to accessory protein binding in non-neuronal cells, (2) different calcium sensitivities for various synaptotagmin isoforms in cells in which these are expressed and mediate exocytosis [128–130], and (3) calcium sensors other than synaptotagmin in non-neuronal cells, and for LDCVs in neurons.

Dodge and Rahamimoff [131] defined a complex, cooperative, and nonlinear requirement for calcium in exocytosis that appears to apply to a wide variety of excitable, exocytotic cells. Thus, exocytosis might require multiple calcium-sensitive proteins, as well as multiple cooperative calcium binding sites on individual proteins. To act as a sensor for calcium triggering exocytosis, a calcium-binding protein would need to bind calcium with an affinity less than calcium channels in resting cells, depend for its efficacy in promoting exocytosis on that binding event, and possess an affinity compounded of on and off rates for calcium consistent with the kinetics of stimulus-secretion coupling. Synaptotagmin itself could potentially act by binding and enabling fusion/prefusion with different binding partners at different calcium concentrations in a stepwise fashion, the last stage of which is the low-affinity one, which is the rate-limiting step for primed, and possibly "prefused," SSVs. This could account for the requirement for four calcium ions in SSV exocytosis, corresponding to several calcium-dependent synaptotagmin interactions, perhaps even including synaptotagmin dimerization, and less than four calcium ions in other secretory systems involving SGs or LDCVs [125].

Several other calcium-binding proteins have been investigated as calcium sensors for exocytosis in a variety of secretory cells. The calcium-activated protein for secretion (CAPS) is an ubiquitous cytosolic protein that nevertheless binds LDCVs, but not SSVs, in a highly specific and calcium-dependent fashion [132]. Its ablation in *Drosophila* demonstrates that it is essential for LDCV exocytosis [133]. CAPS is present in all secretory cells that exhibit calcium-regulated secretion [134] and, interestingly, is absent from the parotid gland [61]. A puzzling aspect of CAPS involvement in LDCV exocytosis is its relatively low affinity (about 250 μ*M*) for calcium [132], since the calcium threshold for exocytosis in, for example, chromaffin and PC12 cells is less than 10 μM [135]. However, calcium affinities *in vitro* and *in vivo* may easily be discrepant, given both the possibility for cooperativity with other calcium-binding proteins in the exocytotic process, and the need to define very carefully the pool of docked vesicles contributing to secretion in a given experimental paradigm, only a small fraction of which is "readily releasable" [58,136].

The neuronal calcium sensor (NCS) or frequenin family of calcium-binding proteins is made up of candidates for transducing calcium signals during exocytosis that are just beginning to be studied in detail. Overexpression of NCS-1 in AtT-20 cells, for example, has complex effects, blunting secretagogue stimulation of ACTH release in intact cells, yet enhancing calcium- and GTP-gamma-S-induced secretion from permeabilized cells [137] and, thus, possibly representing the link between GTP-binding proteins and calcium in the regulation of exocytosis by nonhydrolyzable GTP analogs reported in mast cells and AtT-20 cells. Frequenin's involvement in exocytosis at the neuromuscular junction appears to be specific for calcium influx through N-type VSCCs, suggesting that this calcium-binding protein may regulate calcium at very specific sites of exocytosis corresponding to calcium channel-vesicle nanodomains [138]. Coscaffolding of frequenin with N-type VSCCs within nerve terminals may explain the observation that both NCS and frequenin demonstrate inhibition of exocytosis at high concentrations, a hallmark of effects on cellular processes upon introduction of high concentrations of scaffolding and scaffolded proteins into many cell types [139].

Cyclic AMP enhances secretion in almost all cell types studied (see [141] for a list and the earlier section on calcium and cyclic AMP). The cAMP sensor is almost universally found, or assumed to be, PKA, due to blockade by PKA inhibitors such as H89. What are the targets for phosphorylation by PKA and, therefore, the effective "cAMP sensors" for exocytosis? One of them may be the cysteine string protein (CSP), a synaptic vesicle protein required for neurotransmission, and first identified by Zinsmaier and Benzer in 1994 [140]. Evans and colleagues [141] have demonstrated that CSP is phosphorylated in neuronal and neuroendocrine cells, is a substrate for PKA-catalyzed phosphorylation *in vitro*, and modulates secretion from chromaffin cells when overexpressed as the wild-type, but not the serine-10-mutated (nonphosphorylatable) form. Secretion from the parotid gland by acetylcholine is likewise potentiated by cAMP, and this may be due to PKA-dependent phosphorylation of the IP3 receptor, resulting in greatly potentiated IP3-dependent intracellular calcium release in response to muscarinic receptor activation [142]. PKA-dependent IP3 receptor phosphorylation is reported to inhibit localized calcium release in pancreatic acinar cells [143]. The finding that PKA may be localized to sites of calcium mobilization, perhaps by specific anchoring proteins or AKAPs [144] suggests that both potentiation and inhibition of secretion may be produced, depending on the specific secretagogue. It has been suggested that the role of PKA-dependent phosphorylation is to regulate intracellular calcium, and therefore exocytosis, by altering the calcium signal patterning of a given secretagogue, rather than simply inhibiting or augmenting calcium fluxes [142,143,143a]. Virtually all members of the SNARE/NSF/SNAP complex contain phosphorylation sites for casein kinase II, CaM kinase II, and PKA, and are phosphorylated by them. Of particular interest in regulation of exocytosis is that the affinity of alpha-SNAP phosphorylated by PKA for the core docking/fusion complex is 10 times weaker than dephospho-alpha-SNAP [145].

Protein phosphatases inhibit, and protein kinases enhance, insulin secretion in mouse pancreatic ß cells [54].

A second sensor for cAMP, the cAMP-dependent guanine nucleotide exchange factors (or Epacs) I and II, have recently been identified [146,147]. cAMP-GEF-II is present in the central nervous system and the adrenal medulla, though not in PC12 cells. Its introduction into PC12 cells, however, results in enhancement of forskolin-stimulated secretion [148] and, therefore, its involvement in cAMP-augmented, or cAMP-driven, exocytosis in other secretory cells, including those of the central nervous system, is an open question.

Thus, an emergent picture of secretagogue regulation of exocytosis is a primary role for calcium in exocytosis in most systems, with an augmentation of secretion by cAMP mediated through PKA phosphorylation of a wide variety of substrates directly involved in exocytosis. A possibility not generally addressed is that first messengers without intrinsic secretagogic activity of their own might well have important modulatory influences on primary secretagogue action, most likely through elevation (or suppression) of cAMP levels in the cell.

Role of Signal Summation in Regulated Exocytosis

Besides the immediate triggering of exocytosis itself, calcium and cyclic AMP (and other second messengers) control secretion in a more temporally extended manner by stimulation of cAMP- and calcium-activated proteins that possess intrinsic, sophisticated means of recording the cellular exocytotic history over time, and tailoring the response to immediate stimuli based on past experience. Calmodulin kinase II (CaMKII) is a prototypical regulatory molecule involved in exocytosis regulation. CaMKII possesses a "molecular memory" for calcium such that its stimulation by calcium in the presence of calmodulin results in a greater than 10-fold discrimination between calcium pulsed at 1, 2, and 4 Hz [149]. Consistent with its regulatory properties, CaMKII appears to play a role in long-term facilitation of exocytotic release in several cell types, via refilling of the docked/primed vesicle pool. In pancreatic ß cells, for example, acetylcholine acting through muscarinic receptors, stimulates late-phase exocytosis several fold, via activation of CaMKII, and this effect appears to be at the level of mobilization of vesicles into the RRP [150]. There are several known substrates for CaMKII relevant to the regulation of exocytosis in secretory cells, including synaptotagmin [151] and synaptobrevin/VAMP [145]. The best documented role for CaMKII in modulation of exocytosis is via phosphorylation of the synaptic vesicle-associated protein synapsin, resulting in vesicle untethering from cytoskeletal elements and mobilization into the "docked" pool [152]. The role for synapsin in exocytosis has been documented in neurons and for SSVs [152,153]. It may obtain as well for some endocrine cells, although many do not express synapsins.

It has been reported that both depolarization-induced secretion of gonadotropin-releasing hormone (GnRH) from the median eminence of the hypothalamus and carbachol or potassium-induced release of catecholamines from PC12 cells is dependent on CaMKII activation, based on blockade by the CaMKII inhibitors KN-62 or KN-93 [154,155]. However, inhibition by the KN compounds was less than 50% in these experiments, and confirmation of involvement of CaMKII in secretion in these systems by nonpharmacological means, in the absence of quantitative controls for the well-documented nonspecific effects of KN-62 and KN-93 directly on calcium influx through VSCCs [156], remains to be determined. Involvement of CaMKII in insulin secretion from ß cells has, however, been deduced from inhibition by KN-62, with no effect of KN-04, a compound that shares the calcium influx inhibition, but not CaMKII inhibitory properties of KN-62 [157].

Calcium signaling may also be responsible for the activation of tyrosine kinases that ultimately affect regulated secretion [158], although the molecular steps intervening between elevation of intracellular calcium and activation of, for example, MAP kinases in bovine chromaffin cells, remain unidentified.

Synaptic facilitation, depression, and other forms of synaptic plasticity that involve altered probabilities of secretion for a given action potential stimulus are mediated by protein kinase stimulation by both calcium and cyclic AMP [159]. The precise cellular loci of such long-term effects on exocytosis remain to be determined, but are likely to involve, at least in part, mobilization of the vesicular pool into the RRP or a pool from which vesicles may be recruited into the readily releasable one. A recent report provides evidence that calcium and cyclic AMP antagonistically regulate SNAP-25 phosphorylation through calcineurin and PKA activation, respectively, thus indirectly affecting the kinetics of vesicle movement into the RRP in chromaffin cells [160]. Long-term facilitation in Aplysia sensory neurons, as well as presynaptic components of long-term potentiation in cerebellum and hippocampus in mammals, may represent in part such temporally delayed regulatory effects on the efficacy of stimulus-secretion coupling [161–163].

Role of PKC and Other PMA Targets in Regulated Secretion

Protein kinase C has been invoked as a regulator of the secretion of histamine from mast cells, catecholamines from chromaffin cells [164], neurotransmitters from nerve terminal SSVs [165], amylase from parotid gland and exocrine pancreas [166], insulin from ß cells, and hormones from the anterior pituitary [167]. Several of these experiments have involved implication of PKC through application of phorbol myristate acetate (PMA), on the assumption that this is the major, if not the sole, target for PMA in secretory tissue. Recently, Rhee and coworkers [168] have reported that the synaptic protein Munc13 is a target for PMA and is the substrate through which PMA and DAG augment synaptosomal neurotransmitter release. Thus, while multiple substrates

for PKC, including SNARE complex proteins and cation channels exist, a thorough reassessment of the roles of Munc13, and PKC, in exocytosis can be anticipated. Conventional protein kinases C (PKCc's) possess unique regulatory properties: the C1 (DAG-binding) and C2 (calcium-binding) domains of PKCc's allow this kinase, like calmodulin kinase, to decode frequency-dependent calcium signaling and, in addition, to prolong it when concomitant signaling to phospholipase activation occurs [169]. PKC is implicated in long-term regulation of neurotransmission in the central nervous system through increase in the size of the readily releasable vesicle pool, albeit the extracellular messenger(s) that signal to PKC to cause this event are not identified [165]. The unique response properties and effector functions of PKC and Munc13 make it likely that these two DAG/Ca^{2+} targets function in parallel with each other, and with wholly calcium-dependent processes, in regulating exocytosis.

Negative Regulation of Secretion

Some secretory systems, though dependent on calcium influx or cyclic AMP elevation to trigger secretion, tend to be tonically active *in vivo*, with regulatory function exerted by inhibition of secretion. These systems are highly illustrative both for understanding the triggers of exocytosis and the role of intracellular signaling in negative modulation. Secretion from the prolactin-secreting cells of the anterior pituitary, for example, is vastly increased by removal of the hypothalamic input to the gland [170], raising two questions: How can regulated prolactin secretion also be constitutive and how do factors from the hypothalamus down-modulate this "default-stimulated" system? Lactotrophs exhibit spontaneous action potentials that may drive calcium influx and mobilization in the absence of additional signals, but can be down-regulated by first messengers that act as "antisecretagogues" or inhibitory factors [171]. Dopamine released from the hypothalamus acts through D2 receptors to inhibit prolactin secretion independently of down-regulation of cAMP [172]. Likewise, somatostatin is a critical down-regulator of growth hormone secretion stimulated by GH-RH, ACTH secretion stimulated by CRF, glucagon secretion from α cells of the exocrine pancreas, and neurotransmitter release in the central nervous system. In each case, somatotstatin's actions appear to be exerted via multiple mechanisms involving somatostatin receptor/Gi coupling, including restoration of potassium-mediated hyperpolarization by blockade of secretagogue-stimulated inhibition of these conductances via K-channel phosphorylation, activation of calcineurin leading to secretory vesicle "depriming," and as-yet uncharacterized pleiotropic effects of decreasing intracellular levels of cAMP as well as calcium influx [173–175].

Presynaptic inhibition by the action at nerve terminal autoreceptors of newly released transmitters is a critical negative regulatory mechanism in the nervous system. Catecholamines released from nerve terminals interact with presynaptic α_2 receptors that inhibit further secretion and function as a brake on oversecretion [176,177]. The α_2 agonists are therefore important therapeutic agents for hypertension [178]. Presynaptic inhibition is also important for autoreceptors in striatum that limit the physiological release of dopamine controlling extrapyramidal function [176,179,180]. ATP costored with neurotransmitters in secretory vesicles from a variety of neuroendocrine secretory cells is metabolized to adenosine upon release and can inhibit further exocytosis via activation of adenosine receptors at the nerve terminals or endocrine cells from which release occurs [181].

Upstream Regulation of Secretion

Vesicular organellogenesis and trafficking, hormone packaging, sorting and processing, and vesicular filling with small-molecule transmitters via specific transporters are all steps that precede exocytosis and are critical for delivering to the plasma membrane both the vesicle and its informational contents prior to exocytosis. Some aspects of these processes are constitutive, at least with respect to secretagogue signaling and exocytosis, but, surprisingly, some steps are highly dynamic and may be regulated by the same signals that control exocytosis itself.

Exocrine and endocrine secretory granules, and neuroendocrine and neuronal LDCVs begin life as immature secretory granules formed in the trans-Golgi network (TGN) [182], as first described in detail for the prolactin-containing secretory granule of the pituitary lactotroph [183]. Various mechanisms for sorting of secretory proteins into immature secretory granules (ISGs) have been proposed, including cation-assisted secretory protein aggregation and precipitation into membrane-bound nascently budding structures that become the secretory protein-containing ISGs [184,185]. Chromogranin A has recently been demonstrated as a master switch regulating LDCV granulogenesis in PC12 cells [186]. Prolactin may act similarly to promote self-aggregation and granulogenesis in lactotrophs [187,188]. The regulation of biosynthesis and degradation of these proteins is therefore important not only because they are hormones or precursors for hormones themselves, but because their abundance in the TGN will determine the extent and rate of secretory granule formation.

The content of secretory vesicles is determined, for peptide hormones, via copackaging of processing enzymes that themselves may function as sorting chaperones [189], and by the rate of production of mRNAs, encoding them (see next section). The neurotransmitter content of LDCVs and SSVs is determined by the presence of vesicular transporters that drive the uptake of amine and amino acid transmitters into the vesicle against a concentration gradient, providing enrichment of up to 10,000-fold [190,191]. Surprisingly, neurotransmitter uptake is a rate-limited step: Vesicular amine content can be increased by doubling and decreased by halving the amount of vesicular transporter expressed *in vivo* [192–194]. Furthermore, the phosphorylation state of

amine transporters can regulate their trafficking and, therefore, availability to a specific vesicle type [195]. Amine loading into vesicles may be regulated by transporter phosphorylation [191,195–198]. Finally, the extent of filling of secretory granules in catecholamine-containing secretory cells may directly affect the degree of processing of cosecreted peptide hormones [199], most likely due to inhibition of proteolytic processing enzymes of the secretory granule by high concentrations of catecholamines [200]. Thus, a complex regulatory web connects exocytosis from the onset of granulogenesis until the presentation of vesicular contents to the extracellular milieu upon vesicle exocytosis.

Far Upstream Regulation of Secretion

Secretory cells participate in organismic homeostasis via regulated exocytosis of informational molecules, and they also possess homeostatic mechanisms for reconciling organismic demand and conservation of cellular resources to provide secretory product over time. *Stimulus-secretion-synthesis coupling* is a term coined to describe the concomitant stimulation of secretion and secretory hormone biosynthesis, so that compensatory biosynthesis enhancement to replete secretory stores can occur during episodes of prolonged secretion [201]. Stimulus-secretion-synthesis coupling may occur in one of two ways. First, a single second messenger produced by a secretagogue may affect both secretion and synthesis. Secondly, dual second messenger signaling initiated by a single first messenger may separately regulate secretion and synthesis. Various types of regulation occur in different types of secretory cells. In corticotrophs, isoproterenol stimulates both release of ACTH and POMC mRNA up-regulation, but increasing intracellular calcium stimulates release without affecting biosynthesis [202]. In chromaffin cells, acetylcholine couples secretion and synthesis via calcium [201], histamine couples secretion and synthesis via both calcium mobilization and influx [203–205], and PACAP couples secretion and synthesis via cAMP and calcium acting both synergistically and independently [83,85].

Conclusions and Future Outlook for Signaling in Exocytosis

This chapter has covered stimulus-secretion coupling as it applies to secretagogue stimulation of calcium influx and cyclic AMP elevation leading to calcium sensor activation, protein phosphorylation, and ultimately secretory vesicle fusion with the plasma membrane. The modes of secretagogue signaling have been discussed. These include depolarization via sodium channel activation leading to VSCC opening and calcium influx; modulation of outwardly directed potassium channels leading to altered calcium influx; direct first-messenger stimulation of VSCC opening; cyclic AMP stimulation of secretory granule exocytosis via interactions with components of the calcium pathway; and cooperative roles of intracellular calcium mobilization and calcium influx in exocytosis. Secretion from SSVs ("fast" neurotransmission) and LDCVs ("slow" regulated secretion) and their separate modes of regulation were discussed. Regulation of exocytosis from calcium influx through secretory vesicle docking, priming, and membrane fusion was described, with SG, LDCV, and SSV exocytosis as special cases of a general process of regulated secretory vesicle fusion, which can be distinguished kinetically, and by differences in the rate-limiting step for exocytosis for each type of vesicle. Secretagogue signaling during exocytosis may include modification of protein components of the "fusion machinery" to modulate their phase associations as well as interactions with other proteins. The role of signaling via PKA- and PKC-mediated phosphorylation was characterized as functionally important in physiological secretion and as affecting virtually all aspects of exocytosis from trafficking and filling to mobilization/docking, priming, and fusion of the vesicles. An additional area not obviously a subject for signaling during exocytosis is the regulation of granulogenesis/vesiculogenesis, including proteins that control aggregation, sorting, hormone processing, and small-molecule vesicular filling prior to and during secretion. Stimulus-secretion-synthesis coupling was discussed to illustrate that secretogogue-based regulation involves control of the export economy of the secretory cell, including both the quantity and quality of the neurosecretory quantum of the SSV, the endocrine hormone cocktail of the LDCV, and the zymogen mixture of the exocrine secretory granule.

In the future, secretion is likely to be viewed within a more unitary framework for neuronal, endocrine, and exocrine secretion, but with a better molecular understanding of the diversity among cell types and various types of exocytosed cargo. Temporal aspects of secretion will receive additional attention, both from the standpoint of speed of the exocytotic events (from less than a millisecond to several seconds) and their persistence (from seconds to hours and days). Various types of neuronal plasticity, such as long- and short-term potentiation, and long-term depression, will be increasingly viewed in the context of ultra-slow components of the process of exocytosis itself, resulting from increased or decreased speed of docking and priming events that are initially not relevant in the time frame of a single exocytotic event, but become rate limiting and significant when encoding the neuronal experience of past strong, weak, and coincident stimuli that underlies learning and memory. Frequency and amplitude modulation of secretion will be increasingly understood in terms of the conversion of calcium spikes with local cellular influences that sum to global calcium changes much as dendritic and somal depolarizations sum to action potentials in neurons. Many regulatory molecules involved in exocytosis act only after recruitment from one membrane compartment to another, or from cytosol to vesicle or plasma membrane, in a transfer that depends on local and specific alterations in membrane lipid composition. Regulation of membrane lipid, via raft formation, inositol removal and addition, and release and addition of lipid acyl moieties from the lipid glycerol backbone have been touched on here

only in passing: Their regulation by G-protein coupling, cAMP, and calcium is intimately involved in both vesicular trafficking and cell signaling and creates an important functional interface between these two processes that is beginning to be explored in detail [65,206].

Molecular details of the roles of calcium and cyclic AMP sensors will be learned about in far greater detail by using conditional knock-outs in worms, flies, and mice, including, in particular, the roles of synaptotagmin and other SNARE complex component isoforms, with functions subtly but importantly specialized for performance and regulation in neuroendocrine, endocrine, and exocrine cells [207–210]. The precise order of ATP-, cAMP-, and low-, medium- and high-affinity Ca^{2+}-dependent steps in exocytosis will be determined with greater precision through the use of real-time evanescent wave and other techniques for localizing vesicles with respect to their proximity to the plasma membrane, and through real-time reportage on calcium and cAMP concentrations near a specific protein component of exocytosis using fluorescence resonance energy transfer and other biophysical techniques in conjunction with recombinant reporter molecules. With a greater variety of biochemical, biophysical, pharmacological, and genetic tools, a greater emphasis can be placed on studying secretion from primary cells in intact tissues or *in vivo*, to determine the physiological and pathophysiological conditions under which various secretagogues dominate or even are rate limiting for secretion, for example, before and after meals for secretion from parotid, pancreas, and gut; during processing of sensory information and habit formation in the nervous system; and during metabolic, psychogenic, or physical stress in the neuroendocrine and immunoinflammatory systems. These details will be critical for a mechanistic understanding that will lead to pharmacological intervention in regulated steps of exocytosis to correct malfunctions in fast and slow neurotransmission, in endocrine and exocrine secretion, and in myesthetic and dystrophic, diabetic, and gastrointestinal disease. Regulation of slow transmission, in particular, is a currently underexploited avenue of research into the modulation of brain function in biogenic amine-related disorders including schizophrenia, attention deficit disorder, and depression.

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CHAPTER 327

Nonclassical Pathways of Protein Export

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Introduction

The majority of proteins that are destined to be secreted are usually translated with an NH_2-terminal cleavable signal peptide sequence. This structure is rich in hydrophobic amino acid residues that enable the polypeptide to dock and insert into the lumen of the endoplasmic reticulum for its further transport to the Golgi apparatus and eventual secretion as an intravesicular polypeptide [1]. However, there are groups of extracellular proteins that neither display a signal peptide sequence in their primary structure nor utilize the classical endoplasmic reticulum (ER)–Golgi pathway for their release. In this review, we focus on the mechanisms of stress-induced release of two signaling proteins belonging to this group, fibroblast growth factor 1 (FGF-1) and interleukin 1α (IL-1α). However, before discussing the export of these polypeptides, we briefly review the literature concerning the nonclassical release of other signal peptide-less proteins.

One of the earliest studies of nonclassical protein release was devoted to secretory transglutaminase (TG), an enzyme that cross-links extracellular polypeptides and is released into the extracellular space by cells of the prostate [2,3]. Aumuller and colleagues [3] extensively investigated TG release using immunoelectron microscopy and observed that TG appeared to be synthesized on non-ER-associated ribosomes and was not found in the lumen of the ER and Golgi structures but rather accumulated in cytoplasmic blebs protruding from the apical surface of the secretory cells. Interestingly, the detachment of these apical blebs followed by the release of their contents has long been known as *apocrine secretion* [4]. Although the expression and release of TG is dependent on stimulation by androgens, the regulatory mechanism of TG release has not been investigated, and the mechanism of TG transport to the apical cell membrane as well as the detachment of apical blebs remains obscure. Likewise, the human immunodeficiency virus 1 TAT protein [5] and sphingosine kinase (SK), an enzyme involved in the biosynthesis of shingosine-1-phosphate [6], galectins [78], annexin1 [79], and some other signal peptide-less polypeptides [80] are present in the extracellular compartment. The release of SK and TAT into the extracellular compartment occurs independent of the function of the ER–Golgi apparatus yet their intracellular traffic and export activity has not yet been studied in detail [5,6]. Thioredoxin and S100 proteins are also known to be released by a nonclassical mechanism and these are discussed later in this review.

Fibroblast Growth Factor Export Pathways

FGF-1 and FGF-2 are the signal peptide-less prototypic members of the FGF gene family and they are involved in mesodermal and neuroectodermal induction, angiogenesis, chondrogenesis, neurogenesis, wound healing, and tumor formation [7]. In contrast to the export of SK, TAT, and TG, the release of the FGF prototypes has been studied in some detail. Although the transcripts encoding the FGF prototypes are expressed in all tissues and cells [7], expression of the FGF prototype polypeptides is most evident in mononuclear

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cells especially within macrophages [8,9]. The release of the FGF prototypes into the extracellular compartment is a necessary precondition for their biological activity because their association with the glycosylaminoglycan, heparan sulfate, and their high-affinity tyrosine kinase receptors are requisite for cellular signaling [7,10]. However, the absence of a classical signal peptide sequence within the structures of the FGF prototypes excludes a possibility of their transport through the ER–Golgi pathway. Indeed, *in vitro*, cells transfected with FGF-1 do not release it under normal cell culture conditions, and the addition of a classical signal peptide sequence at the NH_2 terminus of FGF-1 results in the generation of an oncogenic form of FGF-1 as a consequence of an extracellular autocrine signal established by its secretion through the ER–Golgi compartments [11]. FGF-2 also exhibits similar structural and functional behavior [12], yet the majority of the members of the FGF gene family have been characterized as oncogenes as a result of the presence of a functional signal peptide sequence that enables them to function in an autocrine and paracrine manner [10,13].

The mechanisms responsible for the regulation of the release of the FGF prototypes have diverged because we know that FGF-2 is released in a constitutive manner [14–17], whereas the release of FGF-1 utilizes a stress-inducible pathway [18]. Interestingly, the invertebrate FGF homolog genes encode FGFs containing a signal peptide sequence [13,19] and it is not known where or why the vertebrate FGF prototypes lost their signal peptide structures. One can speculate that as evolution selected for species with extended life spans, the maintenance of organ and tissue physiology required the function of nononcogenic forms of the FGFs, and this may explain the divergent nature of the pathways utilized to export the FGF prototypes. However, a common feature shared by the FGF prototypes is their independence of the function of the ER–Golgi apparatus since the release of FGF-1 and FGF-2 is insensitive to treatment with brefeldin A [14,15,18].

Although the export of FGF-2 is constitutive, the mechanism of FGF-2 release involves the function of the Na^+/K^+-ATPase transporter and thus may involve changes in membrane potential for the export of FGF-2 as a functional mitogen [20,21]. In contrast, however, the export of FGF-1 is inducible and involves the function of intracellular Cu^{2+} to assemble a multiprotein complex that enables the release of FGF-1 as a biologically inactive Cys30 FGF-1 homodimer [22]. A variety of cellular stresses have been implicated as the initiation signal for FGF-1 export and these include heat shock, anoxia, hypoxia, irradiation, serum deprivation, and oxidized LDL [7,18,23–25]. Because many of these stimuli are involved in establishing an inflammatory response and FGF-1 expression is exaggerated in mononuclear cells [9], it is likely that FGF-1 is delivered to inflammatory sites for tissue/organ repair by mononuclear cells including macrophages [9]. Because this would involve the function of pro-inflammatory cytokines as signals for this recruitment process [26], it is intriguing to speculate that members of the IL-1 gene family, which also lack a classical signal peptide sequence [27], may be involved in mediating this process. However, we should emphasize that the genes encoding the FGF prototypes are not transcriptionally responsive to environmental stress (S. Friedman and T. Maciag, unpublished results, 1993) and thus the export pathways utilized by the FGF prototypes are regulated primarily by either translational or posttranslational events.

The appearance of FGF-1 in the extracellular compartment in response to cellular stress as a Cys30 FGF-1 homodimer is interesting since the dimeric form of FGF-1 can be formed under cell-free conditions by Cu^{2+} oxidation [28]. Yet FGF-2, its close structural homolog, is unable to form a disulfide-linked homodimer even though two of three cysteine residues are conserved between the FGF prototypes [29,30]. It is also interesting that the nonfunctional FGF-1 homodimer exhibits a significantly reduced affinity for immobilized heparin [28]. Because heparin affinity is a hallmark of the proteins encoded by the FGF gene family and interactions with heparin are known to protect FGFs from proteolytic inactivation [31], the failure to achieve an extracellular reducing environment in response to cellular stress would ensure proteolytic inactivation of the latent FGF-1 homodimer and prevent FGF-1 from being functional at inopportune situations. It is of interest to note that the thioredoxin gene also encodes a signal peptide-less redox regulatory polypeptide that is released from dendritic cells following the interaction with antigen-recognizing T lymphocytes and provides a reducing microenvironment in the extracellular compartment [32]. However, although it is not known whether thioredoxin functions to regulate the redox potential of the latent FGF-1 homodimer, it is clear that an extracellular reducing agent is required to activate the latent FGF-1 homodimer as a heparin-binding polypeptide mitogen.

The Export of FGF-1 as a Multiprotein Complex

Early observations that bovine neural tissues contain high-molecular-weight noncovalent FGF-1 complexes [33] facilitated the identification and characterization of polypeptides that are involved in the export of FGF-1 [34–36]. Indeed, the identities of two of the four polypeptides associated with the high-molecular-weight form of brain-derived FGF-1 were determined to be synaptotagmin (Syt)1 and S100A13 on the basis of microsequencing and immunoblot analysis [36,37]. Syt1 is a 65-kDa transmembrane protein involved in the regulation of exocytotic vesicle traffic, and structural information has implicated the function of the p40 extravesicular fragment of p65 Syt1 as the FGF-1-associated chaperone [38]. The p40 form of Syt1 appears to be the result of proteolytic cleavage at an extravesicular hypersensitive proteolytic cleavage site near the transmembrane domain of p65 Syt1 [39]. In addition, the p40 form of Syt1 contains a single cysteine residue, as well as two Ca^{2+}-binding C2 domains [39]. In contrast, S100A13 is a member of the S100 gene family characterized as EF-hand-mediated Ca^{2+}-binding proteins implicated in a variety of calmodulin- and F-actin-dependent cytoskeletal processes [40]. However, unlike the majority of the members of the S100 gene family,

the structure of S100A13 is novel because it lacks cysteine residues and contains a basic residue-rich domain positioned at its carboxy-terminus [41,42]. The function of both Syt1 and S100A13 in the regulation of the release of FGF-1 in response to cellular stress could be demonstrated by the expression of dominant-negative mutants of either Syt1 or S100A13 that are able to attenuate FGF-1 export [35,43]. However, the Ca^{2+}-binding properties of p40 Syt1 and S100A13 are not likely to be involved in the stress-induced release of FGF-1 because the Ca^{2+} ionophore does not impair or interfere with the release of either FGF-1, S100A13, or p40 Syt1 into the extracellular compartment [35]. Interestingly, a novel feature of both p40 Syt1 and S100A13 is their ability to be constitutively released from cells under normal cell culture conditions [35,43] in spite of the absence of a classical signal peptide sequence in S100A13 and the fact that the signal peptide sequence in p65 Syt1 remains structurally intact within the intravesicular domain (p25) of the transmembrane polypeptide. Although the mechanisms(s) utilized by p40 Syt1 and S100A13 for constitutive export is not known, the stress-induced presence of p40 Syt1 and S100A13 in the extracellular compartment with FGF-1 implies that these intracellular proteins may indeed assemble into a multiprotein complex prior to export (Fig. 1) and that formation of p40 Syt1 from p65 Syt1 may involve the function of an intracellular protease that cleaves p65 Syt1 at its hyperproteolytic site within the extravesicular domain.

Evidence for the self-assembly of the FGF-1:p40 Syt1:S100A13 complex was obtained from cell-free studies utilizing the recombinant forms of these polypeptides. Interestingly, the self-assembly of this multiprotein complex requires the oxidative capacity of Cu^{2+}, and it has been possible to define the molar ratio of the Cu^{2+}-dependent multiprotein complex containing p40 Syt1:FGF-1:S100A13 as 1:2:2, respectively [22]. Additional studies utilizing *in vitro* methods have confirmed this stoichiometry and it appears that Cu^{2+} is able to facilitate the formation of a heterotetramer FGF-1:S100A13 complex containing p40 Syt1, which is assembled and released in response to cellular stress [22].

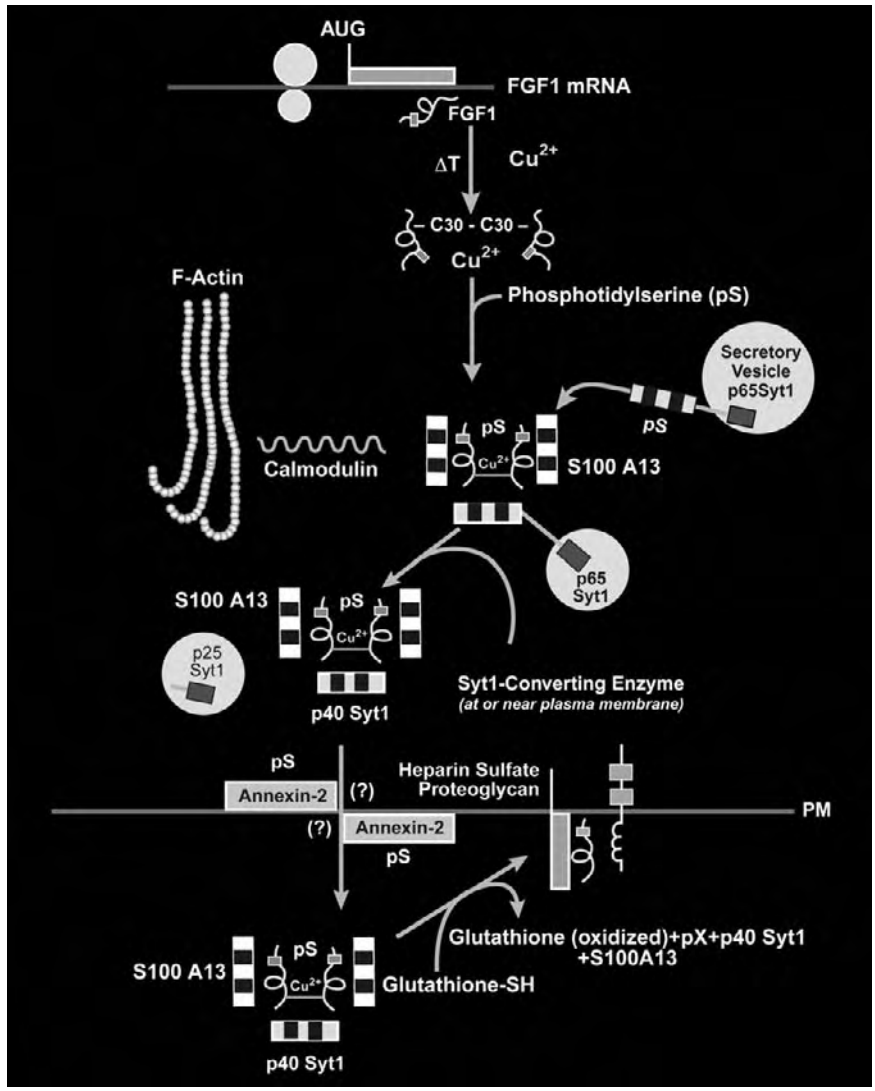


Figure 1 FGF-1 release pathway.

The observations that FGF-1, p40 Syt1, and S100A13 are Cu^{2+}-binding proteins [22,28] and that the Cu^{2+} chelator, tetrathiomolybdate [44], is able to repress the stress-induced release of these polypeptides *in vitro* [22] adds additional credence to the involvement of intracellular Cu^{2+} oxidation in the release of FGF-1.

Interestingly, the overexpression of S100A13 completely repressed the sensitivity of FGF-1 release to both cycloheximide and actinomycin D [43], and this suggests that the Cu^{2+}-dependent assembly mechanism does not require the transcriptional activation and translation of a stress-induced chaperone to facilitate the intracellular assembly of this multiprotein complex. Note that while some members of the S100 gene family are stress-induced genes, neither Syt1 nor S100A13 is transcriptionally activated by cellular stress (T. Lavallee, F. Tarantini, and T. Maciag, unpublished results, 1998). Thus, it is likely that the mechanism utilized by cellular stress to assemble the FGF-1 release complex may involve alterations in the distribution of intracellular Cu^{2+} in order for the divalent oxidant to access S100A13 for the formation of the FGF-1 homodimer. Indeed, recent kinetic data obtained using confocal immunofluorescence microscopy suggest that the intracellular distribution of S100A13, p40 Syt1, and FGF-1 from the cytosol to the inner surface of the plasma membrane in response to cellular stress occurs prior to the assembly of the multiprotein complex [45].

The observation that the assembly of the multiprotein complex follows the intracellular trafficking of the individual components to the inner surface of the plasma membrane [45] suggests a potential role for the cytoskeleton in this process. The first evidence for a role of F-actin filaments in this process came as a result of studies with amlexanox, an anti-inflammatory agent [36,46]. Oyama *et al*. [47], using amlexanox affinity chromatography, purified S100A13 complexed with annexin 2 as an amlexanox-binding complex from lung extracts. Because S100A13 expression is important for the release of FGF-1, the ability of amlexanox to inhibit the release of FGF-1, p40 Syt1, and S100A13 in response to cellular stress was consistent with the ability of amlexanox to associate with S100A13 [36,43]. Interestingly, amlexanox was also able to stimulate the activation of Src and, as a result, collapse the F-actin cytoskeleton [46]. The ability of a dominant-negative Src construct to inhibit the stress-induced release of FGF-1 [46] and the ability of amlexanox to inhibit the translocation of FGF-1 to the inner surface of the plasma membrane [45] is consistent with a role of the F-actin cytoskeleton in this process. However, neither FGF-1, S100A13, nor p40 Syt1 appears to be phosphorylated during either the assembly or release process (T. Lavallee, F. Tarantini, M. Landriscina, and T. Maciag, unpublished results, 1998, 2000) suggesting that the role of Src in FGF-1 export is involved in the maintenance of cytoskeletal integrity. Thus it is likely that the intracellular trafficking of the individual components of the multiprotein complex may utilize the F-actin cytoskeleton for transport to the inner surface of the plasma membrane where the FGF-1:p40 Syt1:S100A13 complex is assembled for export into the extracellular compartment.

The role of Syt1 in the stress-induced release of FGF-1 requires the function of an intracellular protease to convert the transmembrane form of p65 Syt1 to soluble intracellular p40 Syt1. Although the identity of this protease is not known, it is probable that the enzymatic activity of this converting enzyme is regulated by cellular stress. However, it is unlikely that this regulation occurs at the transcriptional level since the Cu^{2+}-dependent assembly of the multiprotein complex prior to export is independent of new transcription and translation [22,43]. It is interesting to note that thrombin induces annexin 2 flipping from the inner surface to the outer surface of the plasma membrane [81] where it functions as the receptor for the fibrinolytic enzymes plasminogen and tissue plasminogen activator [48]. Because S100A13 is able to associate with annexin 2 [47], it is intriguing to speculate that intracellular annexin 2 may not only be the site of residence for the p65 Syt1-converting enzyme but may also be the site of assembly for the multiprotein complex prior to export.

Interleukin 1 Export Pathways

The IL-1 gene family is presently comprised of eight members that are known to function as extracellular receptor-dependent pro-inflammatory cytokines [27,49]. However, the IL-1 gene family contains only a single member with a classical signal peptide sequence, the IL-1 receptor antagonist [27,49]. The majority of the members of the IL-1 gene family are translated as precursor structures and the IL-1 gene family prototypes, IL-1α and IL-1β, require proteolytic modification by either calpain (IL-1α) or the IL-1-converting enzyme (IL-1β) to generate the mature forms of these cytokines [27]. Interestingly, although a primary sequence similarity exists between the FGF and IL-1 prototypes, the crystallographic structures of the IL-1 gene family prototypes are remarkably similar to the crystallographic structures exhibited by the FGF prototypes [50–52] and this structural feature implies that there may be other similarities between the FGF and IL-1 gene families as well. Further, the differences observed between the FGF-1 (stress-induced) and FGF-2 (constitutive) release pathways may be conserved between the members of the IL-1 gene family. Indeed, with respect to nonclassical release, the release of IL-1α, like FGF-1, is regulated by cellular stress and utilizes a pathway similar yet distinct from FGF-1 [53]. The release of IL-1α requires the proteolytic activity of calpain since the mature form of IL-1α but not the precursor form of IL-1α is released in response to cellular stress [53]. The release of mature IL-1α exhibits identical pharmacologic and kinetic properties as those described for FGF-1 [53] and, like the FGF-1 release pathway, IL-1α export requires the function of S100A13 and intracellular Cu^{2+} [82]. Unlike FGF-1, mature IL-1α is released as a functional cytokine and does not contain cysteine residues within its structure that are conserved among IL-1α structures from

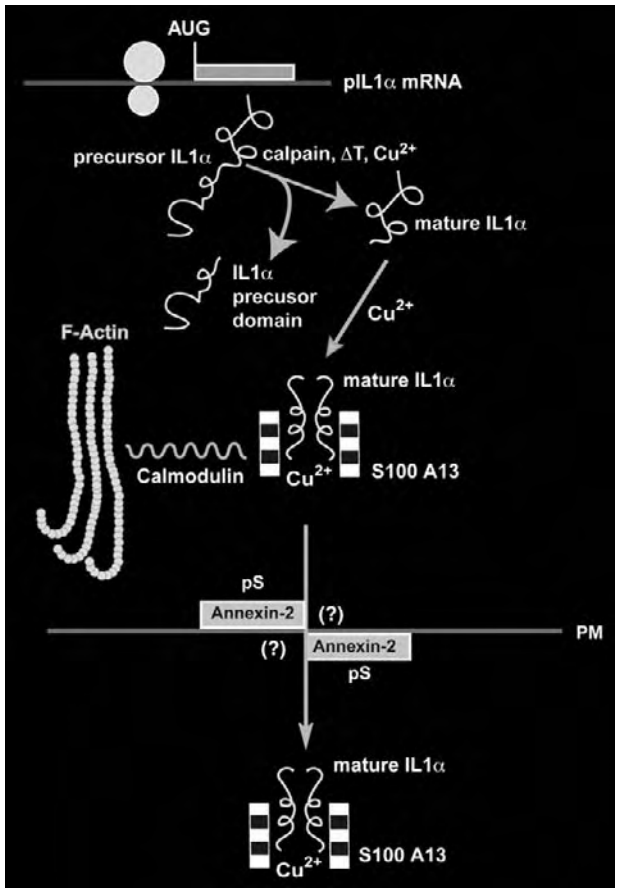


Figure 2 IL-1α release pathway.

other species [54,55]. Thus it is likely that the function of S100A13 and Cu^{2+} is not to establish an intracellular IL-1α:S100A13 heterotetramer by the oxidation of a disulfide bond between two monomeric forms of IL-1α, but rather to form a noncovalent heterotetramer (Fig. 2). The observation that S100A13 can enable the release of a cysteine-free mutant of FGF-1 as a noncovalent heterotetramer [43] is consistent with this premise. However, unlike the stress-induced FGF-1 release pathway, the release of the mature form of IL-1α does not require Syt1 because expression of a dominant-negative mutant of Syt1, which represses FGF-1 export, does not attenuate the release of IL-1α under similar conditions [53]. Thus, if the function of the Ca^{2+}-binding C2 domains in Syt1 is important for the release of both FGF-1 and IL-1α, another intracellular polypeptide containing a C2 domain may be involved. Last, the expression of the precursor but not the mature form of IL-1α represses the release of FGF-1 in response to cellular stress [53] and this observation further argues in favor of the convergent nature of the FGF-1 and IL-1α release pathways.

Because of the convergence between the FGF-1 and IL-1α release pathways, it is possible that other members of the IL-1 gene family may be convergent with the FGF-2 release pathway. However, because the release of the mature form of IL-1β is enhanced by cellular stress including temperature [56] and lipopolysaccharides [57], it is unlikely that the mechanism of IL-1β release will have significantly diverged from the FGF-1 and IL-1α release pathways. Although there is no information about the potential role of S100A13 and/or Cu^{2+} ions on the regulation of IL-1β release, it is noteworthy that IL-1β, like FGF-1 and IL-1α, is a Cu^{2+}-binding protein (S. Bellum, A. Mandinova, and T. Maciag, unpublished results, 2002), yet unlike the release of FGF-1 [58] and IL-1α (F. Tarantini and T. Maciag, unpublished results, 1999), IL-1β export is sensitive to methylamine, an inhibitor of exocytosis [56]. Recent evidence has suggested that IL-1β is contained within intracellular vesicles that express markers of the lysosome but not ER–Golgi, and lysosomotropic agents such as chloroquine and NH_4C1 inhibit the export of IL-1β [57]. A similar inhibitory effect was also observed with sulfonylurea glibenclamide (SG), an inhibitor of ABC1, a mammalian ATP-binding cassette (ABC) translocator [59] and it is known that the nonclassical export pathway employed in unicellular organisms utilizes ABC transporters [60]. However, SG was unable to repress the release of FGF-1 and IL-1α (F. Tarantini and T. Maciag, unpublished results, 2000). Thus, comparative analysis of the IL-1α and IL-1β export pathways suggests that, like the FGF-1 and FGF-2 export pathways, these pathways may have also diverged with the IL-1β export pathway dependent on translocation into the lumen of lysosomes and subsequent lysosomal fusion with the cell membrane possibly using classical exocytotic principles.

Acidic Phospholipids and the Molten Globule Hypothesis

The mechanism utilized by these polypeptides for translocation through the plasma membrane is not known. However, there are a few properties of these proteins that may be consistent with their ability to traverse lipid bilayers. Phosphatidylserine (pS) is an acidic phospholipid that is known to flip from the inner to the outer surface of plasma membranes in response to cellular stress. Although pS flipping is an important component of the intrinsic coagulation system [61] and is widely used in its exaggerated form as evidence for cellular apoptotic behavior as a result of annexin 5-binding [62], all of the known and assumed components of the FGF-1 and IL-1α release pathway are pS-binding proteins. Indeed, IL-1α [63], FGF-1 [64], the S100 gene family [41], p40 Syt1 [39], and annexin 2 [65] are able to associate with pS under cell-free conditions. Although the flipping of pS is known to be ATP dependent [61], as is the release of FGF-1 [35] and IL-1α [53] in response to cellular stress, the molecular basis for pS translocation has not been firmly established [61]. However, FGF-1 [66–68] and IL-1α [82] are known to permeabilize acidic phospholipid-rich vesicles in cell-free systems and are endowed with a *molten globule* character [66–68]. Although the physical protein biochemistry of the molten globule is in its earliest stage of investigation, the term defines the state of a protein that is able to display uncooperative unfolded

states or transitions in which the structure of the protein attains multiple conformational states with high secondary but low tertiary structure [69].

As a result of these changes, the partially unfolded conformations are able to achieve low solubility in aqueous environments, resulting in the association with lipid bilayers that they are able to transverse [70]. Although molten globule states are usually formed at low pH and physiological temperatures, the molten globule character of FGF-1 begins to be exaggerated at temperatures between 37° and 42°C and this is facilitated by the presence of acidic phospholipids in the lipid bilayer [66–68].

Thus it is possible that the combination of pS affinity, pS-flipping, and molten globule character may enable FGF-1 to traverse the lipid bilayer. Whether or not S100A13 and p40 Syt1 exhibit similar biophysical characteristics is not known, but it should be emphasized that cells stably transfected with these genes do exhibit constitutive export of both S100A13 and p40 Syt1 into the extracellular compartment at 37°C [35,43]. Interestingly, the S100A13 and p40 Syt1 expression in a FGF-1 background represses their constitutive release of S100A13 but not p40 Syt1, suggesting that S100A13 but not the extravesicular domain of Syt1 does interact with FGF-1 under stress-free conditions [43]. Indeed, if S100A13 and/or p40 Syt1 do exhibit molten globule characters, it is possible that their presence with FGF-1 in the multiprotein complex may facilitate the formation of multiple conformational states and cooperate to exaggerate the low solubility of FGF-1 in an aqueous environment. Because crystallographic data demonstrate that the cysteine residue at position 30 in FGF-1 is buried [50], and S100A13 is able to expose this cysteine residue to solvent for Cu^{2+} oxidation [22,43], these data are consistent with this hypothesis because they suggest that S100A13 is able to alter the conformation of FGF-1.

The Potential Pathophysiological Implications of Nonclassical Release

Although the biological significance of nonclassical release mechanisms is not well understood, some intriguing observations are noteworthy. Interestingly, the pro-inflammatory and angiogenic character of Cu^{2+} is well appreciated [44,71]; however, it is only recently that efficient and specific Cu^{2+} chelators have been utilized in the clinical management of human disease. Indeed, in two phase 1 clinical trials with stage 4 human cancer patients [72,73] including a study limited to head and neck tumors, which are extremely difficult to clinically manage, the Cu^{2+} chelator tetrathiomolybdate (TTM) has been shown to be a valuable addition to the arsenal of antitumor agents as a palliative treatment for the clinical management of solid human tumor growth. Initially described for the treatment of Wilson's disease [74], a disease of Cu^{2+} excess in man, TTM has been documented as an anti-angiogenic effector both in preclinical [83] and clinical [72] settings.

Although the mechanism utilized by TTM is not fully understood, it is possible that the ability of TTM to inhibit the release of the pro-inflammatory cytokine IL-1α from the tumor microvasculature could prevent the tumor from recruiting FGF-1-rich mononuclear cells [9,75], which would significantly reduce the tumor angiogenic response. Indeed, recent experiments on knockout mice demonstrated that IL1 is required for tumer angiogenesis [84]. Further, TTM could also serve to impair the release of FGF-1 from the tumor itself or its surrounding microenvironment to further impair development of the tumor microvasculature. If this hypothesis is indeed accurate, there are other human pathologies in which inflammatory angiogenesis is thought to play an important role including rheumatoid arthritis [76], Alzheimer disease [86], psoriasis [87], and restenosis [77]. Thus, it may be potentially quite interesting to determine whether these nonclassical export pathways do contribute to the development of human pathology.

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CHAPTER 328

Regulation of Cell Cycle Progression

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Introduction

The fundamental purpose of the mitotic cycle is the generation of genetically identical daughter cells. Because growth, DNA replication, and organelle duplication are physically independent processes, successful division is dependent on ensuring that the duplication and segregation of cellular components is tightly coordinated. Coordination is achieved by placing the execution of all cell cycle events under the control of a family of highly regulated protein kinases. These kinases are known as cyclin-dependent kinases (Cdks) and each phase of the cell cycle is defined by the activation and inactivation of distinct members of the family. The general principles by which the sequential activation and inactivation of Cdks are achieved are as follows: (1) Processes that initiate Cdk activation also lead to the down-regulation or destruction of the activity. (2) The activity of one Cdk-cyclin sets up the conditions needed for the activation of the next. (3) The destruction of cyclins ensures a unidirectional cell cycle. (4) The inhibition of assembled Cdk-cyclin complexes, either by phosphorylation or by the binding of inhibitory protein, allows the accumulation of inactive complex. (5) When cellular or environmental conditions are not favorable, checkpoint signals delay the activation of performed Cdk-cyclin complexes beyond completion of previous cell cycle events.

Being There: Cyclins Define Cell Cycle Phase

As the name implies, Cdks are protein kinases that need to bind a cyclin subunit to be active. Cyclins are synthesized and degraded in a highly coordinated process as cells progress through the cell cycle, thus, the most fundamental level of control exercised over the activity of Cdks is the periodic presence and absence of the cyclin subunit. When resting (quiescent) cells are stimulated to enter the cell cycle, the first cyclin to be expressed is cyclin D. Expression of cyclin D is dependent on sustained and coordinate signaling of growth factors, through receptor tyrosine kinases, and of extracellular matrix components through integrins (reviewed in [1]). Cyclin D expression is also increased by increased mRNA translation and stability in a phosphoinositol-3-kinase-dependent pathway (reviewed in [2]). There are three D-type cyclins in human cells, with D1 being the most ubiquitously expressed [3]. Cyclin D assembles with Cdk4 and Cdk6; the complex enters the nucleus where it is phosphorylated by a Cdk-activating kinase (Cak, see later section) to form an active kinase that phosphorylates the retinoblastoma protein (Rb) and the related pocket proteins p107 and p130 [4]. Cdk4 and Cdk6 are coexpressed in many cell types and are thought to have partially, but not completely, overlapping functions [5]. Both Cdk4 and Cdk6 are constitutively expressed throughout the cell cycle, because the monomeric kinase subunits are relatively unstable and the abundance of cyclin D is limiting for the stabilization and activation of Cdk4 and Cdk6.

The same mitogenic signals that induce cyclin D expression also induce expression of a second cyclin, cyclin E, and of two inhibitors of cyclin-dependent kinases, $p21^{cip1}$ and $p27^{kip1}$. The induction of Cdk inhibitory proteins as cells enter a new round of growth and division seems counterproductive. However, p21 and p27 are actually required for the efficient assembly and nuclear import of cyclin D/Cdk4 complexes. p21 and p27 bind

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to cyclin D/Cdk4 without inhibiting kinase activity [6,7]. By contrast, they are effective inhibitors of cyclin E/Cdk2 activity, thus, the presence of these proteins early in G1 promotes the formation of active cyclin D/Cdk4 complexes at the same time that it delays the activation of cyclin E/Cdk2 complexes.

Cyclin D/Cdk4 plays two distinct roles in promoting cell cycle progression. One function stems from the ability to phosphorylate Rb and other pocket proteins, p107 and p130. Unphosphorylated pocket proteins repress transcription by inhibiting the E2F family of transcription factors and by recruiting transcriptional repressors, such as histone deacetylase and the chromosomal remodeling complex SWI/SNF, to the promoters of specific G_1/S-phase genes. By phosphorylating Rb and the other pocket proteins cyclin D/Cdk4 inhibits the ability of Rb to repress transcription. Free E2F promotes transcription from a number of promoters including those of cyclin E, cyclin A, and the several proteins needed for S phase (reviewed in [8]). The second role cyclin D plays in promoting cell cycle progression is noncatalytic and depends on the ability of cyclin D/Cdk4 to bind p27. As cyclin D expression increases in G_1 the increasing abundance of cyclin D/Cdk4 provides a binding site for p27. p27 Is recruited away from cyclin E/Cdk2, and the amount of cyclin E/Cdk2 relieved from the inhibitory effect of p27 increases. Hence, the continued expression of cyclin D indirectly stimulates the activation of cyclin E/Cdk2, and the transcriptional program initiated by mitogenic stimuli drives two temporally distinct waves of Cdk activation (Fig. 1).

Cyclin E/Cdk2 collaborates with cyclin D-Cdk4/6 in phosphorylating and inactivating Rb and the pocket proteins. This dual phosphorylation is required for the full activation of the S-phase transcriptional program [9]. The shift in cyclin D/Cdk4/6 catalyzed phosphorylation of Rb to that catalyzed by cyclin E/Cdk2 correlates with the transition to a state in which further cell cycle progression will occur even if extracellular growth factors are removed. Once this stage of the cell cycle is reached, the signals to continue with the cycle all come from within the cell. Internal or external events can delay, or prevent cell cycle progression. However, if no signal to stop is received, the initiated cycle of growth, replication, segregation, and division will continue without further extracellular input. If extracellular conditions remain favorable, mitogenic signaling maintains cyclin D expression through the cell cycle, and on completion of mitosis the new daughter cells will be ready to initiate a new round of cell division immediately. If, on the other hand, mitogenic stimuli are not maintained, cyclin D is phosphorylated in a glycogen synthase kinase 3 (GSK3)-dependent process and is targeted for destruction by the proteosome (reviewed in [10]). Progression into another round of replication is thus dependent on sustained, favorable extracellular signals.

The activation of cyclin E/Cdk2 leads to the initiation of DNA replication, and a number of substrates required to carry out the task of duplicating and segregating cellular contents

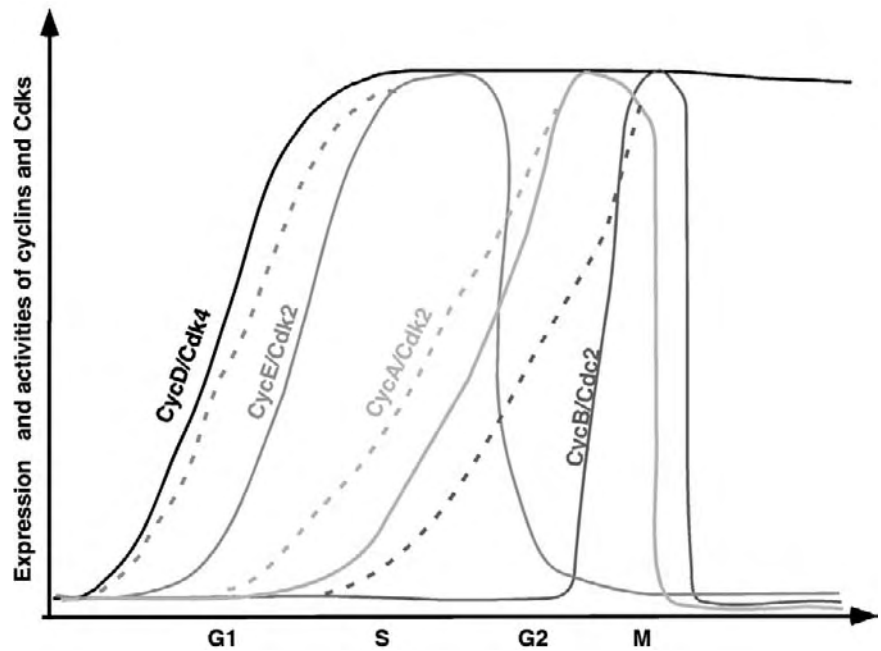


Figure 1 Expression pattern of cyclins and Cdk activities during the cell cycle. Expression of the specified cyclin is indicated by the dashed lines; activity of the specified cyclin/Cdk is indicated by the solid line. Sustained expression of cyclin D is dependent on favorable extracellular conditions as explained in the text. Note that the delay between expression of cyclin E and activation of cyclin E/Cdk2 is primarily due to inhibition of kinase activity by p27. The delay between expression of cyclin B and activation of cyclin B/Cdc2 is primarily due to inhibitory phosphorylation. Note that cyclin D also binds to Cdk4 and that cyclin A also binds to Cdc2.

have been identified. Specifically, cyclin E/Cdk2 phosphorylates components of the centrosome, initiating the process of centrosome duplication and, hence, segregation of the replicated sister chromatids [11]. It is likely that more cyclin E/Cdk2 substrates exist [12,13]. Cyclin E/Cdk2 also phosphorylates the Cdk inhibitor p27. This converts p27 to a form that is recognized by an ubiquitin ligase and is targeted for destruction by the proteosome (see later section) [14]. Thus, activation of cyclin E/Cdk2 triggers the destruction of its negative regulator. Cyclin E/Cdk2 autophosphorylates on cyclin E, targeting the cyclin for degradation [15,16]. This feature of priming an inhibitor for degradation and priming the cyclin subunit for degradation renders the kinase complex both self-activating and self-limiting.

Cyclin A is expressed soon after cyclin E at the G_1/S boundary. Both cyclin E/Cdk2 and cyclin A/Cdk2 activities are essential for the initiation and completion of DNA replication [17–19] and for ensuring that replication occurs only once in each cell cycle. Our understanding of the mechanism by which cells ensure that DNA is replicated once and only once in each cycle derives from experiments in many species, and the exact details may vary among organisms. Nevertheless, the principles by which it is achieved are at least partly conserved. The initiation of DNA replication requires the assembly of replication machinery complexes on DNA. One part of the machinery, the origin recognition complex, is present throughout the cell cycle, whereas other proteins, including the polymerase loading factors Cdc6 (Cdc18), Cdt1, and the MCM proteins, are loaded stepwise in G_1. The loading of these proteins and the activation of replication are under the control of cyclin E/Cdk2 and the S-phase activated kinase Cdc7 [20].

Once replication has initiated Cdc6 (Cdc18) and other essential replication cofactors, including the MCM protein, are phosphorylated and inactivated (reviewed in [21,22]). Inactivating phosphorylation triggers the degradation of some proteins, and it prevents access to the nucleus or to chromatin of others. The prereplicative state, that is, that which allows loading of the replication machinery on to chromatin, cannot be reestablished until division is complete and Cdk activity drops following mitosis [23]. In addition to this, metazoan cells express Geminin, an inhibitor of origin firing in S phase. Expression begins in S phase, and the protein inhibits the initiation of DNA replication until the metaphase-to-anaphase transition when it is degraded by the anaphase promoting complex (APC) (reviewed in [24]). The APC is a multiprotein complex that ubiquitinates several mitotic protein and then targets them for degradation by the 26S proteosome (see later section). In addition to its role in controlling replication, cyclinA/Cdk2 also promotes the efficient execution of S phase by increasing transcription of histone and other genes needed to accommodate replication [10].

After growing and duplicating its contents, the cell is faced with the challenge of dividing itself into two viable daughter cells. This is achieved in mitosis, which begins at prophase, with the condensation of chromosomes and the formation of a mitotic spindle. In prometaphase, the spindle microtubules attach to the two sister chromatids via kinetochores, and the nuclear envelope breaks down. Once everything is correctly aligned, the spindle pulls the sister chromatids apart in anaphase. Following separation of the replicated genomes, the spindle is disassembled, the chromatids decondense, and the nuclear envelope begins to reform, in telophase. Finally, at cytokinesis, the formation of the two daughter cells is completed by the partitioning of the cytoplasm. These dramatic morphological changes are under the control Cdc2 (Cdk1) in association with cyclin A and cyclin B. Expression of cyclin B lags behind that of cyclin A, rising late in S phase and remaining high throughout G_2 and mitosis. The gradual increase in cyclin B protein is not reflected in gradually increased kinase (Fig. 1) because the complex is largely maintained in a phosphorylated inactive form until the G_2/M boundary. Dephosphorylation and activation of cyclin B/Cdc2 correlates very closely with the morphological changes that accompany mitosis. Nuclear lamins, nucleolar proteins, centrosomal proteins, and Eg5 (a kinesin relates motor) have all been described as cyclin B/Cdc2 substrates [10]. Cdc2 in association with a second cyclin B subunit, cyclinB2, which localizes predominantly to the endoplasmic reticulum, may play a role in dispersing the Golgi apparatus so that cytokinesis will provide the two daughters with sufficient components to rebuild the secretory apparatus [25]. Cyclin B/Cdc2 also phosphorylates and activates at least two other kinases in mitosis, polo-like kinase (Plk) and the Aurora-related kinases. Plk and Aurora have roles in centrosome separation, spindle assembly, and chromosome segregation [26,27]. As an activator of Cdc25C, the phosphatase that activates Cdc2/ CycB, Plk1 is thought to play a role in the feedback loop to ensure that cyclinB/Cdc2 is rapidly and fully activated in mitosis (Fig. 2) [26]. Cyclin B/Cdc2 also stimulates its own destruction by phosphorylating and activating components of the APC.

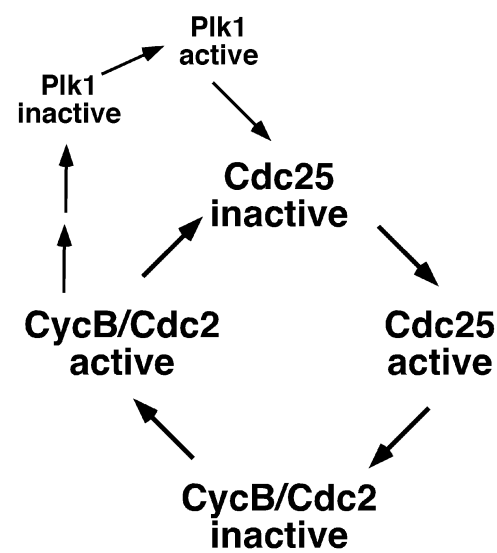


Figure 2 Activation of a small amount of cyclin B/Cdc2 leads to the activation of Cdc25, which promotes the activation of more cyclin B/Cdc2. Stimulation of Cdc25 by Plk1, which is activated in mitosis, could reinforce the positive feedback loop.

Signals to Slow Processes: Regulation of Cdks by Inhibitory Proteins

An important mechanism for negatively regulating Cdk/cyclin is the interaction with small inhibitory proteins. Two distinct families of Cdk inhibitors (CKIs) have been described in mammals. The Ink4 family (p15, p16, p18, and p19), named for the ability to inhibit Cdk4, specifically inhibits the cyclin D-containing Cdks, and the Cip/Kip family ($p21^{cip1}$, $p27^{kip1}$ and $p57^{kip2}$) strongly inhibits Cdk2-containing complexes [4].

The Ink family of CKIs bind to monomeric Cdk4 and Cdk6. The inhibitors bind to the same region of the Cdk as cyclin D, thus, high Ink4 expression blocks formation of the active cyclinD/Cdk4 complex. Because Ink4 prevents cyclin D/Cdk4 complex formation it prevents binding of p27 to cyclinD/Cdk4. Increased Ink4 expression therefore increases the pool of p27 available to bind to and inhibit cyclin E/Cdk2. Hence, up-regulation of Ink4 directly inhibits cyclin D/Cdk4 activity, and indirectly inhibits cyclin E/Cdk2 activity [4]. The regulation of distinct Ink4 family members is complex and not fully elucidated. Nevertheless, the well-characterized pathway by which the cytokine, TGFβ, induces Ink4 elegantly illustrates one mechanism by which signals from outside the cell delay progression if extracellular conditions are not favorable for division [28].

The Cip/Kip family of inhibitors binds to preformed cyclin/Cdk complex blocking substrate access. The first of these inhibitors to be characterized, p21, is up-regulated by p53 in response to DNA damage. The p21 inhibitor predominantly interacts with and inhibits cyclin E/Cdk2 and cyclin A/Cdk2, thereby it delays entry into S phase. Also, p21 was identified as a protein that is up-regulated during the transition to replicative senescence, that is, in cells that remain metabolically intact but do not to under go further rounds of division [31]. The role p21 plays in adaptation to replicative senescence may be comparable with the role it plays in permanently arresting cells that have suffered irreparable DNA damage.

Cdks Are Positively and Negatively Regulated by Phosphorylation

The association of kinase and cyclin subunits results in a partially activate kinase complex. Full activation requires phosphorylation of a threonine residue within the activation or T-loop region of the kinase. Crystallographic studies and biochemical analyses support a model in which cyclin binding to Cdks induces a conformational change in the kinase, exposing the T-loop threonine and making it more accessible to the activating kinase [32]. Phosphorylation of the T-loop induces a second conformational shift that moves the T-loop away from the ATP binding region and thus facilitates catalysis.

The kinases responsible for catalyzing the activating threonine phosphorylation are known as Cak, for Cdk activating kinases. In mammals, Cak itself is a Cdk comprised of a Cdk subunit, Cdk7, and a cyclin subunit, cyclin H [33]. Cdk7 exists in a ternary complex of cyclin H/Cdk7 and a ring finger protein Mat1. Mat1 stabilizes the cyclin H/Cdk7 complex and the kinase complex is active in the absence of T-loop phosphorylation [34]. However, the finding that cyclin A/Cdk2 and cyclin B/Cdc2 activate cyclin H/Cdk7 by phosphorylating residues within the T-loop implies that an intriguing, reciprocal Cdk activation loop may be operational [35]. The activity of cyclin H/Cdk7 is constitutive across the cell cycle and there is little evidence that modulation of CAK activity regulates cell cycle progression. Cyclin H/Cdk7 has a second function in transcription-coupled repair. A pool cyclin H/Cdk7 associates with the transcription machinery and phosphorylates the carboxyl-terminal domain (CTD) of RNA polymerase II, promoting the transition from the initiation-competent form to the elongation-competent form of the complex [36]. The dual functions of cyclin H/Cdk7 in basal transcription and in cell cycle progression may help to coordinate these two processes.

Phosphorylation of Cdks also negatively regulates kinase activity. The activity of Cdc2/cyclin B is perhaps the best characterized example of regulation by inhibitory phosphorylation; however, the activities of cyclin A/Cdk2, cyclin E/Cdk2, and CylinD/Cdk4 are also, at least partially, controlled this way [37,38]. Inhibitory phosphorylation occurs near the amino terminus in the ATP binding cleft of the kinase subunit, at sites corresponding to threonine 14 and tyrosine 15 in human Cdc2. Wee1 is a nuclear kinase that shows a preference for phosphorylating tyrosine 15. Whereas Myt1, a cytoplasmic protein that associates with the membranes of the endoplasmic reticulum and Golgi, shows a preference for phosphorylating threonine 14. The distinct location of these two kinases suggests a mechanism by which Cdc2 can be negatively regulated in response to both cytoplasmic and nuclear conditions. Wee1 and Myt1 are active throughout interphase, and coincident with the activation of cyclin B/Cdc2 in mitosis, both kinases are inactivated [39–41]. Two kinases have been reported to inactivate Myt1 in maturing oocytes [42,43], however the exact mechanism of inactivation of Myt1 and Wee1 in mitotic cells is not known [41].

The inhibitory phosphates on Cdks are removed by the Cdc25 family of dual-specificity phosphatases. Three forms of Cdc25 are expressed in mammalian cells. Cdc25A acts early in the cell cycle to promote the dephosphorylation and activation of cyclin E/Cdk2. Expression of the predominantly cytoplasmic Cdc25B rises in S phase and remains high in G_2. The abundance of Cdc25C is constant; however, its activity increase in mitosis consistent with a function in the G_2/M transition (reviewed in [44]). Curiously the Cdc25 proteins are themselves substrates of the Cdks: Cdc25A is phosphorylated and activated by cyclin E/Cdk2, and Cdc25C is activated by phosphorylation by cyclin B/Cdc2 [45,46]. The activation of Cdc25 by the kinases that it activates led to the idea of a positive feedback loop that stimulates the rapid and full activation of Cdc2 as shown in Fig. 2. In the case of the G_2/M transition, the feedback loop may require the activity

of a non-Cdk kinase, Plk1, which phosphorylates and activates Cdc25 and is itself activated in mitosis [47,48].

Antisense and microinjection of antibodies suggest that both Cdc25B and Cdc25C promote the G_2/M transition [49–51]. Surprisingly, disruption of the mouse genes encoding Cdc25B or Cdc25C is not associated with defects in proliferative rate, cell cycle profile, or checkpoint responses [52,53]. Thus, despite differences in timing of expression and in subcellular location, it is possible that Cdc25B compensates for the loss of Cdc25C and vice versa. The phenotype of the double disruptant is needed to validate the importance of Cdk dephosphorylation in the regulation of cell cycle progression and checkpoint control.

Degradation: The Importance of Being Absent

Each cell cycle phase is defined by the form of the cyclin/Cdk complex that is present and active. It is therefore important that cyclin/Cdks be inactivated and destroyed in a timely manner. Ubiquitin-mediated degradation is the main pathway for ridding cells of cell cycle proteins that have executed their function. The addition of ubiquitin residues to substrate proteins is the signal for degradation by the 26S proteasome, a multisubunit cellular complex that specializes in the unfolding and proteolysis of ubiquitin-tagged proteins. Traffic of cell cycle proteins into the 26S proteosome is controlled by the activity of two structurally and functionally similar complexes, the SCF (Skp-Cullin F box) and APC. The SCF E3 ubiquitin ligase is active in G_1, through S phase, and into G_2. The APC is also an E3 ubiquitin ligase; it is activated in mitosis and remains active in G_1. The specificity of these ubiquitin ligases is controlled by substrate recognition proteins. In the case of SCF a large number of substrate recognition proteins, characterized by the presence of F boxes, leucine-rich repeats, or WD-40 repeats, are known to exist [54]. In the case of the APC, two substrate-specific interacting proteins, Cdc20 and Cdh1, are known, but more may exist [23]. Specificity and appropriate temporal degradation are achieved by a combination of the fact that Cdk catalyzed phosphorylation of target proteins is required for recognition by F-box proteins, and that components of APC are regulated by Cdk-dependent phosphorylation.

As discussed earlier, the degradation of p27 and cyclin E is initiated when these proteins are phosphorylated by active cyclin E/Cdk2. In a similar manner, the transcriptional activator E2F is inactivated by cyclinA/Cdk2-dependent phosphorylation and degradation by the SCF [55]. In both cases degradation is initiated when the protein has been phosphorylated and thus degradation is under the direct control of Cdk activity. Compound mechanisms regulate the traffic of proteins through the APC. The rise in expression of the substrate recognition protein, Cdc20, in mitosis initiates degradation of APC/Cdc20 substrates. Phosphorylation of Cdc20 and core components of the APC by cyclinB/Cdc2 stimulates ubiquitin ligase activity.

A major role of the APC/Cdc20 complex is to promote the degradation of proteins that maintain sister chromatid cohesion and, thus, to allow the metaphase-to-anaphase transition. If all of the chromosomes are not correctly aligned, the presence of an unattached kinetochore generates a signal that inhibits APC/Cdc20. Activated Mad2 and BubR1 have been shown to independently inhibit the metaphase-to-anaphase transition by inhibiting APC function [56,57]. By inhibiting the APC/Cdc20, the spindle checkpoint prevents sister chromatid separation until all chromatids are properly attached to the spindle microtubules that will pull the two sister chromatids to opposite sides of the cell. Expression of the second APC specificity factor, Cdh1, rises in S phase and persists through G_1. However, APC/Cdh1 is inhibited by high cyclinB/Cdc2 activity, thus degradation of APC/Cdh1 substrates cannot occur until after degradation of cyclin B. Once APC/Cdh1 is activated, proteins that prevent anaphase including remaining mitotic cyclins are destroyed. The inhibition of Cdh1 by mitotic Cdks prevents the onset of anaphase if metaphase is delayed. APC/Cdh1 remains active into G_1 ensuring that components of the previous mitosis are destroyed before initiation of the next cycle. The APC also catalyzes the destruction of Cdc20, thereby preventing APC activity until Cdc20 is synthesized again late in the next cycle (Fig. 3).

Location, Location, Location

The substrates of Cdks are predominantly nuclear; thus, the presence or absence of active Cdks to the nucleus is a potentially important mechanism of regulation. Just as the cyclin subunit controls the temporal activation of Cdks, there is evidence that cyclins also control the subcellular location of cyclin/Cdk complexes (reviewed in [58]). For example, monomeric Cdc2 appears to be pancellular; however, as

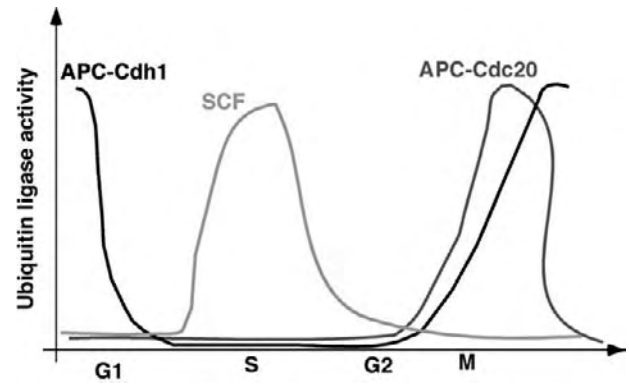


Figure 3 Activity profiles of the ubiquitin ligase complexes that tag cell cycle proteins for degradation. Degradation of cyclins and other cell cycle regulators is required for progression. Degradation of SCF targets is maximal when Cdk2 complexes are available to phosphorylate target proteins. The activity of APC/Cdc20 increases with the abundance of Cdc20 and is stimulated in mitosis by cyclin B/Cdc2 phosphorylation. The APC/Cdh1 degrades both cyclin B and Cdc20, allowing the completion of anaphase and entry into a new G1.

cyclin B expression increases, the complex accumulates in the cytoplasm, predominantly in a perinuclear region. At the start of mitosis, cyclin B/Cdc2 translocates to the nucleus, just prior to nuclear envelope breakdown. The cytoplasmic location of cyclin B/Cdc2 is due both to active retention in the cytoplasm and to active transport out of the nucleus [59]. The signals that mediate cytoplasmic retention and nuclear export are clustered in the amino-terminal region of the protein. Phosphorylation of sites within this region alters the balance of import–export such that during prophase, the bulk of cyclin B/Cdc2 becomes nuclear [59]. The coincident activation and nuclear import of cyclin B/Cdc2 is thought to contribute to the rapid, switch from G_2 into mitosis [60]. However, expression of constitutively nuclear cyclin B mutant is not sufficient to induce premature mitosis, which suggests that regulated localization is perhaps one of the redundant mechanisms used by higher eukaryotes to regulate cell cycle progression [59,61].

An important issue of spatial regulation is how Cdks coordinate nuclear and cytoplasmic functions. In part, the answer lies in the realization that proteins are much less static than immunofluorescence images might suggest. For instance, CycE and CycA complexes appear to be predominantly nuclear when examined in cells that have been fixed. However, live cell analysis shows that they constantly shuttle between the cytoplasm and nucleus [62]. The shuttling of these complexes allows them to phosphorylate both nuclear and cytoplasmic substrates. Thus, for example, cyclin E/Cdk2 phosphorylates nuclear proteins at sites of ongoing replication and cytoplasmic proteins in the replicating centrosome. Shuttling presumably also allows access to regulators if they are present in different compartments.

Checkpoint Signaling

Once it has passed the mitogen-dependent stage, the cell cycle is an autonomous signaling system in which the completion of each step promotes progression through the next. Despite this constant drive to move forward, a problem in any of the subpathways of the cell cycle can generate a signal that stops progression in all other subpathways. The mechanisms that inhibit cell cycle progression in response to intracellular problems are known as *checkpoints.* Checkpoint responses may, in fact, be activated in every unperturbed cell cycle; however, they are most easily seen and best understood in the context of cells that have been subjected to unusual levels of damage or unanticipated nucleotide deprivation. The concept of checkpoint signaling and how they integrate with cell cycle regulation has been reviewed [21,63].

If the genome is damaged, cell cycle progression is delayed until the damage is repaired. In addition to delaying cell cycle progression, the DNA damage response must also activate relevant repair pathways. The most appropriate repair pathway depends not only on the nature of the damage but also on the stage of the cell cycle. Thus, for instance, a double-strand break that occurs late in S phase or in G_2 can be repaired by the process of homologous recombination, which requires the presence of a sister chromatid as a template for repair. If, however, a double-strand break occurs in G_1 the process of end joining is likely to be the only viable repair pathway available [64]. Other repair processes that are active throughout the cell cycle [65] are up-regulated when elevated rates of damage are incurred.

As far as we know all DNA damage checkpoints function through two protein kinases, ATM and ATR [66]. Arrest in the G_1 phase of the cell cycle is mainly mediated by the p53 tumor suppressor, a transcription factor that induces cell cycle arrest, damage repair, and apoptosis [67]. ATM and ATR activate the p53 protein in at least three ways (Fig. 4). First, both kinases directly phosphorylate p53 on serine 15, a site that increases stability and transcriptional activity [68–70]. Second, ATM phosphorylates Mdm2, an ubiquitin ligase that tags p53 for degradation. Phosphorylation of Mdm2 decreases its ability to promote the degradation of p53 [71]. Third, ATM phosphorylates and activates another protein kinase Cds1 (Chk2) that phosphorylates p53 on serine 20, disrupting the association with Mdm2 [72,73]. Stabilized and activated p53 drives the transcription of several genes, including the Cdk inhibitor p21. The induction of p21 in response to DNA damage is the major mechanism enforcing arrest in G_1 in response to DNA damage or nucleotide pool perturbation [29,30]. In addition to the role p53 plays in the G_1 checkpoint, p53-mediated transcription of p21 and a 14-3-3 protein, 14-3-3σ, is required for maintaining a long-term G_2 arrest [74]. The 14-3-3 proteins are a family of ubiquitously and highly expressed phosphobinding proteins (reviewed in [75]). The 14-3-3σ protein is thought to prevent mitosis by retaining cyclin B/Cdc2 in the cytoplasm [76].

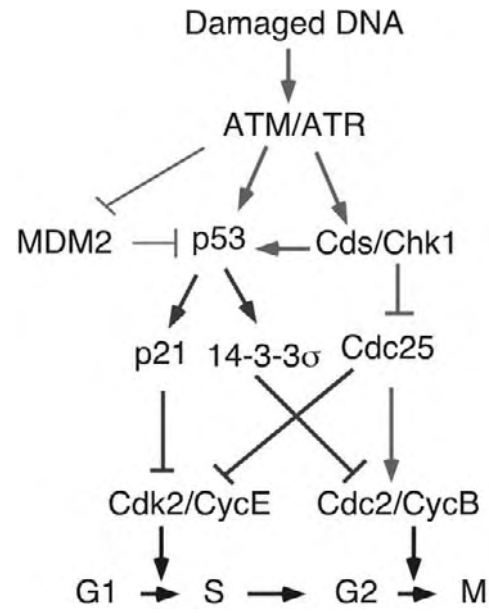


Figure 4 The DNA damage checkpoints inhibit Cdk activity. Phosphorylation and degradation events that are expected to function rapidly following DNA damage are indicated by gray lines and arrows. Processes that require transcription, and are required for the long-term maintenance of the arrest are indicated in black.

In mammalian cells, the initial G_2-damage-induced checkpoint works by maintaining the inhibitory phosphorylation of cyclinB/Cdc2. Cdc2 dephosphorylation and activation can be catalyzed *in vivo* by the two isoforms of Cdc25, Cdc25B and Cdc25C [49]. The checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate Cdc25C on serine 216, a site that induces 14-3-3 binding [77,78]. This site is constitutively phosphorylated throughout interphase, however, it is not phosphorylated in the hyperactive mitotic form of Cdc25C. Phosphorylation of serine 216 by the checkpoint kinases is proposed to reduce Cdc25 function by sequestrating Cdc25 away from its substrate or by preventing its nuclear accumulation [78]. Expressing a mutant Cdc25C that cannot bind 14-3-3 has a modest effect on the G_2 checkpoint [78]; however, expression of a constitutively nuclear mutant of Cdc25 is not sufficient to overcome checkpoint regulation [79]. Thus, additional sites on Cdc25, or other factors may contribute to the G_2 damage checkpoint. The phosphorylation of Cdc25C by Chk1 and Cds1 on sites other than S216 that inhibit phosphatase activity directly may be required to enforce the G_2/M checkpoint [80]. In addition, the possibility that the DNA damage checkpoint additionally up-regulates inhibitory kinases, such as Wee1 or Myt1, has not been discounted in mammalian cells.

Chk1 and Cds1 also play a role in delaying progression through G_1 and in slowing DNA replication. In this case, the checkpoint kinases phosphorylate Cdc25A on a site that targets the protein for ubiquitin-mediated degradation (reviewed in [21]). Thus, it appears that the down-regulation of Cdc25 by multiple overlapping mechanisms is a common theme in preventing Cdk activation following DNA damage. The degradation of Cdc25A presumably acts synergistically with p21 to inhibit cyclin E/Cdk2 and hence delays the G_1/S transition and progression through S phase.

An important factor in checkpoint control is the timescale on which they operate. Checkpoint mechanisms that act through phosphorylation or degradation of key regulators might be expected to operate very rapidly in response to damage and be capable of delaying the onset of the next cell cycle step right up until the moment of initiation. On the other hand, the transcriptional induction of cell cycle inhibitor proteins is a process that functions over the course of hours rather than minutes. It is becoming increasingly clear that quick acting and sustained checkpoint responses are required to effectively inhibit the activity of the cyclin/Cdks that drive cell cycle progression.

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CHAPTER 329

Endocytosis and Cytoskeleton

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Introduction

Signals emanating from membrane receptors regulate a number of fundamental cellular events, thereby dictating whether a cell will differentiate or proliferate, die or survive, rest or migrate. This pleiotropism is achieved through the regulation of a heterogeneous set of signaling molecules, which are organized in complex networks characterized by extensive cross-talk, integration between different linear pathways, and the formation of large signaling units. Additional complexity is brought about by coordinated cellular processes such as receptor internalization, membrane trafficking, and compartmentalization, which govern the duration and the intensity of signals. These latter processes are affected, and at least in part regulated, by the overall architecture of a cell, which, in turn, is dramatically remodeled following membrane receptor stimulation. For these reasons, increasing efforts have been directed toward the identification of the molecular machinery allowing such interplay. Exemplar are the GTPases, such as those belonging to the small GTPase family and the dynamins. The former have emerged as master regulators of several biological processes. They are frequently activated by upstream signals, originating from membrane receptors, and further propagate these signals to downstream effectors. GTPases of the Rho subfamily have been implicated at various steps of the regulation of actin cytoskeleton [1], whereas GTPases of the Rab subfamily govern several aspects of membrane traffic and vesicle dynamics [2]. Evidence suggests integration of these regulatory networks in which Rho GTPases also control endocytosis, and Rab GTPases (in particular, Rab5) participate in the regulation of actin dynamics.

Dynamins, on the other hand, are key endocytic molecules, and act at the step of vesicle fission from the membrane. Recently, however, dynamins have also been implicated in processes as diverse as apoptosis and actin cytoskeleton dynamics, thus emerging as key candidates for integrating multiple signaling pathways.

In this chapter, we cover the emerging evidence supporting the involvement of actin dynamics in endocytosis. We also briefly discuss possible roles for microtubules during the initial and later phases of the endocytic process. Finally, we describe the known molecular circuitries that link and integrate endocytosis and actin dynamics.

Actin Dynamics and Endocytosis

Actin Cytoskeleton and Endocytosis in Yeast

Endocytosis of membrane receptors is governed by a complex structural machinery [3,4], which is in turn controlled by a wealth of accessory regulatory proteins [5,6]. Links between endocytosis and the actin cytoskeleton have been revealed by a number of studies in yeast A variety of the so-called “end” or “dim” mutations, isolated in screening for endocytic defects, also disrupt the actin cytoskeleton (for reviews, see [7–9]). In contrast, many temperature-sensitive mutations in actin and actin-binding proteins display endocytic defects. Notably, a collection of isogenic mutants of the actin gene contains multiple alleles that are defective in endocytosis. Most of these mutations cluster in a region identified as the binding site for fimbrim, an actin filament-binding protein, suggesting that the integrity of actin filaments is required for endocytosis. Additionally, drugs largely used in mammalian systems to disrupt actin dynamics also affect endocytic processes in yeast [8,9]. Moreover, a role in endocytosis for the actin-regulating Arp2/3 complex is emerging. The Arp2/3 complex, which is composed of seven

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subunits, is endowed with the property of nucleating actin filaments and is responsible for the formation of a branched actin network. A variety of activators of Arp2/3, including Las17/Bee1p, Abp1, and the type I myosin Myo3/5p (reviewed in [9]), have been reported to affect endocytosis to various extents. On the other hand, the yeast Eps15 homology domain (EH)-containing protein Pan1p, which is part of the EH domain complex involved in endocytosis [10], can bind and activate the Arp2/3 actin nucleating function [11]. Interestingly, a mutant of Pan1p has recently being described, in which the endocytic defects are separable from those on actin dynamics [11], ruling out the possibility that the effects on endocytosis are the results of the disruption of the actin cytoskeleton.

A certain caution must be exerted when generalizing these observations to mammalian cells, because some important differences exist in the endocytic process between yeasts and mammals. The most striking difference is that no dynamin-like protein seems to participate in the uptake step in yeast. Similarly, clathrin, which is a fundamental component for the formation of a coated pit in mammals, is only "mildly" required in yeasts, because it is dispensable for cell growth, protein secretion, or receptor endocytosis [7].

Actin Dynamics at Various Steps of the Endocytic Process in Mammals

Links between actin cytoskeletal organization and endocytosis in mammalian cells also exist. For example, drugs or proteins that disrupt the actin cytoskeleton by perturbing actin treadmilling, also display a variety of effects on early steps of receptor-mediated endocytosis (reviewed in [12]). These studies have converged to establish a model in which *de novo* actin polymerization (or actin remodeling) is required in endocytosis, while preexisting actin filaments are not. Actin remodeling might participate in the early phases of endocytosis in various ways (Fig. 1). In the case of the internalization of the B-cell receptor, for example, actin polymerization appears to define the topology of endocytosis by restricting the localizations at which receptor internalization can take place [13].

A complex role of actin dynamics in the process of SV-40 internalization has been recently uncovered. SV-40 is endocytosed through caveolae instead of clathrin-coated pits. Entry of viral particles induces a transient breakdown of stress fibers [14]. Actin is then recruited, at the entry site, in patches, which then serve as actin nucleation foci for "comet tail" formation (see later discussion). Prevention of stress fiber breakdown by the actin filament stabilizing drug jasplakinolide reduces virus internalization and infection by inhibiting the dissociation of actin stress fibers, with a consequent block in the formation of actin patches and in the polymerization of actin tails. Conversely, treatment with latrunculin A, an actin monomer-sequestering agent, reduces viral internalization by preventing the formation of actin tails, but not of actin patches, thereby inhibiting viral passage deeper into the cytoplasm [14]. Interestingly, dynamin, like actin, is also recruited to virus-containing

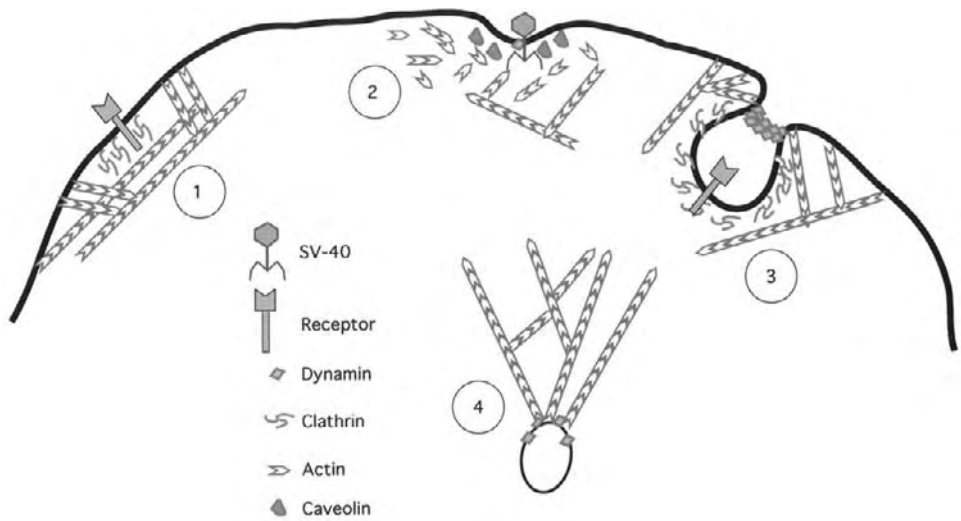


Figure 1 Actin dynamics at various steps of the endocytic route. The illustration shows the potential steps in the endocytic process that might be controlled by actin cytoskeleton remodeling. (1) Actin cytoarchitecture might impose topological constraints on the lateral mobility of the endocytic machinery, resulting in the determination of sites (hot spots) on the plasma membrane where endocytosis take place, as in the case of B-cell receptor internalization [13]. (2) Endocytosis of SV-40 occurs through caveolae. This requires the depolymerization of preexisting actin filaments [14]. (3) Actin might facilitate the fission of vesicles from the plasma membrane as indicated by the increasing molecular connections between the activity of dynamin and dynamin-associated proteins and the actin polymerizing machinery. (4) Actin might participate in later endocytic events by propelling endocytic vesicles in their cytosolic routes. Actin tails have been seen in association with newly formed endocytic vesicles, "rocketing endosomes," with Golgi-derived clathrin-coated vesicles, and with SV-40-containing, caveolae-enriched vesicles.

caveolae and interference with its function reduces virus internalization. Thus, both actin and dynamin are important for the closure of the caveolae by membrane fission and for the movement of vesicles deep inside the cytoplasm.

The participation of actin in the pinching off of vesicles from the plasma membrane (Fig. 1) is further supported by increasing molecular connections between the activity of dynamin and the actin polymerizing machinery (see later discussion) and by studies of myosin VI, the first motor protein implicated in clathrin-mediated endocytosis. Although the mechanisms whereby myosin VI participates in the endocytic process remain to be investigated, myosin VI's ability to move toward the pointed end of actin filaments suggests the intriguing possibility that it may be involved in pulling away the newly formed vesicle along actin filaments, which extends their plus end toward the plasma membrane, leaving their minus ends buried in the cytoplasm (Fig. 1) (see [12] for review).

In addition to this possible role in the early steps of endocytosis, actin might also participate in later events by propelling endocytic vesicles in their cytosolic routes. Actin tails have been seen in association with newly formed endocytic vesicles, "rocketing endosomes," and with Golgi-derived clathrin-coated vesicles [15,16]. These actin comet-like tails are similar to those that drive the movement of intracellular parasites, such as *Listeria* and *Shigella* [17]. This process has been extensively characterized and it requires the recruitment of the basic machinery of actin polymerization, which is in turn regulated by its association with the GTPases Cdc42 and to phosphatidylinositol 4,5 phosphates (PIP2) [18]. Interestingly, PIP2 was also shown to regulate several steps of the formation of the clathrin coat, suggesting that the localized production of this phospholipid, required for the dynamic assembly of the clathrin coat, may also stimulate actin polymerization and drive the movement of vesicles derived from membranes [18].

Role of Microtubule Cytoskeleton in Receptor Endocytosis

Microtubules are long filaments that are involved both in maintaining a precise spatial organization of intracellular organelles and in the transport of cargo between organelles [19]. Movement of some cargo-laden vesicles can be driven by diffusion. However, especially in highly polarized cells, it predominantly occurs along microtubular "tracks" powered by molecular motor proteins like kinesins, dyneins, and myosins. This ensures that vesicles are transported efficiently (at a speed of around 1 μm/s) and for a relatively long distance. In nonpolarized cells, the minus ends of microtubules are located toward the cell center near the centrosome, and the plus ends point radially to the cell periphery. In highly polarized cells, the plus ends of microtubules are localized basolaterally, whereas the minus ends are at the apical side. Movement along the microtubular tracks occurs in both directions. Kinesins are the motor proteins responsible for the movement toward the fast growing plus ends, whereas dyneins are involved in the movement toward the minus ends [20].

The role of microtubules in the endocytic process has been established mainly with the use of microtubule depolymerizing drugs, such as nocodazole (for a review, see [21]). Although the large majority of these studies showed that the microtubule cytoskeleton is not essential for the first step of clathrin-mediated endocytosis, examples are seen in which nocodazole treatment impairs adsorptive and fluid phase endocytosis. Moreover, in cells kept in suspension, but not in cells grown on substrates, nocodazole was reported to reduce by 40% the initial rate of transferrin receptor internalization (for a review, see [21]). This suggests that the overall structural organization of a cell might affect microtubule organization and dynamics, leading to alterations in membrane plasticity, and consequently affecting the ability of a receptor to be recruited to the invaginating clathrin-coated pit.

A much broader consensus has been reached on the requirement of microtubule dynamics at later steps of the endocytic route. For example, the transport of receptors from early to late endosome, their delivery to lysosomes, and their recycling back to the plasma membrane are all severely impaired in nocodazole-treated cells [21]. This is mirrored by the finding that cytoplasmic dynein, in conjunction with the dynactin complex that mediates the association of cargo structures with microtubules, facilitates the inward movement of endocytic vesicles from early to late endosome (for a review, see [22]). It remains unclear, however, to which extent alterations in microtubule dynamics may affect the overall biological activity of membrane receptors, and whether signaling emanating form membrane receptors may in turn modulate microtubule-based vesicle movements. A possible connection is represented by the small GTPase Rab5, which regulates endosome interaction with the microtubule network [23]. *In vitro*, Rab5 was shown to stimulate the association of early endosomes with microtubules and their motility toward the minus ends. *In vivo* Rab5-positive endosomes were shown to move along microtubule tracks. Moreover, the Rab5 effector Vps34, a phosphoinositide-3 kinase, is required for this function, indicating that GTP-loading of Rab5 mediates this effect. Because Rab5 activity is directly controlled by RTKs (see later discussion), it is intriguing to speculate that signaling emanating from activated receptors may also coordinate microtubule-dependent late steps in the endocytic route.

Physical and Functional Interactions of Dynamin and Dynamin-Interacting Proteins with the Actin Cytoskeleton

Dynamin and Actin Cytoskeleton

Dynamin is a GTPase that plays a critical role in endocytosis, by determining the fission of vesicles from the plasma membrane. A number of recent observations, obtained in neuronal cells, functionally linked dynamin to the actin

cytoskeleton (reviewed in [24,25]). In addition, expression of a dominant-negative mutant of dynamin-1, in HeLa cells, causes redistribution of actin stress fibers to the cell cortex [26]. Further links were obtained with studies of podosomes and actin comet tails. In the former case, a dynamin isoform, dynamin-2aa, was found to colocalize with filamentous actin at membrane ruffles [27] and at podosomes. A temperature-sensitive mutant of dynamin-2aa disrupted podosome formation [28]. Interestingly, another dynamin mutant (K44A), which is impaired in GTP hydrolysis and potently inhibits endocytosis, only delayed actin dynamics at podosomes, but did not prevent their formation [28]. This latter result suggests that the interference of dynamin with actin remodeling is not due to secondary effects of an endocytic block, but rather to a direct functional role on the actin cytoskeleton.

Two recent reports identified dynamin 1 and 2 as components of actin comet tails generated by *Listeria*, an intracellular pathogen known to utilize an actin tail for movement within the cytoplasm, and by type I PIP kinase. An inactive (K44A) dynamin mutant significantly reduced comet number, length, velocity, and efficiency of movement, suggesting that dynamin is part of a protein network that controls nucleation of actin from the membranes [29,30].

A further intriguing connection emerged from the functional characterization of the putative tumor suppressor protein nm23H1 (Fig. 2). The nm23 protein belongs to a set of structurally conserved nucleoside diphosphatase kinases (NDKs), a family of enzymes that synthesizes triphosphates from their respective nucleotide diphosphates [31]. In *Drosophila*, nm23, regulates synaptic vesicle internalization at a stage where the function of the dynamin GTPase activity is required [32], suggesting a model whereby the NDK activity of nm23 is required locally to increase the concentration of GTP, in order to facilitate the loading of dynamin, which has an unusually low affinity for GTP. Thus, interference with the function of nm23 should impair receptor internalization, resulting in prolonged exposure of the receptor in the plasma membrane and sustained signaling, a possibility compatible with its postulated metastasis suppressor role. This effect may be further strengthened through an additional activity of nm23H1, which also inhibits Tiam-1-induced production of GTP-bound Rac-1 [33]. Tiam-1 is a Rac-specific GEF originally identified as an invasion and metastasis-inducing gene [34]. Nm23h1 interacts directly with Tiam-1, and blocks its GEF activity *in vivo* [33]. Thus, given the inhibitory effects exerted by GTP-loaded Rac in receptor internalization (see later section), the lack of nm23h1 may result in enhanced Rac activation, contributing to the prolonged permanence of receptors on the plasma membrane. Concomitantly, Rac activation may enhance cell migration, thereby further contributing to the onset of the phenotypic features typical of metastatic cells.

Dynamin-Interacting Proteins and Actin Cytoskeleton

Clues as to the molecular mechanisms through which actin dynamics might influence the function of dynamin are emerging from studies of dynamin-interacting proteins (Fig. 2). Syndapin, for example, binds to numerous proteins involved in the endocytic process (dynamin, synaptojanins, and synapsins) and in the regulation of actin dynamics (N-Wasp, Sos-1). Accordingly, syndapin affects both endocytosis and actin cytoskeleton by a mechanism that coordinates vesicle fission and actin nucleation, thereby triggering a burst of actin polymerization around the vesicle neck, which favors detachment from the plasma membrane and propulsion into the cytoplasm.

Amphiphysin I and II represent another class of dynamin-binding proteins with a dual involvement in endocytosis and actin cytoskeleton. Amphiphysins are thought to target dynamin to the plasma membrane (reviewed in [35]). In addition, expression of their SH3 domains reduces receptor endocytosis, probably by preventing the formation of the dynamin ring [35]. Treatment of primary hippocampal neurons with amphyphysin I antisense oligonucleotides inhibits neurite outgrowth, an effect similar to that exerted by suppression of dynamin expression in the same system [36]. Although the mechanism remains to be determined, it is tempting to speculate that suppression of neurite outgrowth might reflect a role of amphiphysin in actin cytoskeleton, possibly exerted through cooperation with the amphiphysin-interacting protein synaptojanin, an inositol-5 phosphatase that cleaves phosphoinositides, which are in turn able to regulate various aspects of actin remodeling [37].

A final example is provided by Abp1, which binds to F-actin and it is associated to dynamic actin structures in lamellipodia and filopodia, in a fashion controlled by the Rac1 GTPase [38]. Abp1 binds to dynamin both in vitro and in vivo, via its SH3 domain. Ectopic expression of the Abp1-SH3 domain leads to a reduction in receptor-mediated endocytosis, an effect reverted by the simultaneous overexpression of dynamin. Mammalian Abp1 is also enriched at sites where both dynamin and actin are enriched [38]. This supports the possibility that it may serve as a link between the endocytic machinery, via its SH3 domain, and the actin cytoskeleton, through its N-terminal F-actin binding region. The recent discovery that Abp1 modulates actin polymerization through its binding and regulation of the activity of the Arp2/3 actin nucleation complex [39] further strengthens the possibility that actin dynamics may play an active role in driving the completion of clathrin coated vesicle budding.

Integration of Signals in Endocytosis and Actin Dynamics by Small GTPases

Rho GTPases in Actin Cytoskeleton Remodeling and Endocytosis

Actin cytoskeleton remodeling induced by membrane receptors requires the sequential activation of small GTPases of the Rho subfamily, which are in turn connected through a complex network of interactors-effectors. The function of several of these proteins is also required in

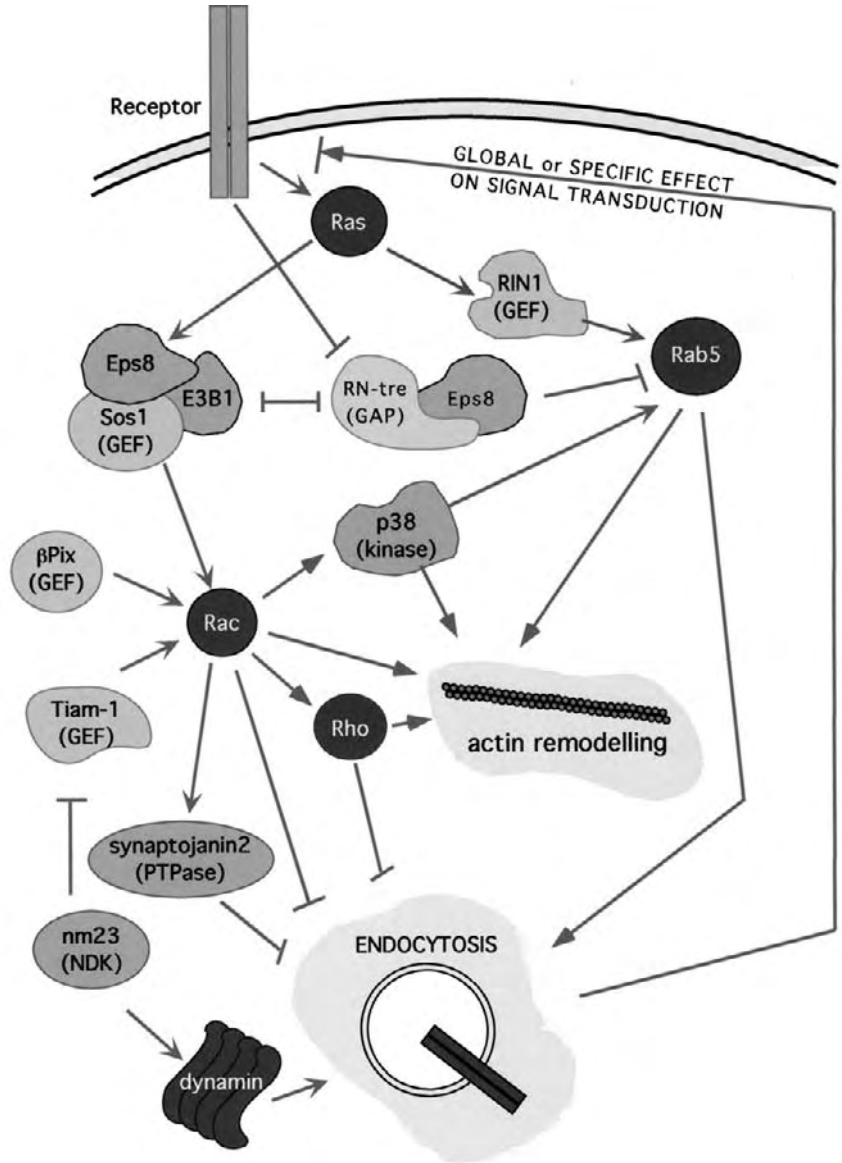


Figure 2 Molecular circuitries connecting actin cytoskeleton and endocytosis. This illustration demonstrates how signaling pathways leading to actin cytoskeleton remodeling and endocytosis are connected and integrated. The scheme is intended to represent a combination of biochemical, genetic, and biological evidence, and it is not exhaustive of all reported interactions. (From [12], Fig. 5, p. 47.)

endocytosis, suggesting that they could represent molecular bridges between cytoskeleton reorganization and vesicular trafficking. Initial indications for a role of Rho GTPases in membrane trafficking derived from the analysis of their intracellular localization. Rho B localizes to late endosomes/lysosomes [40], whereas Cdc42 is associated, in part, with the Golgi [41]. Furthermore, biological studies demonstrated how receptor-mediated endocytosis is inhibited by constitutively active mutants of Rho A and Rac1 [42]. Similarly, activated Rac1 inhibited apical and basolateral endocytosis in polarized epithelial cells [43], while Cdc42 controlled secretory and endocytic transport to the basolateral membrane [44]. This might lead to important consequences. For example, activation of Rho-GTPases by membrane receptors can be predicted to inhibit both receptor internalization and the ensuing inactivation by degradation, thus allowing the sustained and prolonged signaling required for cell proliferation. In addition, increasing evidence is also supporting a requirement for the endocytic process in order to allow full activation of certain signaling pathways, such as those leading to activation of ERKs (reviewed in [45]). Thus delayed endocytosis, obtained through signaling components such as Rho-GTPases, might serve the purpose of delaying the activation of certain signaling, while permitting

others, thereby controlling the temporal progression of signaling (Figs. 1 and 2).

How actin dynamics plays a role in this remains to be elucidated. The molecular mechanism of the action of Rho GTPases in endocytosis is not clear. On the one hand, it is possible that the influence of these GTPases on endocytic process is simply a "by-product" (albeit biologically relevant) of their role in actin cytoskeleton remodeling. On the other, a more direct involvement in the molecular machinery of endocytosis is likely, as witnessed by the observation that Rac1 interacts directly with synaptojanin 2, a polyphosphoinositide phosphatase implicated in the uncoating of endocytic clathrin-coated vesicles [46]. Rac may therefore contribute to the regulation of endocytosis with a dual mechanism: directly, by targeting synaptojanin 2 to the plasma membrane, and indirectly, by controlling actin polymerization. The coordination of these events might dictate the final output and contribute to the regulation of the duration and of the intensity of signals (Fig. 2). Another intriguing example supporting a more complex role of GTPase is provided by the diverse effect exerted by RhoA and RhoB on membrane receptor endocytosis. These GTPases are highly related and display virtual identity at the level of their effector-binding region, suggesting that they may interact with the same subset of downstream molecules. Their intracellular localization is, however, remarkably different. RhoA is mainly cytosolic and relocalizes to the plasma membrane upon RTK activation; RhoB is strictly localized to the cytosolic face of endocytic vesicle [40,47]. This is mirrored by the differential effects that RhoA and RhoB exert on endocytosis. An activated mutant of RhoA inhibited constitutive transferring-receptor internalization [42]; however, it did not affect ligand-induced EGFR-endocytosis, at least in nonpolarized cells [47]. This suggests that RhoA-mediated interference on endocytosis is unlikely due to a general perturbation of the actin cytoskeleton. The identification of RhoA effectors capable of mediating its endocytic function(s) will, however, be required to provide a molecular understanding of this putative endocytic-specific function. RhoB, on the other hand, probably acting through its target PRK1 (serine/threonine kinase) retarded the traffic of EGFR from endosome to a prelysosomal compartment [47], where receptors are degraded. Interestingly, RhoB has been recently shown to be activated by EGF stimulation through the guanine nucleotide exchange factor, Vav2 [48]. This suggests the possibility of a positive feedback loop whereby signals triggered by activated RTK also regulate its trafficking, thus determining the duration and intensity of the biological response. Finally, RhoB transcription was shown to be rapidly induced following EGF stimulation [49], indicating that RhoB may be involved in mediating both acute and adaptive response to EGF stimulation.

Linking Rab5 and Rho GTPase Signaling Pathways

The recognized function of Rab5 is to regulate endosome fusion [2]. However, an important role for this small GTPase in endocytosis is also emerging [12]. Studies of the Rab5:GDI (guanine-nucleotide dissociation inhibitor) complex demonstrated its indispensability for receptor sequestration into clathrin-coated pits [50]. GDI is able to extract from membranes the inactive GDP-bound form of Rab proteins [51] and to deliver them to their membrane site(s) of action where nucleotide exchange occurs. In the case of Rab5, it has been proposed that GDI delivers it to the forming pit after coat assembly, to promote efficient sequestration of ligand by an as yet unknown mechanism [50]. Rab5 has also been implicated in actin cytoskeleton remodeling. A constitutively activated form of Rab5 (Rab5Q79L) induces lamellipodia formation in NIH3T3-A14 cells [52] and enhanced cell migration.

Not surprisingly, the dual function of Rab5 and Rho-GTPases in the control of actin cytoskeleton and endocytosis is mirrored by common levels of regulation and actual "integration" of the two signaling systems (Fig. 2), mediated by the small GTPase Ras. One of the best studied functions of active Ras is to activate actin remodeling via physical interaction with phosphatidylinositol 3-kinase (PI3-K) with ensuing activation of Rac [53]. Interestingly, RTKs activate Rab5 via Ras [54]. Such activation seems to be due to RIN1 [20], which possesses Rab5-specific GEF activity [56]. Binding of RIN1 to Ras-GTP potentiates its GEF activity toward Rab5 *in vitro*, presumably increasing the levels of Rab5-GTP *in vivo.* Consistent with the biochemical data, it was shown that overexpression of RIN1 stimulates internalization of the EGFR, whereas its dominant-negative mutant inhibited it [56]. Because overexpression of RIN1 also increases Rab5-dependent endosome fusion, it is reasonable to assume that the effects of RIN1 on endocytosis are due to activation of Rab5. Thus signals emanating from Ras-GTP coordinately activate Rab5 and Rac (Fig. 2).

Similar levels of coordination apply to the deactivation phase of these GTPases, which is controlled by GAPs (Fig. 2). In the case of Rab5 also, this step appears to be under the influence of RTK-originated signals, as demonstrated by studies of RN-tre, a specific Rab5 GAP. RN-tre, whose function is negatively modulated by EGF treatment, inhibits endocytosis by keeping Rab5 inactive [57]. At least in the case of EGFR internalization, RN-tre requires binding to Eps8 to attenuate endocytosis [57]. The best characterized function of Eps8 is to activate Rac by stimulating the Rac-GEF activity of Sos-1, a dual GEF displaying both Ras-GEF and Rac-GEF activities [58]. Thus, Eps8 represents yet another intersection of pathways regulating actin remodeling (through Sos-1) and endocytosis (through RN-tre).

Further coordination is exerted at the level of GDI (Fig. 2). It has recently been demonstrated that the ability of GDI to extract Rab5 from membranes is regulated via its phosphorylation. The kinase responsible for GDI phosphorylation is the stress-induced MAP kinase p38 [59]. It stimulates the formation of the Rab5:GDI complex, which is limiting in steady-state conditions, thereby accelerating endocytosis [59]. However, it has recently been found that the p38 MAP kinase is also necessary for the formation of membrane

ruffles induced by βPix, another GEF protein for Cdc42/Rac [60]. Overexpression of βPix induces both ruffle formation and increased activity of p38 MAP kinase, which are abrogated by specific inhibitors of p38, strongly suggesting that the activity of p38 is required for the βPix-induced ruffling [60]. Thus, p38 might positively modulate both the activity of the Rab5 cycle and the cascade that from activated Cdc42/Rac leads to actin reorganization (Fig. 2).

Conclusions

Following biological and genetic evidence of a functional link between endocytosis and actin dynamics, the identification of a number of proteins that connect the two pathways is starting to provide the necessary molecular knowledge to define how these cellular functions are integrated. Small GTPases, through their action as molecular switches, seem to act as the master players in this scenario. Given the research efforts and rate of discoveries, it is reasonable to predict that it will not be long before a final and coherent picture is obtained.

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CHAPTER 330

Molecular Basis for Nucleocytoplasmic Transport

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Introduction

Transport of proteins and RNAs between the cytoplasm and the nucleus in eukaryotic cells proceeds through the nuclear pore complex (NPC), a massive proteinaceous structure that spans the nuclear envelope (Fig. 1; reviewed in [1–5]). During the past 15 years, the protein components of the NPC have been completely cataloged by a combination of traditional approaches and proteomic analysis. Moreover, permeabilized cell transport assays developed in higher eukaryotes [6], in combination with *in vivo* and genetic approaches in *Saccharomyces cerevisiae* and other organisms, have led to the identification and characterization of major factors and steps in protein and RNA translocation through the NPC. Finally, X-ray crystallography [7,8] has started to reveal the structures of individual components of the transport machinery at an atomic level, providing complementary information for understanding transport mechanisms.

Macromolecular transport through the NPC is a complex multistep process that involves the movement of cargoes over a distance of ~200 nm. The overall process of nuclear transport is energy dependent. Because individual NPCs are simultaneously engaged in nuclear import and export, one of the key questions about the mechanics is how the NPC achieves selectivity of transport in the face of enormous cargo flux in the import and export directions. Moreover, the transport of specific cargoes is usually highly directional, often occurring against a concentration gradient. Four major determinants of nuclear transport have been described: (1) a transport signal in protein or RNA cargoes, (2) nucleocytoplasmic shuttling receptors that recognize transport signals and carry cargoes through the NPC, (3) the GTPase Ran, and (4) specific proteins of the NPC (nucleoporins) that facilitate transport. In this review, we discuss these four transport determinants, with emphasis on the underlying molecular and structural mechanisms. As discussed later, the majority of nuclear transport receptors are part of a receptor superfamily related to importin/karyopherin β [5]. This family contains 14 members in *S. cerevisiae* and is likely to be larger in higher eukaryotes.

Transport Signals

Nuclear transport signals direct the passage of cargoes through the NPC by specifying binding to specific transport receptors or adaptors, which dictate the directionality of nuclear import and export [11]. Transport signals in proteins most commonly consist of short amino acid stretches, such as the classical basic amino acid-rich nuclear localization signal (NLS) or the leucine-rich nuclear export signal (NES). Some RNAs, such as tRNAs and type D retroviral mRNAs, contain nuclear transport signals within specific nucleotide stretches.

Nuclear Import Signals

Import signals can be recognized directly by an import receptor or by a specific adaptor that in turn binds to the import receptor. The best studied import signals are recognized by the receptor importin β. Two known classes of signal associate with importin β (Fig. 2A): *classical* NLSs, which are recognized by the importin β-binding adaptor

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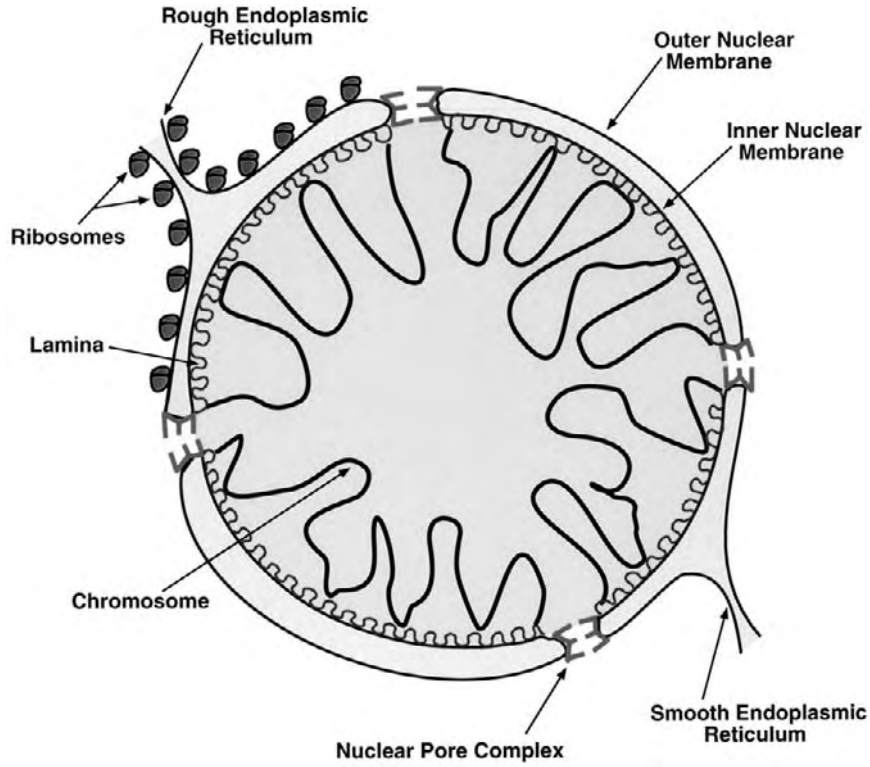


Figure 1 Organization of the nuclear envelope. The nuclear envelope, which separates the nucleoplasm from the cytoplasm, consists of a double membrane that is continuous with the endoplasmic reticulum. The inner and outer nuclear membranes are joined at nuclear pore complexes, which provide the only known transport route across the nuclear envelope. A filamentous network of proteins, known as the nuclear lamina, lines the inner nuclear membrane and serves to anchor chromosomes to the nuclear periphery.

importin α, and *nonclassical* NLSs, which associate directly with importin β. Classical NLSs (cNLS) can be monopartite, as originally discovered in the large T antigen of SV40, or bipartite, as in nucleoplasmin. A classical monopartite NLS consists of approximately seven residues, four or five of which are lysines (or arginines). In contrast, bipartite NLSs contain two discrete basic amino acid stretches separated by approximately 10 amino acids (Fig. 2A). Atomic structures have been determined for several NLS peptides in complex with the adaptor importin α. Both monopartite and bipartite NLSs display an extended conformation (Fig. 2B) [10,11], with the conserved side chains of Lys and Arg engaging in specific electrostatic interactions with the acidic surface of the importin α adaptor. Despite the shortness of the signal, such as in the case of monopartite cNLSs, the binding specificity and affinity for the adaptor can be very high. In the case of the SV40-NLS, the dissociation constant for the adaptor importin α is approximately K_d ~10 n*M* [12].

In contrast to classical NLSs, which associate with importin β via the adaptor importin α, adaptor-independent NLSs are recognized directly by importin β. Two subclasses of adaptor-independent NLSs for importin β have been described thus far: those similar to the IBB domain and those distinct from IBB. The IBB domain was originally described as the *i*mportin β-*b*inding domain of importin α, which mediates the heterodimerization of importin α/β during nuclear import. It consists of approximately 40 amino acids arranged as a long α-helix after binding to the receptor importin β (Figs. 2B and 3B) [13], but it is unfolded in the unbound state [14,15]. An IBB domain also has been found in the adaptors snurportin [1,2] and XRIPα [16]. Several other cargoes like the parathyroid hormone-related protein (PTHrP) [17] and ribosomal protein L5 [18] (Fig. 2A) bind importin β in the absence of an adaptor, but apparently utilize a cargo binding site that is distinct from the region recognized by the IBB domain and thus are expected to have unique properties. Intriguingly, the nonclassical NLS of ribosomal protein L25 (also known as the BIB domain) can be recognized by at least four receptors of the importin β superfamily, namely, importin β-1, RanBP7 (importin 7), importin 3, and importin 4, which can promote, *in vitro*, efficient transport of the cargo into the nucleus [18]. This finding suggests that the recognition of a transport signal by a receptor is not absolute and multiple receptors may cooperate to import the same cargo.

A second well-characterized signal, the so-called "M9" domain (Fig. 2A), is recognized by transportin (karyopherin β-2), a member of the importin β superfamily of import receptors. The M9 signal was originally described in the mRNA binding protein hnRNP A1 and is a 38-residue-long sequence poor in basic residues and rich in glycines and serines. Unfortunately, the binding site and molecular details

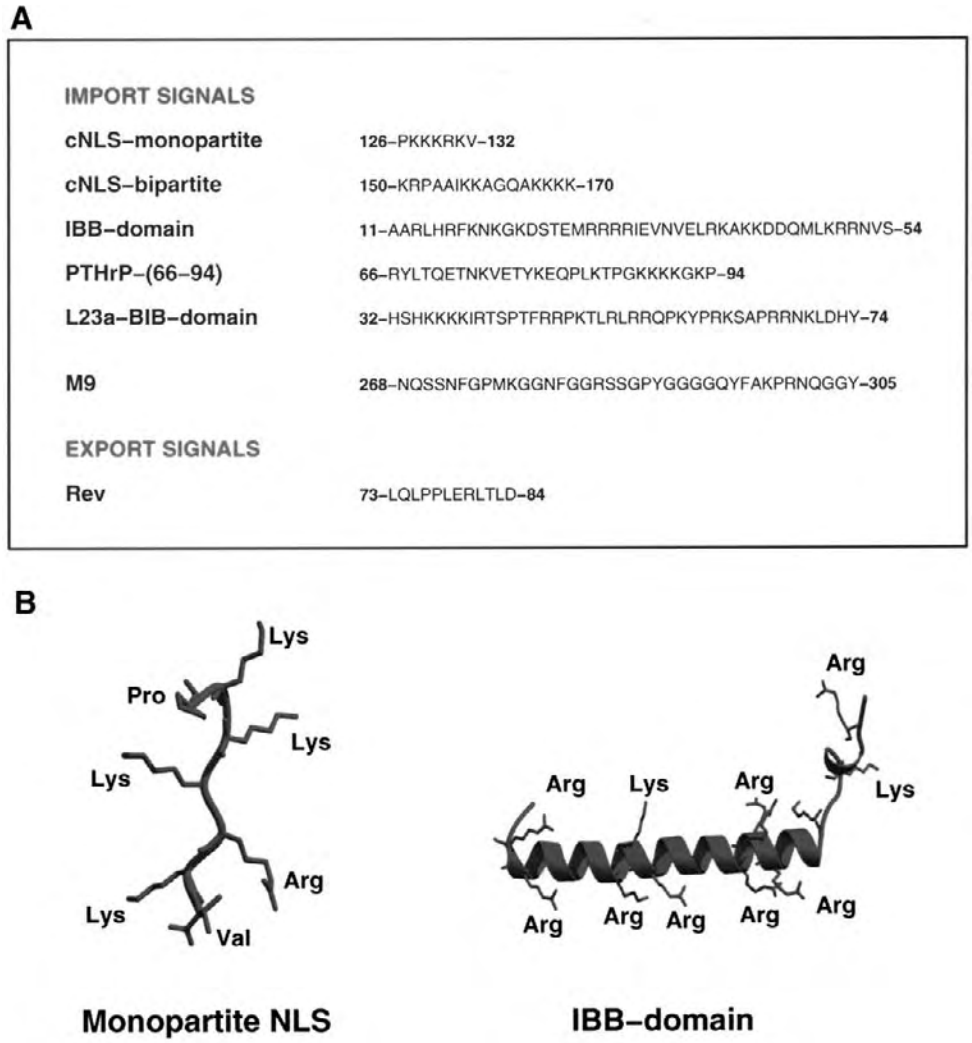


Figure 2 Transport Signals. (A) Signals for nuclear import and export. (B) Structure of classical monopartite SV-40 NLS [10] and IBB domain [13].

of the interaction between the M9 peptide and transportin have not yet been elucidated.

Nuclear Export Signals

NESs are less well characterized than NLSs and lack a strong unique consensus sequence. NESs were originally identified in the inhibitor of the cyclic AMP-dependent protein kinase (PKI) and the HIV Rev protein and subsequently were found in several other cellular and viral proteins [1–5]. All of these originally defined NESs, which are recognized by a single member of the importin β superfamily termed Crm1, are rich in leucine and other hydrophobic residues (Fig. 2A), although both the relative spacing among leucine residues and number of leucines can vary from protein to protein. Certain NESs of this class do not contain multiple leucines, as with the influenza NES protein or the NFAT transcription factor [19], suggesting that it is not the leucine *per se* that confers the molecular recognition, but rather the presence of hydrophobic residues distributed within a short stretch of amino acids.

Transport Receptors

Nuclear transport receptors are soluble proteins that recognize a transport cargo via a direct or indirect interaction with its transport signal and, consequently, are able to carry the cargo through the NPC by virtue of specific interactions with nucleoporins. These transport receptors can be classified into two major classes: members of the importin β superfamily, and receptors unrelated to importin β.

Importin β Superfamily

Receptors of the importin β superfamily have been identified on the basis of their similarity to importin β at the primary structure level [5], and they may have a common evolutionary origin. The members of this superfamily share a number of interesting biochemical properties, including a predominance of α-helical structure, which is present in a tandem array of HEAT repeats (Fig. 3B), a preponderance of acidic over basic amino acid residues, an N-terminal RanGTP

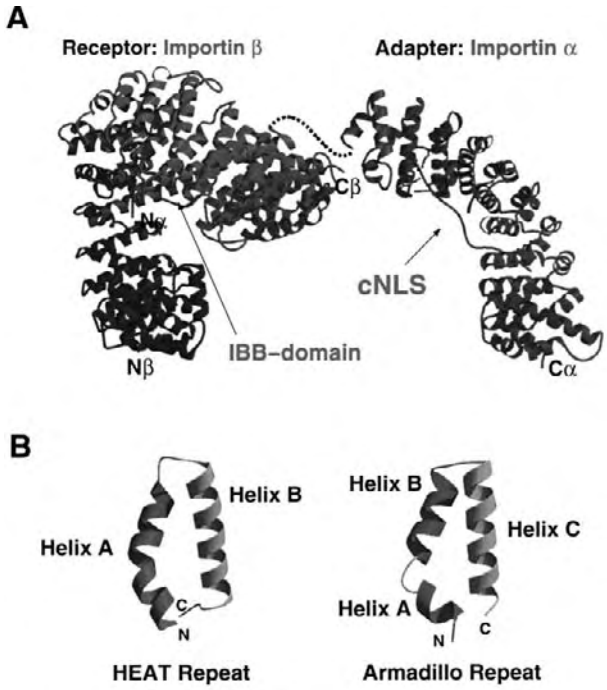


Figure 3 Model for importin α/β heterodimer:cargo complex. (A) The model was built from the structure of importinβ:IBB domain complex [13] (left) and importin α:bipartite NLS complex [10] (right), respectively. The angle between the two proteins could vary according the size of the cargo bound to importin α adaptor. (B) Characteristic HEAT and Armadillo motifs found in importin β and importin α, respectively.

binding domain, and nucleoporin binding sites. Even though the sequence similarity among different members of the family is often difficult to detect, it is possible that all receptors of the importin β superfamily may exhibit a very similar three-dimensional structure.

IMPORT RECEPTORS

Importin β is the best characterized nuclear transport receptor. In complex with the adaptor importin α (Fig. 3A), it mediates nuclear accumulation of cargoes bearing classical NLSs. Importin β presents a characteristic modular structure consisting of 19 tandemly repeated HEAT motifs (Fig. 3B). Each HEAT repeat is formed by two α-helices (helices A and B) connected by a turn. When packed in importin β, the HEAT repeats are arranged to form a superhelix of helices [13]. A-helices are located on the exterior of the protein and form a convex (outer) surface where nucleoporin binding sites are located (Fig. 4A). In contrast, B-helices are arranged inside the protein to create a concave (inner) face, which is involved in binding to cargoes and RanGTP. The IBB domain of importin α interacts with HEAT repeats 7–19 of importin β, and folds as a long α-helix (residues 23–51) followed by a short N-terminal coiled moiety (residues 11–21). The protein–peptide interaction is largely electrostatic in nature and contains highly specific cation-π interactions between critically conserved tryptophan residues of importin β and conserved Lys and Arg residues of the IBB domain.

The dissociation of the importin α/β heterodimer and the subsequent release of the NLS cargo into the nucleoplasm is mediated by the small GTPase Ran (see later section), which also plays a critical role in releasing the transport receptor from nucleoporin binding sites. RanGTP binds to the inner surface of importin β between HEAT repeats 1 and 8, in a region that overlaps minimally with the IBB binding domain [20]. It is plausible that Ran induces dramatic conformational changes in importin β that allow the simultaneous release of cargo and nucleoporins. This model is supported by the structure of importin β-2 (karyopherin β-2, transportin) complexed with RanGppNHp [21]. Transportin is an importin β superfamily protein involved in the import of hnRNP A1 into the nucleus. Similar to importin β, it is a fully helical protein and consists of tandemly repeated HEAT motifs. In complex with RanGppNHp, transportin displays a rather open conformation consisting of two perpendicular arches. RanGppNHp binds to the N-terminal arch, whereas the cargo is predicted to bind to the C-terminal arch.

Several other import receptors have been described [22–24], and the importin β superfamily is likely to grow in the next several years. Based on the presence of tandemly repeated HEAT repeats, it has been predicted that the molecular interactions of these proteins are similar and strongly dependent on the surface polarization between the convex and concave surfaces. The need for adaptor molecules appears as a specific feature of importin β, rather than as a general requirement for all transport receptors.

TRANSPORT ADAPTORS

As previously mentioned, importin β binds classical NLS-bearing cargoes via interaction with the adaptor importin α. The crystal structures of both murine [10] and yeast [11] importin α reveal an elongated banana-like structure (Fig. 3A) consisting of 10 and 11 Armadillo (Arm) repeats, respectively. Each Armadillo motif (Fig. 3B) is formed by three α-helices connected by a loop, having high structural resemblance to the HEAT repeats found in importin β. The NLS binding domain of importin α consists of an acidic binding groove formed by tandemly repeated Arm repeats. Monopartite NLSs can bind to two distinct binding sites in this cavity, a major one located within Arm repeats 2–3, and a minor within Arm repeats 6–7 [10]. As expected, a bipartite NLS peptide spans both cargo-binding sites and thus adopts an extended conformation [11]. Arrays of conserved Trp residues in importin α are fundamentally important for sandwiching the aliphatic portion of conserved arginine and lysine side chains of the NLS. In the murine importin α structure, a portion of the IBB domain occupies the NLS binding site, suggesting a mechanism for auto-inhibition of importin α in absence of a bound NLS [15].

Interestingly, six distinct isoforms of importin α are found in higher eukaryotic cells, which show characteristic tissue-specific expression patterns and altered affinities for distinct NLS cargoes [25]. The functional specialization of the adaptor importin α contributes to an increase in the

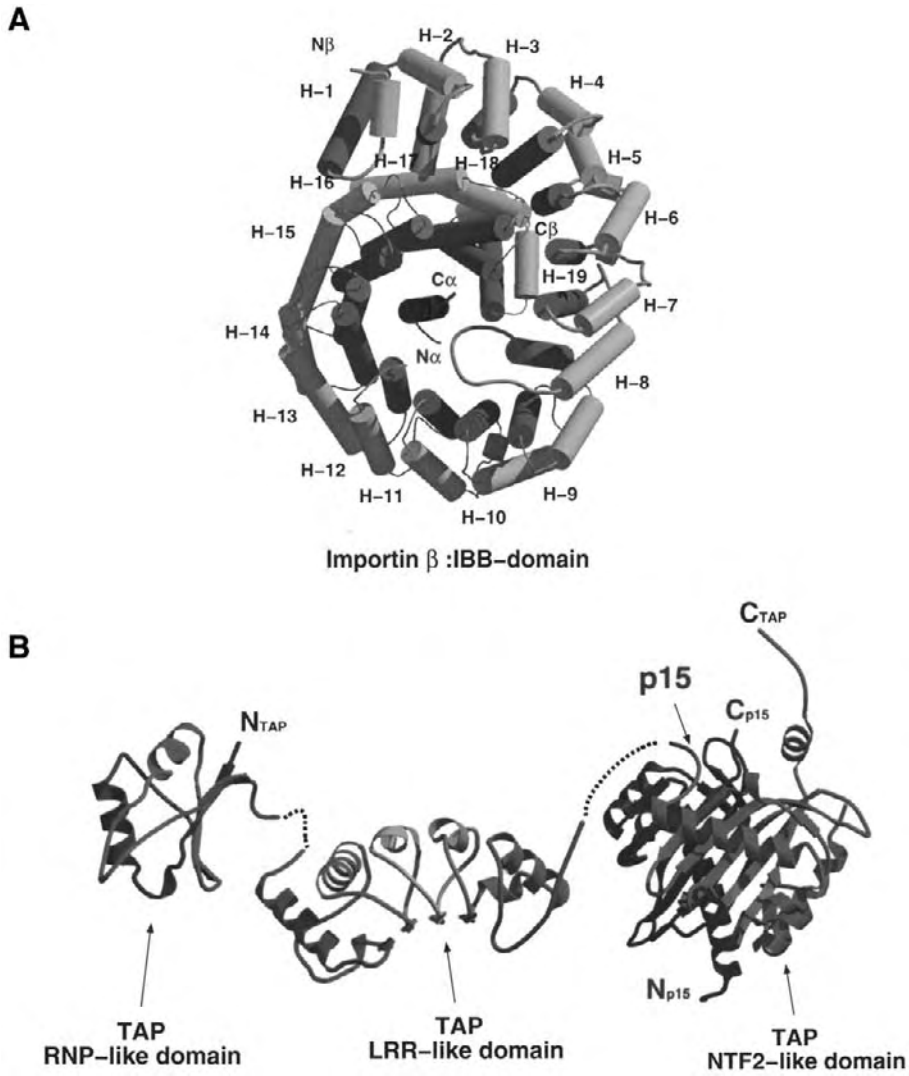


Figure 4 Ribbon representations of nuclear transport receptors. (A) Importin β (1–876) is bound to the IBB domain of importin α. The A-helices of HEAT repeats are located outside the protein, whereas B-helices are arrayed inside. The IBB domain is shown as a rod intimately bound to B-helices of HEAT repeats 7–19. (B) Modular organization of TAP. The model for full-length TAP was created using the structure of TAP fragments reported in [31,34].

range of signals that can be transported by importin β. Furthermore, the definition of receptor and adaptor is not always strict. The transport receptor RanBP7 (Importin β-7), which is involved in nuclear import of ribosomal proteins, also can act as an adaptor for importin β in the import pathway for histone H1 [1].

EXPORT RECEPTORS

A number of export receptors of the importin β superfamily have been described in higher eukaryotes. Although some of these proteins have been extensively characterized by functional and biochemical approaches, no three-dimensional structural information on these proteins has yet been obtained. Export receptors of the importin β superfamily have been described for various cargoes [1–5,26]: tRNAs, dsRNA binding protein, importin α and various proteins bearing leucine-rich NES.

Whereas RanGTP triggers the dissociation of cargo from import receptors of the importin β superfamily, RanGTP has the opposite effect on export receptors of this family, because the binding of RanGTP cooperatively enhances the affinity of the export receptor for the cargo [1–5]. It is possible that export receptors may fully expose the cargo-binding site after binding to RanGTP. Unloading of the cargo in the cytoplasm is mediated by the combined action of RanBP1/RanBP2 (see later discussion) and RanGAP1, which in turn trigger hydrolysis of GTP, disassembly of Ran from the export complex, and subsequent release of the cargo.

Crm1 is the export receptor for cargoes with leucine-rich NESs. Crm1 was originally identified as a protein essential for maintaining chromosome structure in the fission yeast *Schizosaccharomyces pombe*. The interaction between Crm1 and at least some cargoes is stabilized by Ran-binding protein 3 (RanBP3), which binds directly to Crm1 and enhances

the affinity for both RanGTP and cargo [27]. Therefore, in some cases the actual export complex moving through the NPC out of the nucleus into the cytoplasm is a quaternary complex containing Crm1, RanBP3, RanGTP, and cargo. Crm1 is also the specific export receptor of snurportin [1], an import adaptor involved in import of m3G-capped U snRNPs. In this case, the interaction with the cargo is not mediated by a short peptide like a leucine-rich NES, but rather by a large domain of the cargo. Moreover, Crm1 binds snurportin with approximately 50-fold higher affinity than the Rev protein and a 5000-fold stronger affinity than the minimum Rev-NES.

Interestingly, the compound leptomycin B (LMB) can block both *in vivo* and *in vitro* Crm1-dependent nuclear export of NES cargoes. The inhibition is caused by the selective alkylation of cysteine 529 [28] of Crm1 by the α, β-unsaturated δ-lactone group of LMB.

Transport Receptors Distinct from the Importin β Superfamily

TAP is part of a larger family of conserved proteins (known as the NXF family) unrelated to importin β [29], which exhibits the two essential properties of an mRNA export receptor: binding to mRNA and to nucleoporins. In the case of human TAP it has been shown that it acts as an export receptor of the *cis*-acting constitutive transport element (CTE) of simian type D retrovirus mRNA and as general mRNA export receptor [30]. Whereas TAP directly binds to the CTE, the binding of TAP to cellular mRNAs is promoted by other unidentified cellular proteins.

Human TAP has a modular structure and consists of at least four structural domains (Fig. 4B) [27]. The minimal CTE-binding fragment has been mapped in the N-terminal domain of the export factor and consists of a ribonucleoprotein fold-like domain (known as RNP or RBD) and a leucine-rich repeat domain (LRR) [31], similar to those originally observed in the ribonuclease inhibitor structure [32]. Both the RNP and LRR domains are required for efficient binding to the CTE element of simian type D retroviral mRNA. In contrast, the mRNA export activity of TAP is directed by the LRR domain and additional cellular proteins such as p15 (NXT1), a transport factor that shares significant homology with NTF2 [33].

A recent crystal structure of the C-terminal half of TAP in complex with the p15 (Fig. 4B) [34] has revealed that the C-terminal domain of TAP contains an NTF-2 like domain, which heterodimerizes with p15, to closely resemble the NTF2 homodimer [35]. NTF2 is a small transport factor involved in the nuclear import of Ran and known to move through the NPC by interaction with nucleoporins. The NTF2-like domain of TAP, but not that of p15, interacts specifically with Phe-Gly (FG)-rich nucleoporins [36], providing a scaffold for the interaction with the NPC components and providing an explanation for the translocation-promoting properties of TAP. In contrast to receptors of the importin β superfamily, TAP does not seem to bind Ran directly in either nucleotide state. However, the TAP-interacting protein p15 can associate with the GTP-bound state of Ran, and this may influence export complex disassembly.

The Small GTPase Ran

Ran Structure

Ran is a small GTPase (~25 kDa) of the Ras superfamily that plays a key role in nucleocytoplasmic transport [1–5,7,8]. Ran contains the characteristic G-domain fold of other GTPases (Fig. 5A), consisting of a six-stranded β-sheet surrounded by five helices. The overall structure of Ran closely resembles other guanine nucleotide-binding proteins involved in several cellular processes such as Ras, Rho, Arf, and Sar1 [37].

Similar to other proteins of the Ras superfamily, Ran undergoes dramatic conformational changes upon hydrolysis of the nucleotide in two regions of the protein that are referred to as switch regions I and II (Fig. 5A). However, unique among all Ras superfamily GTPases, Ran also contains a long C-terminal extension, which consists of a linker without secondary structure followed by a 16-residue-long helix (also named switch III), situated opposite the switch I region. The end of this helix contains a highly acidic C-terminal DEDDDL motif (residues 211–216), whose charge is conserved in Ran for all species examined.

In the GDP-bound state, both switch regions I and II are in a more open conformation and extend far from the nucleotide, whereas the C terminus is packed as an α-helix against the G domain. In contrast, in the GTP-bound state, switch regions I and II close up to interact with the γ-phosphate of the GTP, and the carboxy-terminal tail is flipped away from the G domain (Fig. 5A). In this structural conformation, the binding of Ran to importin β superfamily receptors is about 10^4-fold higher than in the GDP-bound state [37].

Cellular Asymmetry of Ran

A fundamental feature of Ran as GTPase is the intrinsic low rate of GTP hydrolysis in the absence of effectors. As a direct consequence of this weak activity, the cellular distribution of Ran in the GTP- or GDP-bound state strongly reflects the cellular distribution of Ran effectors. The GTP-bound state of Ran occurs predominantly in the nucleoplasm of eukaryotic cells [38], where the guanine nucleotide exchange factor RCC1 is localized and largely bound to chromatin. Conversely, the GDP-bound form of Ran predominates in the cytoplasm, where RanGAP and Ran binding proteins (RanBP) 1 and 2, which stimulate the GTP hydrolysis activity of Ran, are localized. Of note is that the soluble pools of both RanGAP1 and RanBP1 are concentrated in the cytoplasm at steady state due to the presence of a strong NES in each protein. In higher eukaryotes, a major portion of RanGAP1 also is anchored to the cytoplasmic face of the

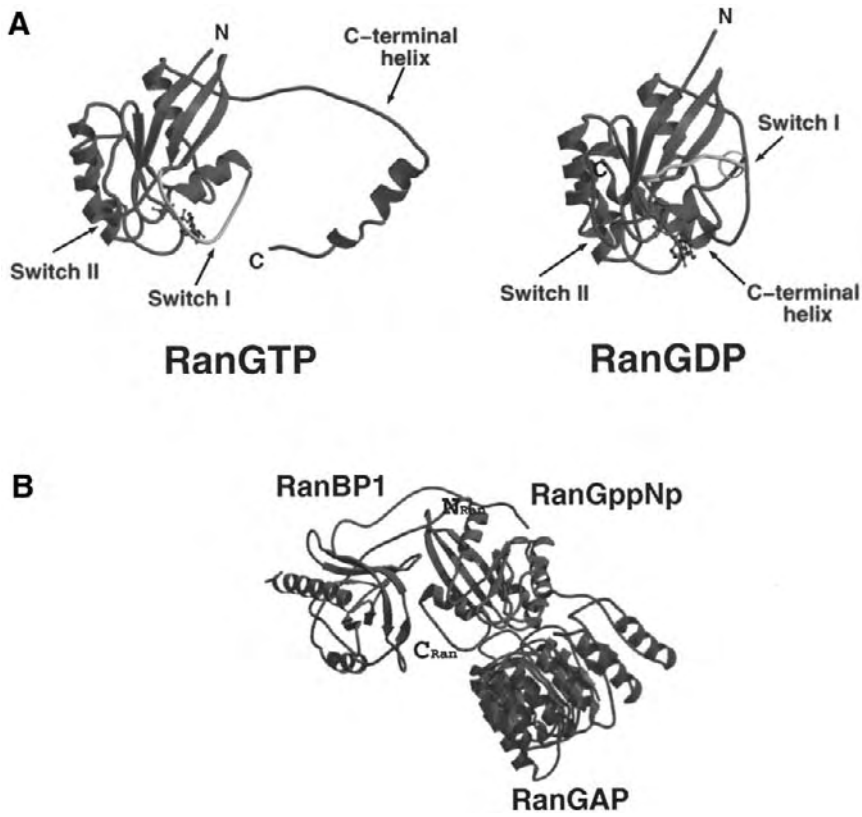


Figure 5 The small GTPase Ran. (A) Conformational changes taking place in Ran in going from a GTP to a GDP-bound state. Switch I and II are indicated by arrows. (B) Structure of RanGppNp complexed to RanBP1 and RanGAP1 (38). The C-terminal arm of Ran, also known as switch III, is flipped away from the G domain and embraces RanBP1.

NPC by the small ubiquitin-related modifier SUMO-1 [1–5]. Similarly, four RanBP1-like modules (termed RanBDs 1–4) are found in the gigantic nucleoporin RanBP2, which localizes at the cytoplasmic face of the NPC. The pool of RanGAP1 and RanBDs localized at the cytoplasmic face of the NPC presumably is involved in the disassembly of export complexes [1–5].

The detailed molecular mechanism of the GTPase stimulation of Ran by the combined action of RanGAP1 and RanBP1 has been revealed by the three-dimensional structure of a Ran–RanBP1–RanGAP ternary complex (Fig. 5B) [39]. RanBP1 does not participate directly in the RanGAP1-stimulated hydrolysis of the GTP by Ran. In fact, the carboxy terminus of Ran wraps around RanBD1 and contacts a basic patch on RanBD1 via its acidic C terminus. This molecular opening of RanGTP facilitates GTP hydrolysis by the GTPase-activating protein RanGAP. However, the molecular action of RanGAP1 is different from all of the GTPase-activating proteins (GAPs) previously characterized, which increases the rate of GTP hydrolysis by insertion of a so-called "arginine finger" into the phosphate-binding pocket. RanGAP1 stimulates the rate of Ran's GTP hydrolysis by stabilizing and positioning the catalytically essential residue glutamine-69 of Ran. In other words, RanGAP1 offers an overall structural scaffold to Ran, but does not intervene directly in the enzymatic mechanisms of catalysis.

Nuclear Import of Ran

In addition to the asymmetric distribution of RanGTP, which is more abundant in the nucleoplasm than in the cytoplasm, much of the total cellular pool of Ran is localized to the nucleoplasm. Similar to other several small proteins, Ran (~25 kDa) could, in principle, diffuse through the nuclear pore complex (see later discussion). Nevertheless, nuclear accumulation of Ran appears to be regulated by the soluble transport factor NTF2 (nuclear transport factor 2, also known as p10), which facilitates the diffusion of Ran by engaging in simultaneous interactions with nucleoporins and RanGDP. In the cell, NTF2 exists as a homodimer, where each monomer is formed by six β-sheets and two α-helices [35]. The tight structure of the NTF2 homodimer opens at one end to form a distinct hydrophobic cavity, forming a potential binding site for certain hydrophobic nucleoporins rich in the FG motif. RanGDP [40] binds the opposite sides of NTF2 homodimer structure, suggesting that NTF2 facilitates the movement of Ran through the pore simply by offering simultaneous binding to nucleoporins and Ran itself. The specificity of NTF2 for RanGDP is also confirmed by the crystal structure of the NTF2–RanGDP complex [40], where switch region II of Ran interacts largely with NTF2. The conformational changes of this region caused by the presence of GTP would sterically impair the binding to NTF2.

Nuclear Pore Complex

The NPC is a large proteinaceous structure spanning the nuclear envelope and providing a passageway for molecular traffic between the cytoplasm and the nucleoplasm [41–44]. The NPC ranges in size from ~125 MDa in *Xenopus* oocyte to ~60 MDa in *S. cerevisiae*. The architecture of the NPC has been extensively investigated by electron microscopy, which reveals a strong eightfold symmetry about the nuclear–cytoplasmic axis (Fig. 6). Three morphologically distinct regions of the vertebrate NPC can be distinguished: (1) the central ring-spoke assembly of 120×80 nm that is roughly symmetrical about the pore midplane and that surrounds a central transport channel, (2) flexible ~50-nm-long fibrils emanating into the cytoplasm from the cytoplasmic ring, and (3) a nuclear basket consisting of fibrils joined at their distal ends, which protrudes ~50–100 nm into the nucleoplasm from the nuclear ring. The central channel provides the primary site for restriction of diffusional movement through the NPC, and it is the region where transport receptors move through the NPC. Its exact composition is unknown, and often it appears to contain polymorphic material trapped in the act of being transported.

Based on its molecular mass and dimensions, the NPC originally was thought to contain as many as 100 different proteins (nucleoporins). However, in yeast [45] and mammalian cells [46] recent proteomic analysis has shown that the NPC consists of only about 30 different polypeptides, each of which is repeated multiple times.

Subcomplexes of nucleoporins that are moderately stable to chemical extraction have been biochemically defined including the Nup62 complex [47,48] and the Nup84 complex [49] in metazoans and yeast, respectively. The Nup62 complex contains four proteins, Nup62, Nup58, Nup54, and Nup45, forming a donut-shaped particle that presumably contains one copy of each of the four subunits. The Nup62 complex has been shown to localize on both sides of the NPC near the central channel and appears to be critically involved in nuclear import [50,51].

The existence of subcomplexes of nucleoporins suggests that NPC architecture may be assembled in a hierarchical way. Moreover, it appears that only two membrane-spanning nucleoporins in higher eukaryotes are involved in NPC anchoring to the nuclear membrane. Thus, most NPC organization appears to be based on extensive protein–protein interactions between subcomplexes. These probably remain partially assembled during mitosis in higher eukaryotes, when the NPC disassembles. The molecular mechanisms of NPC assembly and disassembly are not understood, but may be controlled by phosphorylation of nucleoporins, at least in part [41–44].

Several vertebrate nucleoporins are posttranslationally modified with O-linked *N*-acetylglucosamine and directly participate in nuclear protein import. All O-linked glycoproteins contain multiple repeats of the FG motif, which are often integral parts of larger motifs such as FxFG or GLGF. A large body of evidence suggests that these proteins function as binding sites for nuclear transport receptors [1–5,41–44]. FG-rich nucleoporins are also found in yeast and are localized on both cytoplasmic and nuclear faces of the pore complex. In virtue of the eightfold rotational symmetry of the pore and the repetition of FG motifs within the same nucleoporin,

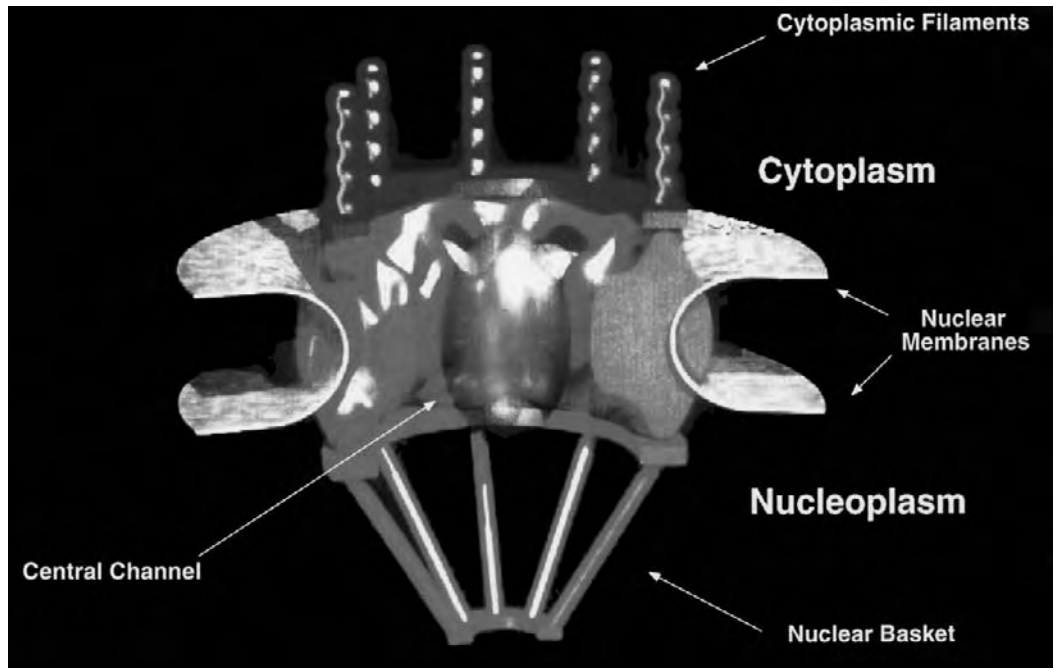


Figure 6 The architecture of the nuclear pore complex in 3D. The volume inside the pore is the location of the central channel, which represents the major diffusional restriction for passage of macromolecules. (Courtesy of Dr. U. Aebi.)

the effective FG-motif concentration in the NPC may be very high.

Mechanism of Transport

Passive and Facilitated Diffusion through the NPC

The NPC provides a selective barrier that restricts the passive diffusion of most proteins and simultaneously acts as an efficient conduit for receptor-mediated translocation of small and large cargoes. Passive diffusion through the NPC has been extensively investigated *in vivo* by cell injection of inert tracers, which suggests that the NPC has an ~10-nm-diameter diffusional channel [41–44]. Passive diffusion through the NPC does not occur at a significant rate for most molecules larger than 20–40 kDa, restricting these molecules to receptor-mediated transport. However, even cargoes that are smaller than 40 kDa often use transport receptors to accelerate their transport rate, which could promote vital processes like DNA replication and/or transcription. In addition to facilitating their rate of transport, transport receptors of the importin β superfamily can serve as chaperones to keep small basic proteins from aggregating [24].

The NPC can support an extraordinary rate of receptor-mediated transport. In an *in vitro* assay where the nuclear import of cargo-free transportin was studied in the absence of competing receptors, a maximum of ~700 transport events NPC^{-1} sec^{-1} was measured [52]. The movement of cargoes through the NPC can occur against a concentration gradient, which results in active accumulation of substrates in either cytoplasm of nucleoplasm. This transport of cargo up a concentration gradient is supported by GTP hydrolysis by Ran, which maintains the nuclear–cytoplasmic RanGTP gradient.

Interestingly, the requirement for GTP hydrolysis by Ran, which is a necessary feature of transport through the NPC *in vivo*, does not appear to be an obligatory step in NPC transit of certain cargoes *in vitro* under conditions in which receptor recycling is not required. Both Crm1-mediated export and importin β import of small protein cargoes [1–5] can proceed in the presence of nonhydrolyzable analogs of GTP. Furthermore, transportin-mediated protein import required neither Ran nor GTP [1–5]. These findings provided strong support for the model that transport receptors can move through the NPC by a facilitated diffusion mechanism, involved repeated binding and dissociation from a series of nucleoporins (see later discussion). However, in contrast to studies with the small cargoes, both Ran and GTP hydrolysis are required for efficient importin β and transportin-mediated import of certain large cargoes [53]. In this case, the presence of RanGTP could promote the facilitated diffusion process by increasing the off-rate of receptor–cargo complexes from nucleoporins [53]. In addition to size, factors such as hydrophobicity [54] and charge of the cargo also can play a role in dictating movement though the NPC. Moreover, different structural conformations of a specific transport receptor when bound to different cargoes may influence the affinity for nucleoporins, which in turn could affect the RanGTP requirement.

Interaction between Transport Receptors and FG-Rich Nucleoporins

Both yeast and higher eukaryotic nucleoporins contain multiple dispersed repeats (up to 30 or more) of the Phe-Gly motif, which provide binding sites for transport receptors of the importin β superfamily as well as for the structurally unrelated TAP. RanGTP is usually required to induce the dissociation of the receptor from nucleoporins during transport when the affinity of the former is substantial. However, even for receptors like transportin that have only a low affinity for nucleoporins, RanGTP is required for transport of large cargoes [55].

Two structural analyses of transport receptors bound to FG-rich peptides were recently reported. In one case, the structure of an N-terminal fragment of human importin β (1–442) was solved in complex with a yeast nucleoporin fragment of Nsp1p containing 5 FxFG repeats [55]. This structure reveals two major FxFG binding sites located on the outer convex surface of the importin β, between HEAT repeats 5–4 and 7–8. The interaction between the transport receptor importin β and the FG peptide is primarily hydrophobic and involves almost exclusively the two Phes of the FxFG core, which are buried in a hydrophobic pocket generated by the A-helices of HEAT repeats 5–6.

In a second case, the structure of a C-terminal NTF2-like domain of the export receptor TAP:p15 heterodimer [34] was solved in complex with a FG-bearing peptide derived from the nucleoporin Nup214. In this case, the interaction is driven by a single Phe residue of the FG peptide, buried inside a highly hydrophobic pocket of TAP. The binding pockets of importin β and TAP interacting with the FG motif are structurally different and arise from distinct classes of transport receptors. Nonetheless, the FG nucleoporin repeat assumes a similar conformation upon binding to the two receptors, mostly likely due to the structural flexibility of the glycine residue and the ability of the phenylalanine residue to insert into hydrophobic pockets. It is possible that FG binding sites on nucleoporins may act synergistically, thus strengthening the affinity of a specific FG-rich nucleoporin for a certain receptor. However, it does not appear that the number of FG repeats in a nucleoporin correlates directly to the affinity for a certain transport receptor, such as importin β. The critical parameter is likely to be in the accessibility of an FG sequence rather than in the number of FG repeats.

Models for Translocation

Cargo movement through the NPC (over a distance of ~200 nm) is thought to occur by the sequential binding and release of the receptor–cargo complex to a series of nucleoporins that extend across the NPC [1–5]. This model raises the problem of how directionality of the movement is achieved [56]. It is clear that one of the key determinants of transport

directionality is the compartmentalization of RanGTP and its effectors (see earlier discussion). RanGTP promotes the disassembly of import complexes when they reach the nucleus and also promotes the assembly of export complexes. Moreover, RanGAP and RanBP1/RanBP2 promote the disassembly of export complexes once they reach the cytoplasm.

An additional mechanism to promote transport directionality could involve a progressively higher affinity of a transport receptor for the nucleoporins along its transport pathway. This could promote vectorial transport by guiding the receptor to a terminal binding site in the pathway. To investigate this model, the affinity of the interaction between the transport receptor importin β and several bacterially expressed FG-containing nucleoporins has been analyzed by solid phase binding assays using recombinant nucleoporins [51]. The observation was made that the affinity of importin β for nucleoporins increases progressively in a cytoplasmic to nuclear direction, from a K_d of ~200 n*M* for a nucleoporin in the cytoplasmic fibrils (Nup358) to a K_d of ~10 n*M* for nucleoporin of the nuclear basket (Nup153). It is not known whether there is a similar affinity gradient for other importins, but there may be an affinity gradient in the reverse direction for Crm1 export complexes [57].

As discussed earlier, the lack of an energy requirement for the NPC transit of certain receptor–cargo complexes *in vitro* suggests that receptors can move through the NPC by facilitated diffusion. However this does not explain how the NPC selectively transports only receptor-bound macromolecules, while retaining a diffusion barrier for macromolecules lacking transport signals. To explain the movement of a receptor–cargo complex through the diffusionally restricted central channel of the NPC, Rout *et al.* [45] proposed a Brownian affinity gate translocation model, which suggests that the NPC is not physically gated, but is instead screened by a high concentration of flexible NPC filaments as a result of their Brownian motion. Transport receptors would be concentrated within this FG mesh in virtue of their affinity for FG repeats, in turn, strongly increasing the probability of entering and diffusing through the pore.

In contrast, Ribbeck and Görlich [52] have proposed that the NPC contains a central plug that constitutes a hydrophobic phase in which transport receptors can partition. This hydrophobic phase is proposed to be formed by an internal FG-rich nucleoporin meshwork that is held together by the mutual attraction between FG repeats. Accordingly, the network of cross-linked nucleoporins would give rise to a molecular sieve, allowing only the passage of molecules smaller than the mesh size, and restricting the movement of larger molecules. Transport receptors could "solubilize" the hydrophobic nucleoporin phase by engaging in interactions with FG repeats in nucleoporins, thereby allowing the partitioning of the receptor–cargo complex in the hydrophobic phase of the NPC and permitting translocation through the pore. This model assumes low-affinity interactions among nucleoporins and between nucleoporins and receptors and the physical existence of a hydrophobic nucleoporin phase in the central channel.

Future Directions

The mechanism of nucleocytoplasmic transport is a key problem in cell biology. Because many of the signals, soluble factors, and nucleoporins involved in nuclear transport have now been described, research is entering a new phase that is directed at obtaining a detailed mechanistic understanding of the transport process. To achieve this goal, we need to understand the three-dimensional structure of the NPC in much greater detail. This is technically very challenging, because the NPC has a massive and inherently very flexible architecture. It is likely that analysis of NPC substructures by a combination of X-ray crystallography and three-dimensional reconstruction based on electron microscopy will be needed to achieve this objective. It will be especially important to understand the organization of the central channel, because this is the major site of transport selectivity. In parallel, it will be important to develop new biophysical approaches and models for understanding the individual steps involved in transport of the receptor–cargo complexes between specific nucleoporins. Closely linked to the question of NPC architecture is the issue of how the NPC is assembled, both during interphase and at the end of mitosis. These questions can feasibly be addressed with a combination of genetic studies and *in vivo* and *in vitro* nuclear assembly analyses. Lastly, the nuclear transport machinery is clearly regulated in different functional states of the cells. Understanding how NPC regulation occurs in response to cell signaling and understanding how nuclear transport is integrated with other cellular pathways are major goals for the future.

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CHAPTER 331

Apostosis Signaling: A Means to an End

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Introduction

Programmed cell death is a fundamental biological process of all multicellular organisms and plays important roles in tissue homeostasis, host defense, development, metamorphosis, and morphogenesis. In animals, programmed cell death occurs via apoptosis, a morphologically defined form of cell death that has a number of biochemical features. In addition to its physiological roles, apoptosis contributes to several pathological conditions, such as cancer, AIDS, aging, and cardiovascular, neurodegenerative, and autoimmune diseases. The mechanisms that govern a cellular decision to live or die are complex and tightly regulated by a plethora of molecules with distinct roles in the signaling process. Generally, cell death occurs after an initial apoptotic signal spurs a cascade of subsequent events from which a cell cannot recover.

Apoptosis is defined by its morphological features of membrane blebbing, cellular shrinkage, and chromosomal condensation. Endonuclease activation that results in a characteristic 200-bp nucleosomal DNA ladder is a common feature of apoptosis, although not definitive [1]. Intense research has indicated that the cellular events leading to apoptosis are complex and varied, often depending on the cell type and stimulus utilized. However, several aspects of these death pathways are common among various stimuli and cell types.

The End of the Road

It is the cleavage of key cellular substrates and not a general proteolytic digestion that orchestrates the morphological and biochemical changes that characterize cell death by apoptosis. The degradative phase of apoptosis is mediated by a highly conserved family of cysteine proteases (caspases) that cleave specific proteins, including other caspases, at the C-terminal end of aspartic acid residues [2]. Figure 1 classifies caspases 1–10 and depicts several structural motifs. Most, if not all, caspases exist intracellularly as inactive zymogens. Following an apoptotic signal, the inactive zymogen is cleaved between what will be the large and small subunits of the mature enzyme to generate an active caspase [3,4]. Caspases-3, -6, and -7 execute the degradative events and are, therefore, classified as effector caspases [2]. The inactive proforms of these appear to preexist as dimers, which can only be activated by proteolytic cleavage to create a mature executioner caspase with two active sites [5]. The activation of an executioner caspase by proteolytic cleavage is illustrated in Fig. 2.

The active executioner caspases then cleave key substrates in the cell to promote apoptosis. For example, a complex of a nuclease and its chaperone/inhibitor is activated when caspase-3 cleaves the inhibitor of CAD (iCAD), releasing the nuclease CAD (caspase-activated DNase), to cleave DNA [6]. Activated caspases also promote the blebbing of a dying cell through the cleavage and activation of several molecules, including gelsolin, p21-activated kinase, and ROCK-1 [7,8]. Perhaps most importantly, caspases promote the exposure of phagocytosis markers on the surface of a dying cell, such as the externalization of phosphatidylserine [9], which binds to specific receptors on phagocytic cells. However, exactly how caspases induce phosphatidylserine externalization remains unknown. Active caspases are therefore crucial for this specialized form of cell death that ensures that the dying cells will be packed and

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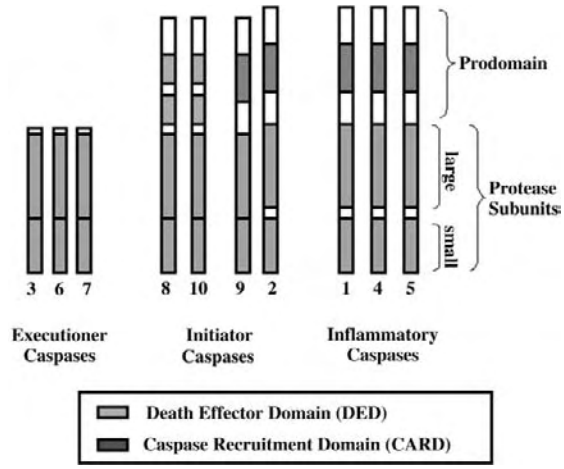


Figure 1 Caspase classification. Caspases 1–10 can be divided into three categories based on function and structural composition. Executioner caspases-3, -6, and -7 contain the large and small protease subunits and a small prodomain. In contrast, initiator caspases, such as caspases-8, -9, and -10, possess large prodomains consisting of protein–protein interaction motifs, including death effector domains (DEDs) and caspase recruitment domains (CARDs). Caspases-1, -4, and -5 also contain CARDs within large prodomains, but are termed inflammatory caspases for their role in inflammation. Caspases are cleaved after aspartic acid residues that are located between the prodomain and the protease subunits and the large and small protease subunits.

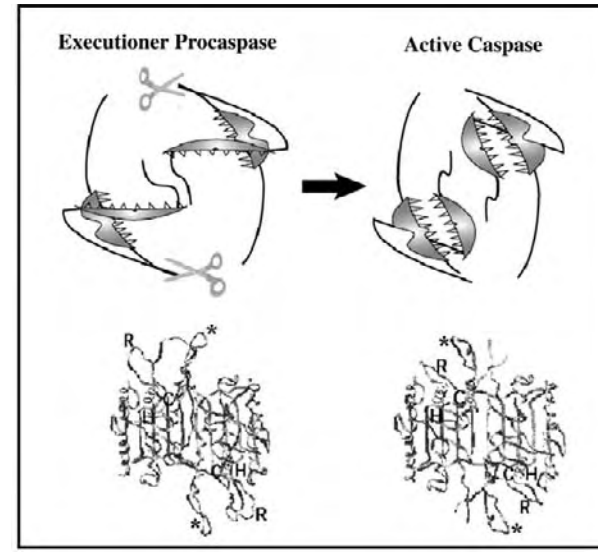


Figure 2 Activation of executioner caspases. Executioner caspases exist as inactive dimers that are activated following cleavage between the small and large subunits of each monomer. Although separated, the large and small subunits remain intimately associated and both contribute residues to the active site that are necessary for substrate binding and proteolysis. The drawing depicts where cleavage between the subunits occurs and the resultant conformational change. The crystal structure of an executioner caspase (caspase-7) shows the formation of the active sites (represented by asterisks) that occurs following cleavage. The amino acids critical for the active site (C, H) and for the aspartate specificity (R) of the protease are indicated.

marked for clearance by professional phagocytes, including macrophages and surrounding epithelial cells, to avoid the induction of an inflammatory response by released intracellular molecules.

Although inhibition of caspase activity prevents many phenotypic characteristics of apoptosis, such as DNA fragmentation, cell death may still ensue in the absence of caspase activation [10–12]. Caspase-independent cell death proceeds following the loss of mitochondrial function and may involve the release of alternative death-inducing molecules, suggesting that mitochondrial damage may signify a "point of no return" for dying cells [10–12].

A family of naturally occurring inhibitors of apoptosis proteins (IAPs) blocks the activity of caspases and may target active caspases for degradation, thereby averting apoptosis at critical commitment steps of the pathway [13]. IAP orthologs have been identified in several species, including yeast, nematodes, and flies. The mammalian family members c-IAP1 and 2 function to inhibit the activation of caspase-8, an initiator caspase responsible for the cleavage and activation of downstream effector caspases. X-linked IAP (XIAP) inhibits both effector caspase-3 and caspase-9, another initiator caspase (Fig. 3). Whereas certain family members appear to directly bind specific caspases (namely, caspases-3, -7, and -9), the mechanism by which IAPs inhibit caspase enzymatic activity has yet to be clarified. Some of the IAPs (such as yeast Bir-1 and possibly mammalian survivin) function in cell cycle regulation rather than by controlling caspases or apoptosis [14].

The initiator caspases bear long prodomains containing one or more protein interaction motifs (Fig. 1) that solicit binding by adaptor molecules and subsequent activation by dimerization [2]. The process through which initiator caspases are activated often characterizes the pathways that lead to apoptosis. There are two general types of initiator caspase activation that are best understood: one is activation by receptor ligation and another occurs following the release of mitochondrial intermembrane proteins into the cytosol. The initiator caspases that are predominantly involved in these two pathways are caspase-8 and -9, respectively.

Caspase-8 Activation via Death Receptors

The death receptors are a subset of the tumor necrosis factor receptor (TNFR) superfamily and include Fas (CD95), TNFR1, TRAIL receptors-1 and -2, and death receptor-3 (DR3). These relay extracellular apoptotic signals through binding of specific ligands to trimeric receptors [15]. Death domains (DDs) found in the cytoplasmic portion of the death receptor interact with DDs on adaptor molecules, namely, Fas-associated death domain (FADD) or TNFR-associated death domain (TRADD), through homotypic interactions (Fig. 3). FADD also associates with TRADD by DD interactions, providing a common scaffold for death receptor-induced caspase activation. Engagement of DDs on FADD exposes another domain termed the death effector domain (DED) that interacts with DEDs present in the prodomains

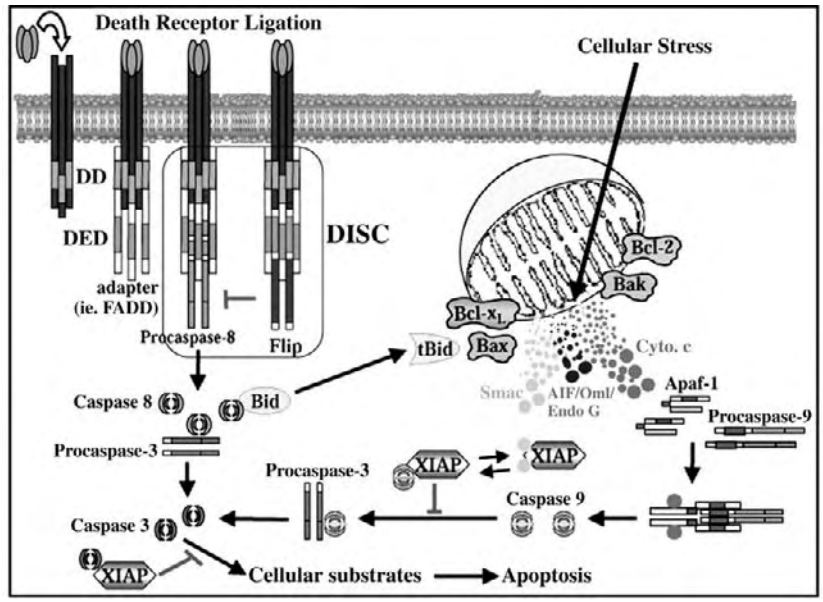


Figure 3 Apoptotic signaling from two distinct pathways. The death receptor (i.e., Fas) pathway involves ligand binding, recruitment of adaptor proteins (i.e., FADD) through death domain (DD) interactions, binding of procaspase-8 through death effector domain (DED) interactions, and proteolytic activation of the initiator caspase-8. c-Flip expression blocks caspase-8 activation by inhibiting binding with FADD at the DISC. Caspase-8 may directly activate executioner caspase-3 or may cleave Bid to target the mitochondria. The mitochondria are regulated by the pro-apoptotic (Bid, Bax, Bak) and anti-apoptotic (Bcl-2, Bcl-x_L) Bcl-2 family proteins, and disruption of the mitochondrial membrane results in the release of cytochrome c, Smac/Diablo, AIF, Omi/Htra2, and Endo G. The binding of cytochrome c to Apaf-1 and recruitment of procaspase-9 forms the apoptosome, which allows aggregation and activation of caspase-9. Caspase-9 then activates caspase-3 (and caspase-7) to cleave key cellular substrates and dismantle the dying cell. Proteolytic activity of caspase-9 or -3 may be inhibited by XIAP and cytosolic Smac reverses this inhibition upon binding XIAP.

of procaspases-8 and -10. Although distinct in sequence, the structures of the DD and DED are very similar, forming a so-called "death fold." The result is the formation of a DISC (death-inducing signaling complex), which leads to rapid aggregation and activation of caspase-8 or -10 [16]. When two procaspase-8 molecules are brought together, each cleaves after the aspartic acid residues in the other's chain, separating the large and small subunits, removing the prodomain, and forming a mature and fully active caspase with two active sites [17]. It is likely that caspase-10, a similar caspase, is activated in this way as well [18]. The mature initiator caspases can now cleave and activate the executioner caspases to promote apoptosis.

The regulation of the DISC is complex and not fully understood. A naturally occurring inhibitor of DISC formation, termed c-Flip (also known as Casper, Clarp, Flame-1, I-Flice, Cash, Ursurpin, and Mrit), impedes the binding of procaspase-8 to FADD, thereby preventing apoptosis induced by death receptors [19] (Fig. 3). Decreased Flip expression sensitizes cells to Fas and TNFα-induced apoptosis; however, the precise mechanism by which this occurs remains to be elucidated [20–22]. Evidence suggests that Flip is a caspase regulator that may also function to promote caspase activation by facilitating caspase aggregation under some circumstances [23]. It appears that the level of Flip expression, relative to other members of the DISC, may determine its role in apoptosis.

Cells that are resistant to apoptosis induced by the death ligands TNFα, Fas ligand, or TRAIL can often be sensitized by inhibiting macromolecular synthesis (e.g., by addition of actinomycin D or cycloheximide), indicating that an active pathway of protection exists. In the case of TNFR1, the receptor induces both an apoptotic signal through TRADD and a survival signal through the activation of the transcription factor nuclear factor κB (NFκB) [24]. Although the inhibition of NFκB often sensitizes cells to death by TNFα, exactly how NFκB activation blocks TNFα-induced apoptosis remains unclear. The ability of NFκB to block TNFR1 signaling may be in part through the expression of cIAP1 and cIAP2, which bind to TNFR1 adaptor molecules [25]. Moreover, NFκB has recently been implicated in the regulation of c-Jun N-terminal kinase (JNK) activity, which may play a role in TNFα-induced apoptosis [26,27]. Although NFκB can interfere with apoptosis in some cases, in others, such as Fas-induced apoptosis [28], it does not. There are also instances in which NFκB activation actually enhances apoptosis, indicating that the response to NFκB activation may be stimulus specific [29]. The balance between signals for survival versus death appears to be crucial for determining a cell's ultimate fate.

Cells can be loosely categorized by the apoptotic pathway employed following death receptor ligation [30] (Fig. 3). In short, ligation of a death receptor triggers apoptosis through activation of initiator caspases-8 or -10, which can cleave and activate executioner caspases that orchestrate the death of the cell. Alternatively, activated caspase-8 may cleave and activate the pro-apoptotic Bcl-2 family protein Bid [31,32], which induces mitochondrial membrane permeabilization and the release of apoptotic factors from mitochondria [33] (discussed later). Certain cell types, termed type 1 cells, generate effective DISC formation and rapid, abundant caspase-8 cleavage that can result in direct activation of caspase-3. In contrast, type 2 cells form small amounts of DISC with only slight caspase-8 activation, necessitating an amplification of the apoptotic signal through Bid cleavage and disruption of the mitochondrial outer membrane [30].

Mitochondria and the Activation of Caspase-9

The activation of the second class of initiator caspase, caspase-9, represents a fundamentally different pathway from that involving caspase-8. Procaspase-9 also has a large prodomain containing a protein interaction domain called a CARD (caspase recruitment domain) (Fig. 1) that structurally resembles DD and DED and also forms a death fold [34]. During its activation, the prodomain of caspase-9 is not removed. Unlike the other caspases discussed, cleavage between the large and small subunits is not necessary for activation of procaspase-9 [35]. Instead, procaspase-9 appears to be activated by binding to an adaptor, Apaf-1 (apoptotic protease activating factor-1) [36].

Inactive Apaf-1 is present as a monomer in the cytosol. Cytochrome c released from mitochondria binds the inert Apaf-1 and promotes Apaf-1 oligomerization and activation [37]. This is illustrated in Fig. 4. In its active configuration, Apaf-1 forms a complex of seven Apaf-1 molecules with exposed CARDs in the central "hub." The CARDs of Apaf-1 bind the CARDs within the caspase-9 prodomains, allowing for aggregation and activation of the protease, which must remain associated to be active [38]. This complex has been termed the *apoptosome* and its formation leads to caspase-9 multimerization and a conformational change that permits only one of the two active sites in the caspase-9 tetramer to be active at a time [39]. Evidently, the conformational change necessary for the formation of a specificity-determining groove pulls the other active site out of alignment (Fig. 4). It is proposed that the binding of Apaf-1 to the prodomain of caspase-9 allows this conformational change to occur. Once caspase-9 is activated, it cleaves and activates executioner caspase-3 to induce the degradative events of apoptosis.

The release of cytochrome c from mitochondria is a critical event for apoptosome formation [36]. Cellular stresses, such as DNA damage, cytoskeletal damage, and metabolic disruption, induce pro-apoptotic Bcl-2 family molecules (discussed later) to compromise the barrier functions of the mitochondrial outer membrane, ultimately leading to the dissipation of the inner transmembrane potential and release

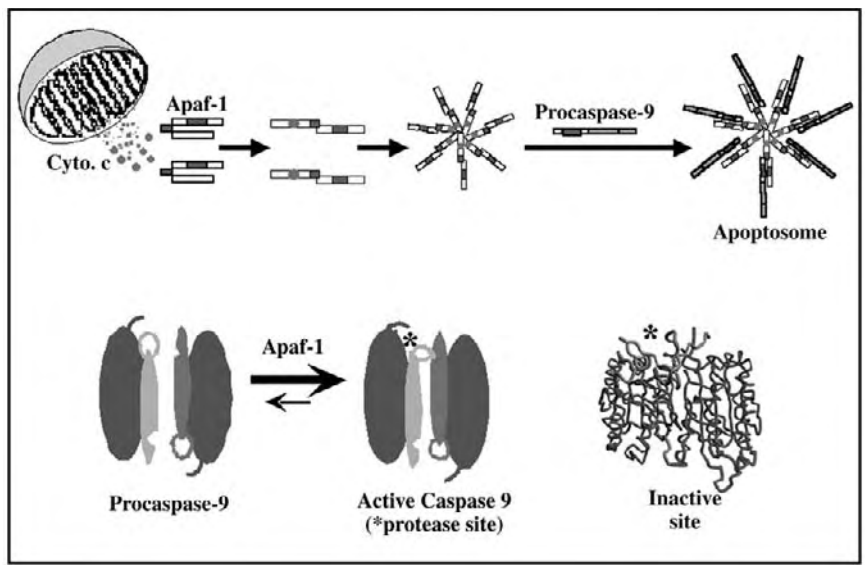


Figure 4 Apoptosome formation and activation of caspase-9. Cytochrome c (cyto. c) released from mitochondria binds to inactive Apaf-1 and induces a conformational change and oligomerization of seven Apaf-1 molecules. This assembly exposes the caspase recruitment domain (CARD) of Apaf-1 and allows interaction of Apaf-1 CARD with the CARD of procaspase-9. The resultant formation is termed an apoptosome. Within the apoptosome, bound procaspase-9 is cleaved between the large and small subunits, which remain associated and form a proteolytic active site. Multimerization of caspase-9 induces a conformational change that allows for only one proteolytic site to be active at a time (identified by the asterisk).

of cytochrome c [40]. Normally found in the intermembrane space of the mitochondria, cytochrome c must be released into the cytosol to induce apoptosome formation. Embryonic cells lacking cytochrome c did not undergo apoptosis in response to several inducers, demonstrating the importance for cytochrome c in the mitochondrial death pathway [41]. Cells from mice deficient in either Apaf-1 or caspase-9 display similar phenotypes (resistance to a variety of apoptotic stimuli) to cells lacking cytochrome c, suggesting that these three molecules serve a cooperative function in this pathway of apoptosis [42,43].

In addition to the release of cytochrome c, other mitochondrial proteins, including, Smac (second mitochondria-derived activator of caspase)/Diablo (direct IAP binding protein with low pI), Endo G (endonuclease G), Omi/Htra2, and AIF (apoptosis-inducing factor), are expelled into the cytosol following mitochondrial membrane disruption and potentially play a role in apoptosis. Because the function of cytochrome c in apoptosis appears to be fundamentally distinct from its role in electron transport, it is possible that several of the basic mitochondrial functions necessary for cell survival may also signal apoptosis when mitochondria are disrupted. Thus, various molecules associated with mitochondria may serve dual roles to maintain cellular function as well as to induce death, depending on cellular conditions. For instance, AIF, which possesses redox activity in the mitochondria, translocates to the nucleus and may promote DNA damage and cell death via a caspase-independent mechanism [44,45]. Endo G, an endonuclease potentially involved in mitochondrial replication, is released from mitochondria and has been suggested to mediate nuclear DNA damage in the absence of caspases [46]. Similarly, Omi/HtrA2, a mitochondrial serine protease, may promote caspase-independent cell death via its protease activity (distinct from that of caspases) when released into the cytosol [47]. These proteins, along with cytochrome c, are conserved from mammals to yeast and apparently retain a potential to kill cells independent of their role in mitochondria function.

While some of the death-inducing factors released from the mitochondria contribute to caspase-independent cell death, others promote caspase activation to enhance apoptosis. Both Smac/Diablo [48,49] and Omi/HtrA2 [47,50–52] can block IAP-mediated caspase inhibition by binding XIAP via an AVP(I/S) sequence at the N terminus and disrupting the association of caspase-9 with XIAP (Fig. 3). Thus, these mitochondrial proteins function in the cytosol to negate IAPs' anti-apoptotic function. Moreover, Smac/Diablo may serve an additional pro-apoptotic role that is independent from its ability to bind IAPs [53].

Mitochondrial Outer Membrane Permeabilization

The coincidental release of proteins from the mitochondrial intermembrane space during apoptosis suggests the occurrence of a mitochondrial outer membrane permeabilization. Although the onset of cytochrome c release may vary depending on the initial apoptotic stimulus utilized, once release occurs, it is rapid and complete [54]. In most instances, the molecules discussed earlier (e.g., Smac/Diablo, AIF) are also expelled into the cytosol with cytochrome c; however, the mechanism for this event remains unclear and it is possible that selective release can occur.

Essentially three models (reviewed in [55]) account for outer membrane permeabilization during apoptosis: (1) the pore-forming model in which permeabilization is mediated by a change in the outer membrane, permitting protein release without involving the inner membrane [33]; (2) the permeability transition model, in which an opening of the adenine nucleotide transporter (ANT) in the inner membrane causes matrix swelling, leading to outer membrane disruption [56]; and (3) the voltage-dependent anion channel (VDAC) closure model, in which metabolic signals trigger a closure of VDAC in the outer membrane, resulting in inner membrane perturbations that cause matrix swelling and outer membrane disruption [57]. Models 2 and 3 propose an active role for mitochondria in apoptosis, whereas model 1 defines this organelle as a repository of apoptogenic factors that are released by the actions of mediators on the outer membrane (discussed later). However, the function of the electron transport chain, which is essential for most mitochondrial functions including ATP generation, is lost following permeabilization, and this loss is greatly facilitated by caspase activation [54,58]. Mitochondrial dysfunction may have a fundamental role in apoptotic cell death, but the extent to which the loss of mitochondrial function versus the activity of death-promoting proteins accounts for the death of cells following mitochondrial outer membrane permeabilization is currently unresolved.

The Bcl-2 Family

Although the precise nature of mitochondrial membrane permeabilization remains elusive, some of the factors controlling it are beginning to be understood. A group of related molecules known as the Bcl-2 family proteins can target the mitochondria and regulate membrane permeabilization [59]. Bcl-2-related proteins are categorized by their ability to either induce or inhibit apoptosis. Apoptotic inducers (Bax, Bad, Bak, Bik, Bok, Bim, Bip, Bid, Diva, Hrk, or Blk) and protectors (Bcl-x_L, Mcl-1, A1, and Bcl-w) that share homologous regions (BH domains) with anti-apoptotic Bcl-2 have been identified (Fig. 5) [60]. Anti-apoptotic members Bcl-2 and Bcl-x_L prevent mitochondrial outer membrane permeabilization, whereas pro-apoptotic members Bax and Bak produce it [59]. Thus, the balance of anti-apoptotic and pro-apoptotic Bcl-2 family members may be responsible for maintaining membrane integrity.

The pro-apoptotic Bcl-2 family members have been further divided into a subset termed *BH3-only* proteins (Bid, Bim, Bik, Blk, Bmf, Bad, Hrk, BNIP3, Puma, Noxa) that contain only the BH3 of the four BH domains and may function as "sensors" to assess the status of death or survival signals.

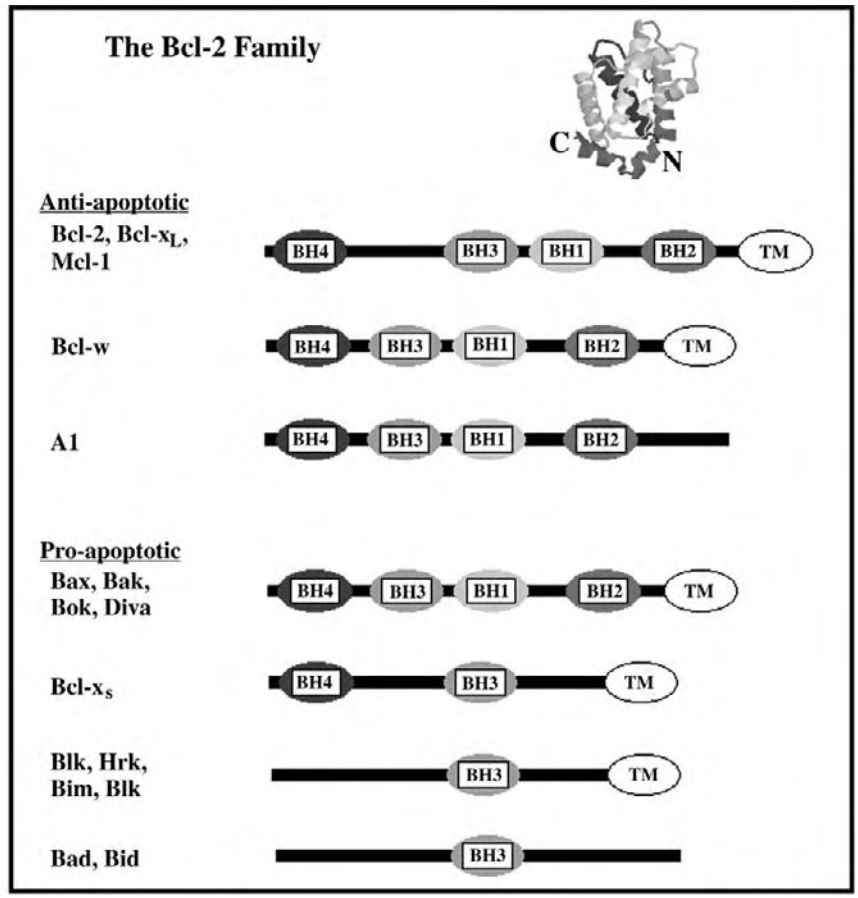


Figure 5 The Bcl-2 family. The proteins belonging to the Bcl-2 family are related based on the presence of Bcl-2 homology (BH) domains, but are categorized by function. The family members are divided based on their ability to inhibit (anti-apoptotic) or induce (pro-apoptotic) apoptosis. The Bcl-2 family is further sorted by the presence or absence of the four BH domains. For instance, the BH3-only proteins are a subdivision of the pro-apoptotic molecules that contain only the BH3 domain. Several of the family members also contain a hydrophobic transmembrane (TM) domain that allows for membrane insertion and localization to the mitochondria. The presence of common BH domains allows for oligomerization of family members, including binding between pro- and anti-apoptotic proteins, which may determine the effect on mitochondrial integrity.

For example, Bid "senses" protease activation and becomes activated itself through proteolytic cleavage by caspases [61], the cytotoxic lymphocyte protease granzyme B [62], and lysosomal proteases [63]. Bim [64] and Bmf [65] appear to "sense" cytoskeleton status, and Bad "senses" the status of growth factor receptor signaling. Noxa [66] and Puma [67,68] "sense" the activation of the pro-apoptotic transcription factor p53 through a p53-dependent increase in their expression. Although they may not directly induce mitochondrial outer membrane effects, the BH3-only proteins interact with other pro-apoptotic Bcl-2 family members, namely, Bax and Bak, to induce mitochondrial membrane permeabilization [69]. Bax and Bak have three of the four BH domains and are called *multidomain* or *BH-123* proteins. In this scenario, the BH3-only molecules sense a particular cellular apoptotic signal and direct Bax and Bak to disrupt the mitochondria. Isolated mitochondria from cells lacking both Bax and Bak (but not single knock-outs) are strikingly resistant to the induction of mitochondrial permeabilization and this pathway of apoptosis [70], suggesting that these two proteins are intimately, perhaps directly, involved in the mechanism of membrane permeabilization.

The ability of Bax and Bak to induce permeabilization may depend on oligomerization. Bax and Bak, which are highly homologous, exist as inactive monomers that can be induced to oligomerize by BH3-only proteins [71,72]. Oligomerization correlates with induction of membrane permeabilization; however, the precise role of oligomerization has not been directly demonstrated. It is possible that oligomerized pro-apoptotic Bcl-2 proteins form a pore or alter the lipids of the mitochondrial outer membrane to induce permeabilization. In support of this idea, Bcl-2, Bcl-x_L, Bax, and Bid contain similar structures that resemble the pore-forming chain of some bacterial toxins and have been shown to have weak channel-forming activity for small ions through lipid membranes [55]. It has yet to be determined if these molecules form pores on their own or through interactions with other proteins in the outer membrane, such as VDAC [55,73].

Furthermore, the anti-apoptotic Bcl-2 proteins may act by inhibiting oligomerization and possible pore formation by the pro-apoptotic Bcl-2 proteins [74]. However, whether or not Bcl-2 family members directly regulate apoptosis in this manner remains controversial.

Cell Cycle versus Apoptosis

To maintain homeostasis, cell death and proliferation must be precisely balanced, and communication between these two distinct signaling pathways is critical. Several proteins that engage the cell cycle, including c-Myc and E2F1, sensitize cells to apoptosis by increasing susceptibility to mitochondrial membrane permeabilization. In response to apoptotic stimuli, such as growth factor withdrawal or DNA damaging agents, c-Myc activation stimulates a caspase-independent release of cytochrome c from mitochondria [75]. Furthermore, activation of c-Myc may provoke Fas-mediated apoptosis, possibly through a c-Myc-dependent up-regulation of Fas ligand [76,77] and/or sensitization to death receptor signaling [78].

Anti-apoptotic Bcl-2 family members may function not only to protect mitochondria from membrane permeabilization, but also to repress cell cycle entry. The ability of Bcl-2 to prevent or delay entry into G_1 appears to be independent of its role in maintaining mitochondria integrity, as point mutations in Bcl-2 have been described that eliminate the cell cycle-inhibitory activity without affecting the anti-apoptotic activity [79]. Therefore, while entry into the cell cycle does not directly induce apoptosis, these two processes are linked and a variety of proteins have coevolved to play distinct roles in each pathway. The connection between induction of the cell cycle and cell death, termed *antagonistic pleiotropy* [80], is a fundamental mechanism to prevent cancer and may be "hard wired" in animals in which cell proliferation is required for tissue homeostasis. Nevertheless, this does not mean that entry into the cell cycle is a requirement for apoptosis, nor that blockade of the cell cycle will necessarily promote (or inhibit) cell death via apoptosis. These are distinct but evolutionarily linked cellular processes.

Conclusions

When properly regulated, apoptosis is essential for maintaining homeostasis of several cellular systems, including development, tissue turnover, immune regulation, and control of oncogenesis. However, inappropriate cell death influences several disease states, because too much (i.e., Alzheimer's disease) or too little (i.e., cancer, autoimmunity) apoptosis creates an adverse imbalance. Considering the vast number of molecules involved in orchestrating an apoptotic response, defining the precise mechanisms and targets that may contribute to apoptosis in a specific setting is proving to be extremely challenging. In addition, many apoptotic signaling pathways are stimulus and cell-type specific, further complicating the process. Novel proteins and pathways are being discovered at a fervent pace and although tremendous gains have been made in elucidating the mechanisms of apoptosis, this is a highly complex process that remains only partially understood.

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CHAPTER 332

Signaling Down the Endocytic Pathway

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Introduction

Intercellular signaling has long been recognized to be initiated at the cell surface, and receptor clearance from the plasma membrane by internalization mechanisms has correspondingly been associated with termination of signaling. Recognition of the existence of multiple pathways for internalization as well as the complexity of intracellular itineraries that ligands and receptors can utilize clearly indicates that cellular signaling has not only temporal but also spatial properties. As ligand–receptor complexes proceed down the endocytic pathway, they encounter unique components and environments at both the cytoplasmic and lumenal interfaces. Relevant "geographical" effects are only recently beginning to come into focus, as potential intracellular sites of action are revealed. Several reviews of the interface between signaling and endocytosis that the reader may wish to consult have appeared [1–3]; the focus here is on aspects of signaling following internalization of receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs).

RTK Signaling from the Cell Surface

Receptor tyrosine kinases have been investigated for more than 25 years. Ligand binding to epidermal growth factor receptors (EGFR), a particularly well-studied system, induces dimerization and autophosphorylation, followed by recruitment of multiple adaptor proteins and activation of several signaling systems at the cell surface including Ras, Jak/Stat, and PLCγ pathways (reviewed in [1,4,5]). Sustained occupancy of cell surface EGFRs, achieved either through continuous supply of external ligand or through inhibition of removal of surface ligand–receptor complexes, is known to be required for mitogenesis. Thus, while the wild-type form of the EGF receptor (EGFR) is rapidly internalized and is not oncogenic, a mutant form that is not endocytosed exhibits a more pronounced transformation phenotype in the same setting [6]. Another RTK, the insulin receptor, exhibits a corollary phenomenon. When insulin receptor endocytosis is blocked, certain activities initiated at the cell surface such as glucose and amino acid transport are increased [7].

RTK Signaling from Endocytic Compartments

Although the observations just mentioned establish that signaling occurs on the cell surface, they do not exclude the possibility of signaling from internal compartments, and the two possibilities are not mutually exclusive. Indeed, there have been substantive indications that signaling from internal compartments does occur. One line of investigation has revealed that signaling is attenuated or altered when internalization is blocked by inhibitors of endocytosis. For example, upon expression of a mutant form of dynamin that blocks endocytosis, EGF binding induces Shc recruitment to the stimulated receptor but MAP kinase activation is blocked [8]. Indications of the potential complexity of interpreting these experimental designs are the observations that Ras, Raf, and MEK1 are actually phosphorylated under these conditions, suggesting that the block in MAP kinase activation may actually result from a failure to internalize MEK1 [9], and that high-affinity EGFRs disappear from the cell surface [10]. Yet an additional

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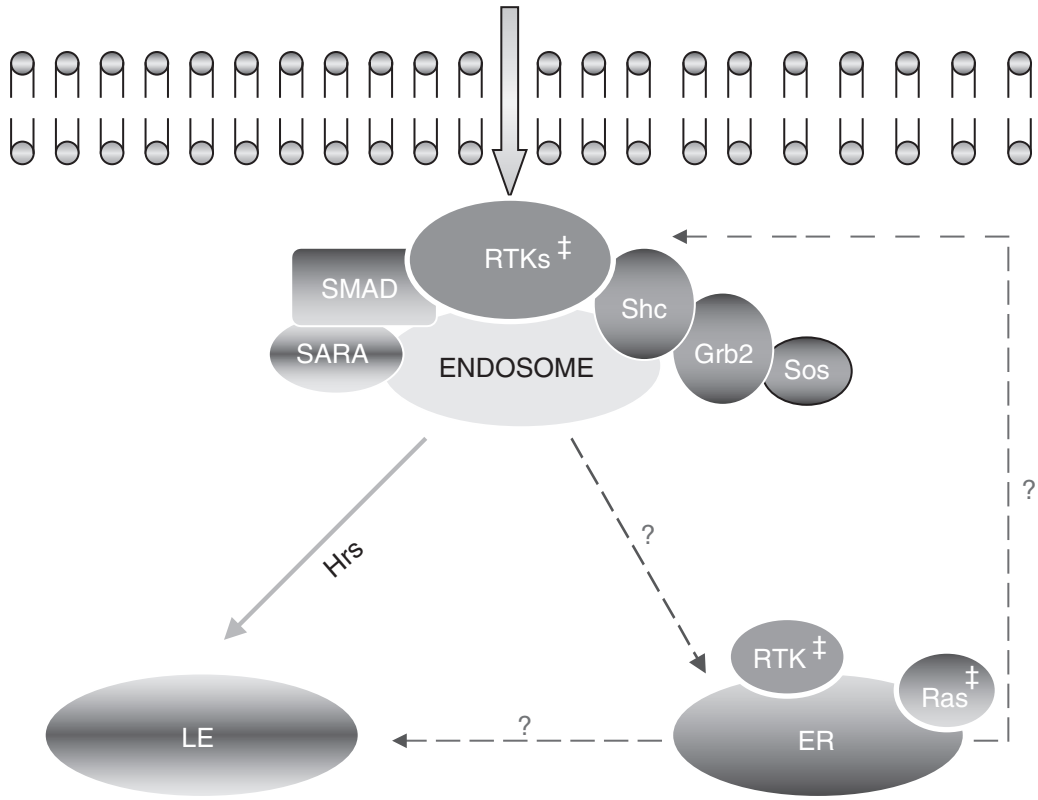


Figure 1 Receptor tyrosine kinase signaling pathway components in internal compartments.

Activated (phosphorylated) receptor tyrosine kinases (RTKs‡) and other functional signaling molecules, some of which are shown here, can be detected in endosomal compartments. Degradation of internalized receptors occurs in multivesicular bodies of the late endosomal (LE) system in which the RTK substrate Hrs plays a major role. There is also evidence for the presence of activated receptors and signaling molecules such as Ras on the endoplasmic reticulum (ER) though their precise origin, function and fate remains uncertain. See text for details.

wrinkle is suggested by the observation that agonist-dependent trafficking of Ras and Raf1 may be required for activation of the MAP kinase cascade [11]. Finally, inhibition of endocytosis has also been reported to block delivery of the RTK (EGFR/PDGFR) activated transcription factor Stat3 to the nucleus [12]. Colocalization of Stat3 with AP-2 decorated vesicles (usually restricted to the plasma membrane) reported in this study is surprising, but the possibility that transcription factors use the endocytic pathway for inward vectorial transport is intriguing. A related observation is the report that EGFR itself can function as a coactivator or transcription factor; this is of particular significance because the receptor is frequently found in the nucleus in various cell lines and tissues [13].

Another line of study that has implicated signaling from cytoplasmic compartments following RTK activation is the observation that activated EGFRs are present on internal compartments [14]. Furthermore, biochemical preparations derived predominantly from endosome-enriched fractions had markedly different phosphotyrosine patterns than those derived from plasma membrane preparations [15]. This has subsequently been confirmed and extended by morphological studies at both the light and ultrastructural levels that visualize specific localization of phosphospecific antibodies [16,17]. Signaling from internal compartments might also be expected to require the presence of a known cast of adaptor molecules (Fig. 1). Indeed, endosome preparations from stimulated cells have been shown to contain Grb2, SOS, and Shc in association with activated EGFR [18] and more recent reports have identified other signaling molecules including Src [19], MEK, and Raf [20].

Though the interpretation of these kinds of biochemical experiments is necessarily dependent on the purity of the endosomal preparations—no minor hurdle given the pleomorphic nature of the endosomal compartment—they have found support in two additional lines of study. There is evidence that the internal pool of EGFR is intrinsically competent for signal initiation because activation of MAP kinase [21], PLCγ [22], and Ras [23] could be demonstrated in broken cell lysates. Elegant fluorescence resonance energy transfer (FRET) experiments utilizing tagged Ras and Ras binding domain proteins provide evidence for pools of activated Ras on the endoplasmic reticulum and Golgi in response to serum activation [24]; while endocytosis of activated receptors did not seem to be required in this system, its role in activating endogenous levels of Ras remains a possibility. And in an exogenous expression approach in live cells, Jiang and Sorkin [25] have shown that Grb2, Shc, and Ras can be visualized in pairwise combination accumulating on endosomes following EGF stimulation. Finally, Haj and colleagues [26]

have demonstrated that phosphorylated and endocytosed EGFR and PDGF receptors interact on the endoplasmic reticulum with the protein tyrosine phosphatase PTP1B. Collectively, these results are provocative in demonstrating that the machinery for implementing and terminating signaling is present on internal compartments.

Accessibility to appropriate internal substrates for signaling might be expected to be subject to tight regulation. For example, activated EGFRs can be shown to persist after internalization (consistent with the distinct phosphotyrosine patterns noted earlier), as do tyrosine-phosphorylated and activated forms of its downstream effector phospholipase Cγ; however phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis appears to occur only at the plasma membrane [22]. Using fluorescent reporters, evidence for phosphotyrosine production at internal sites could be visualized, but PIP_2 localization was restricted to the plasma membrane localization [27], suggesting that accessibility to appropriate downstream substrates for signal generation is tightly regulated in the intact cell.

Signaling unique to internal compartments might also be expected to involve additional specialized components not present on the cell surface. Recent findings indicate that the protein Hrs (hepatocyte growth factor regulated tyrosine kinase substrate) fulfills this role [28]. Hrs is a tyrosine kinase substrate for several RTKs [29] that is phosphorylated only after endocytosis [30]. It contains a phosphatidylinositol 3-phosphate (PI3P) binding FYVE domain that contributes to its localization to early endosomes [29], and it is the mammalian homolog of the Class E yeast Vps27p, which regulates sorting of proteins to vacuole. Interestingly, an *hrs* null mutant in mice exhibited enlarged early endosomes [31]. More recent studies of *Drosophila* larvae expressing mutant Hrs reveal an impairment in the formation of inwardly budded vesicles in endosomes that are thought to give rise to multivesicular bodies (MVBs), missing in these cells [32]. Although cells expressing this mutant *hrs* possess the ability to internalize EGFR, they fail to degrade it or other RTKs. Consequently, the cells exhibit increased levels of activated EGFR, which are paralleled by increased levels of MAP kinase activation and an expansion of cells whose proliferation in the larvae is controlled by EGFR signaling. Thus Hrs appears to be a "housekeeping" component critical for EGFR degradation, and its absence provides evidence that functional signaling can emanate from internal compartments under physiological conditions. These results then provide compelling evidence that specific components and associated mechanisms are found in internal cellular compartments to regulate signaling, that these factors are under tight regulation, and that their inactivation results in aberrant signaling.

Although less extensively investigated than the EGFR, there is also recent support for critical steps in intracellular signaling following activation of receptors belonging to the transforming growth factor β (TGF-β) superfamily [33]. These homodimeric and heterodimeric receptor complexes phosphorylate a family of SMAD proteins, which in combination with a cell-specific complement of DNA binding proteins yields the range of signaling possibilities characteristic of this ubiquitous signaling system [34]. For some of these receptors, phosphorylation of the SMAD proteins is regulated by the SARA (SMAD anchor for receptor activation) protein. Interestingly, SARA contains a FYVE domain that binds tightly to PI3P [35], and has been localized to early endosomes [36]. Although still controversial [37], it seems likely that TGF-β receptors can encounter and activate SMAD proteins on endosomes as the receptors are known to undergo rapid endocytosis [38], and mutant SARA proteins lacking the FYVE domain have been reported to inhibit downstream signaling [36].

GPCR Signaling Paradigms and Desensitization

The superfamily of G-protein-coupled receptors mediates intracellular signaling to a diverse array of stimuli including light, odorants, neurotransmitters, lipids, and various peptides and proteins (reviewed in [39]). GPCR activation promotes interaction with heterotrimeric GTP binding proteins (G-proteins) resulting in GDP/GTP exchange and subsequent dissociation of the α-GTP and βγ subunits. These subunits function to regulate the activity of multiple effector proteins including adenylyl cyclases, phospholipases, phosphodiesterases, ion channels, and phosphatidylinositol 3-kinase (PI3K). Thus, GPCRs regulate numerous biological functions including sensory perception, neurotransmission, chemotaxis, development, cellular proliferation, differentiation, and survival.

Although GPCR activation of heterotrimeric G-proteins was initially identified some 30 years ago, more recent studies have revealed that GPCRs can also interact with a myriad of additional proteins (reviewed in [40,41]). These include various protein kinases and a number of adaptor and scaffolding proteins. Here we briefly discuss those interactions that have been implicated in GPCR signaling and endocytosis. Although GPCR activation initiates signaling via heterotrimeric G-proteins, many activated GPCRs also interact with two additional protein families, G-protein-coupled receptor kinases (GRKs) and arrestins (reviewed in [42,43]). GRKs specifically bind to activated GPCRs, leading to increased receptor phosphorylation and enhanced arrestin binding. Arrestins have been implicated in the process of receptor desensitization, that is, the reduced ability of a receptor to respond to hormones following prolonged stimulation. Indeed, GRKs and arrestins were first identified in rod cells and implicated in quenching phototransduction. Arrestins bind to activated-phosphorylated GPCRs and thereby attenuate further G-protein interaction and signaling. Although a role for arrestins in GPCR desensitization is well established, recent studies reveal that the two nonvisual mammalian arrestins (termed arrestin-2 or β-arrestin-1 and arrestin-3 or β-arrestin-2) also function in regulating various aspects of GPCR trafficking and signaling (reviewed in [44]). These functions of arrestins are discussed in more detail later.

Additional interactions that may contribute to GPCR signaling and provide a potential link with endocytosis are more receptor specific and have been most extensively characterized for the β-adrenergic receptors (βARs) (reviewed in [41]). For example, β_1AR interaction with endophilins enhances agonist-promoted internalization of the receptor [45], whereas interaction with PSD-95 attenuates receptor internalization and facilitates interaction with *N*-methyl-D-aspartate receptors [46]. Interestingly, GRK5 phosphorylation of the β_1AR dramatically reduces PSD-95 interaction, suggesting that this association is also regulated by the activation state of the receptor [46]. The β_2AR interacts with the PDZ domain-containing protein Na^+/H^+ exchanger regulatory factor (NHERF) via a C-terminal PDZ binding domain [47]. NHERF interaction with the β_2AR positively regulates Na^+/H^+ exchange but also appears to regulate the sorting of internalized β_2ARs [48]. Interestingly, interaction of the β_2AR and NHERF appears to be negatively regulated by GRK5 phosphorylation of the β_2AR, again providing a link with the activation state of the receptor [48]. *N*-Ethylmaleimide-sensitive factor (NSF) also interacts with the C-terminal region of the β_2AR and facilitates receptor internalization and recycling [49]. In addition, the scaffolding protein gravin (also termed AKAP250) interacts with the β_2AR and has been implicated in regulating receptor resensitization and internalization [50,51]. Thus, multiple proteins function to dynamically regulate GPCR signaling and trafficking.

Control of RTK and GPCR Trafficking Leading to Degradation

Although multiple mechanisms contribute to receptor trafficking, most GPCRs endocytose via clathrin-coated pits (Fig. 2). As discussed previously, GPCR activation often results in GRK-mediated phosphorylation, arrestin binding, and desensitization. Several years ago, two reports suggested a role for arrestins in GPCR trafficking [52,53]. Initial mechanistic insight into this process revealed that nonvisual arrestins specifically interact with the clathrin heavy chain via a LϕXϕE motif (where ϕ is a bulky aliphatic residue) found within a C-terminal insert unique to arrestin-2 and -3 [54]. The interaction of arrestin and clathrin appears to play a critical role in mediating GPCR trafficking [54]. Additional arrestin interactions that have been implicated in GPCR trafficking include phosphoinositide [55], the adaptor protein AP2 [56,57], NSF [58], and ADP-ribosylation factor 6 and its exchange factor ARNO [59]. While detailed mechanistic insight into how these various interactions are coordinated is lacking, it is evident that arrestins play an important role in mediating GPCR trafficking.

For many GPCRs, prolonged activation results in down-regulation, a process that involves increased receptor degradation and an overall reduction in receptor levels (reviewed in [60]). Although multiple mechanisms contribute to receptor down-regulation, a number of studies have suggested

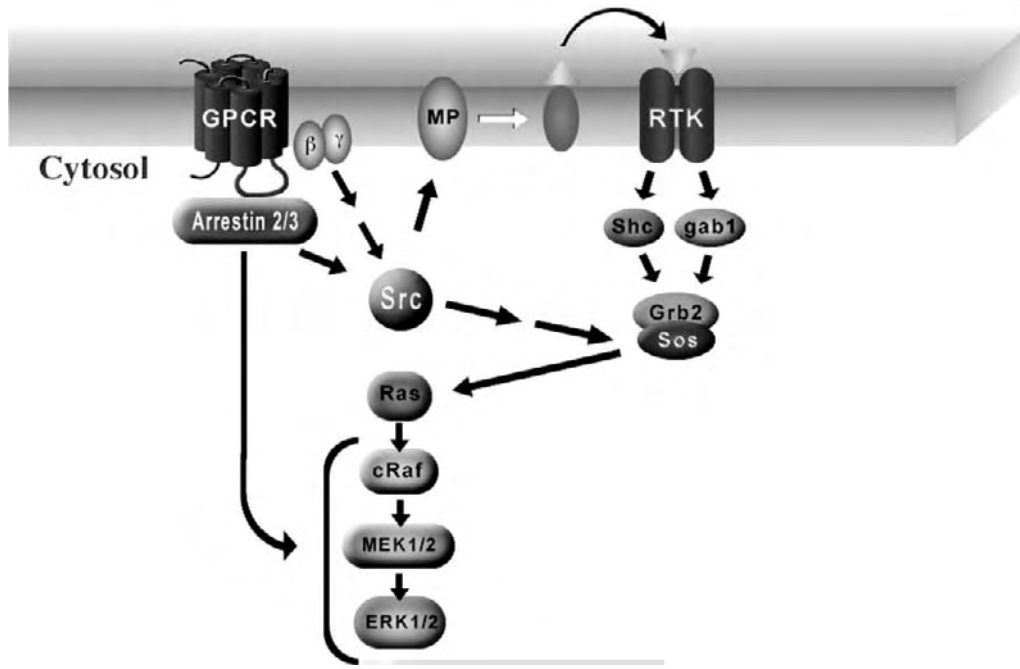


Figure 2 G protein-coupled receptor activation of MAP kinases.

Multiple pathways may provide a link between GPCR activation of MAP kinases and endocytic processes. These include the ability of arrestins to function as scaffolding proteins for various MAP kinases including Raf and ERK1/2 thereby providing a direct connection between GPCRs and MAP kinase localization and activation. Arrestins also function to link GPCRs with activation of Src and Src-like kinases providing a potential mechanism to connect endocytic processes, via the phosphorylation of dynamin, and stimulation of MAP kinases, via the activation of Ras guanine nucleotide exchange factors. GPCRs can also activate MAP kinases via their ability to transactivate receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor which in some cases involves the activation of metalloproteinases (MP) that result in the release of RTK ligands.

that GPCR endocytosis and subsequent sorting to lysosomes play an important role in this process [61–64]. Interestingly, two recent studies suggest that receptor ubiquitination contributes to the sorting and degradation of mammalian GPCRs [64,65]. Interesting correlates to these findings are the mating factor receptors in *Saccharomyces cerevisiae*. Previous studies have revealed that the mating factor receptors undergo agonist-dependent phosphorylation, ubiquitination, endocytosis, and degradation (reviewed in [66]). Although yeast do not contain arrestins, significant overlap may occur in the mechanisms used to sort and degrade mammalian and *S. cerevisiae* GPCRs.

Molecular mechanisms utilizing receptor ubiquitination have also recently been shown to play a role in termination of signaling and down-regulation of RTKs. In the case of the EGFR, recent results have shown that the E3 ubiquitin ligase c-Cbl can bind the receptor through its RING domain and induce receptor ubiquitination, thereby targeting the receptor for intracellular degradation [67,68]. A virally coded form of v-Cbl that lacks the RING domain does not down-regulate the receptor, resulting in increased signaling [69,70]. Furthermore, a role for c-Cbl in inducing rapid internalization as well as shunting internalized receptors for degradation has also recently been reported. Cbl was shown to associate with endophilin through CIN85 to induce rapid internalization of EGFR/c-met receptors; inhibition of this association blocked internalization and again enhanced biological signaling [71,72]. Collectively, these observations point to ubiquitination of the type I RTKs as critical in their down-regulation and, therefore, in overall decrease in their signal generation.

GPCR Activation of MAP Kinases

It is well established that many GPCRs can activate mitogen-activated protein kinases (MAPKs), including members of the extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38 families (reviewed in [73]). Although multiple mechanisms mediate GPCR activation of MAPKs, three pathways in particular have been extensively characterized: (1) transactivation of RTKs, (2) formation of focal adhesion complexes, and (3) direct scaffolding of signaling proteins. These pathways are reviewed in [74] so we primarily discuss them here from the perspective of the potential link between signaling and endocytosis.

RTK transactivation by GPCRs was initially identified by Daub and coworkers [75,76] who showed that Gq and Gi-coupled receptors can activate ERK via a pathway that is dependent on activated EGFR, SHC, and Src. These findings were extended by Luttrell and colleagues [77] who implicated a role for G-protein $\beta\gamma$ subunits in EGFR transactivation. The mechanism of RTK transactivation has not been completely elucidated although recent studies suggest that GPCR activation results in activation of a metalloproteinase that in turn leads to the release of heparin binding EGF (HB-EGF) [78]. HB-EGF can then act in either an autocrine or paracrine fashion to activate cell surface EGFRs. Thus, GPCR activation of MAPKs that occurs via RTK transactivation might be expected to show the same correlation with endocytosis as outlined earlier for RTKs [79].

The other major pathway that may provide a link between GPCR signaling and endocytosis involves the ability of GPCRs to function as scaffolding proteins. In several instances this may involve a direct interaction of a GPCR with a signaling molecule such as Src interaction with the β_3-adrenergic receptor [80]. However, arrestins also appear to interact directly with Src family members, thus providing a broader link between GPCRs, Src, and MAPKs since most GPCRs bind arrestins. Initial studies in this area identified formation of an agonist-promoted complex containing the β_2AR, arrestin-2, and c-Src [81]. Inhibition of complex formation by mutation of proline-rich regions in arrestin-2 resulted in a reduction in β_2AR activation of ERK1/2. Activation of the chemokine receptor CXCR1 results in formation of an arrestin-2 complex with the Src family kinases Hck and c-Fgr [82], whereas formation of an endothelin-1 receptor complex with arrestin-2 and Yes mediates endothelin promoted GLUT4 translocation [83]. Because Src has been implicated in dynamin phosphorylation and endocytosis [84], formation of GPCR/arrestin/Src complexes may provide an important link between signaling and endocytosis.

Several recent studies also suggest an important role for arrestins in linking GPCRs, MAPK activation, and endocytosis. As discussed previously, nonvisual arrestins bind to activated phosphorylated GPCRs and target the receptors for clathrin-coated pit mediated endocytosis. Surprisingly, arrestins also appear to function as direct scaffolding proteins for MAPKs. DeFea and coworkers [85] initially identified formation of a complex containing a GPCR (proteinase-activated receptor 2 or PAR2), an arrestin (arrestin-2), a MAPK kinase kinase (Raf-1), and a MAPK (ERK1/2). Similar studies identified an agonist-promoted complex containing the neurokinin-1 receptor, arrestin-2, Src, and ERK1/2 [86]. Luttrell and coworkers [87] also observed such complexes containing, for example, angiotensin II type 1a receptors, arrestin-3, Raf-1, the MAPK kinase MEK1, and ERK2 [87]. In addition, McDonald and colleagues [88] also found that arrestin-3 can form a specific complex with the MAPK kinase kinase ASK1 and JNK3. Two interesting aspects of these studies are that formation of these complexes appears to be enhanced by receptor activation and that the resulting receptor/arrestin/MAPK complex appears to be endocytosed. Although a direct requirement for GPCR endocytosis in mediating MAPK activation remains controversial (reviewed in [74]), this may provide a mechanism for endocytosing and retaining an active signaling complex in the cytoplasm, thereby preventing nuclear translocation of the MAPK and potentially altering substrate specificity.

Endocytic Signaling in Developmental Systems

Vesicular trafficking and signaling on the "supracellular" level has also recently been recognized as playing a critical role in establishing and regulating morphogen gradients (reviewed in [89–91]). Morphogens have been defined as

secreted molecules that convey signaling information that affects cell fate determination in developing systems, often in a concentration-dependent manner. Originally considered to be spread from cellular sites of synthesis to target cells by simple diffusion, it is now recognized that internalization and intracellular trafficking pathways in the peripheral cells play critical roles in regulating local morphogen concentrations along developmental axes. For example, establishment and maintenance of concentration gradients of transcriptional activators such as decapentaplegic (Dpp), Wingless, and Hedgehog in the fly embryo are controlled by endocytosis and degradation of morphogen. Somewhat more remarkably, the extent of spread of some morphogens is actually amplified by internalization and transcytosis across target cells, apparently establishing longer range gradients than would be effectively established by diffusion alone. This then raises the possibility that the balance between recycling and degradation will control not only intracellular signaling but also the spatial range and vectoriality of cell fate determination, an area of active investigation [92].

Signaling between Neuronal Cell Body and Terminal

Neurons represent an extreme spatial case in which cellular signaling initiated at the terminal region may culminate in biological effects at the cell body, often millimeters to tens of centimeters away. Indeed neurotrophins such as nerve growth factor (NGF), which bind to TrkA receptors at the terminal region, elicit either cell survival or differentiation effects mediated by nuclear events. An intriguing "signaling endosome" hypothesis [93] has been put forward to explain this signal transmission event, invoking internalization of neurotrophin–Trk complexes, and their retrograde transport down the axon to the cell body. Considerable evidence supports the transport of activated receptors (reviewed in [94]). Vesicle-bound forms of activated phosphorylated TrkA receptors can be detected throughout the axon of stimulated neurons [95], and receptor internalization is required for neuronal differentiation though not for Akt-mediated survival [96]. Activated TrkA receptors, NGF, and components of the Ras-MAP kinase signaling pathway as well as clathrin and AP-2 are recovered in clathrin-coated vesicle fractions prepared from stimulated neurons, but not the early endosomal marker EEA1 [97]. This may suggest that in neurons signaling components have been recruited to coated membranes as well as endosomes or that neuronal signaling endosomes have unique characteristics. Dissection of the role of the retrograde transport of hormone–receptor complexes, as well as nonvesicular transport, which may also contribute to extremely rapid signal transmission [95,98], promises to be an exciting task.

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PART V

Cell–Cell and Cell–Matrix Interactions

E. Brad Thompson, Editor

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PART V

Introduction

E. Brad Thompson

In Part V of the Handbook we provide overviews that integrate the *cis*-signaling systems involved in the regulation of many major organs or tissue systems. For the most part, these chapters will not attempt to repeat the information concerning the pathway details that have already been covered in previous sections. Instead, the theme here is to provide overviews of the multiple signaling molecules and pathways that must be integrated at the tissue and organ level in order for cellular homeostasis to be maintained. This part begins with an overview of cell–cell and cell–matrix interactions that introduces readers unfamiliar with the concepts to the various types of ways in which signals are delivered. These include endocrine, paracrine, autocrine, and intracrine signal systems. These various ways in which signaling molecules are presented allow for control of the signals in a three-dimensional, geographic sense. The use of some signals are restricted locally to individual cells or nearby cells, while others are delivered from one set of cells to others at varying degrees of distance. Following this introductory discussion, individual tissues and systems are considered in the succeeding chapters.

General overviews of the various major signals to lung, bone, prostate, breast, gastrointestinal tract, B-lymphoid cells and T-lymphoid cells, pancreas, testis and ovary, and the hematopoietic system are presented. Two aspects of signaling in the nervous system are covered. Neurotrophic factors and how their responses are integrated are discussed in the chapter by Perez-Polo. A recently discovered aspect of nervous system signaling, namely, retrograde signaling by way of dorsal root reflexes, is dealt with in the chapter by Willis. Among the many signaling systems that play on the liver, the stress response and its variation in the young versus the aged animals is discussed in the chapter by Papaconstantinou. The cardiovascular system is represented by two chapters: one dealing with the signaling pathways controlling cardiogenesis, and the second with the regulation of angiogenesis in the postnatal state. The latter obviously has relevance not only to normal development, but also to malignancy, since every growing tissue must be supplied with blood in order to continue to grow.

In most of these discussions, the reader will find that the focus of recent research has been on the discovery of increasing numbers of signaling molecules and how they play upon each tissue or system. Much remains to be done to understand how, in any particular tissue, all the known signals create an integrated response. How the multiple signaling systems interact to achieve the overall homeostatic regulation of an organ has not yet been determined in most cases. Our state of knowledge is one in which we are uncovering the regulatory molecules and the pathways to which they individually act. In very recent years, we have begun to discover the interactions among those pathways, but exactly what the interplay of pathways is that results in the normal regulation of the cell or a tissue is not yet understood, in most cases. This next order of understanding will doubtless be the pursuit of the coming generation of scientists, but it must be based on knowledge of the entire repertoire of signaling molecules that are relevant to that tissue, the pathways through which they act and precise knowledge of how those pathways govern cell behavior molecularly, as well as precise knowledge of the interpathway interactions. A combination of cell biology, biochemistry, molecular biology, and mathematics will ultimately, in the opinion of this editor, be required to solve this large and intricate problem.

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CHAPTER 333

Overview of Cell–Cell and Cell–Matrix Interactions

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Cell-signaling pathways are not simply linear, but in fact form extensive interactive networks. Indeed it is the overlapping and interconnecting nature of these that provides the distinctive features distinguishing many of the response properties of specific tissues and organs. The complexity of these networks will require a great deal of research before their organization is understood in detail, but some generalities are beginning to emerge [1]. The advent of the techniques of genomics, including microchip arrays of genes and proteomics, will stimulate much more rapid development of understanding of pathway interactions as we see how a given signal reverberates through the tissue and cellular systems. Indeed, a considerable amount is already known, and in many systems the overall patterns are beginning to be made clear. Completing this knowledge will undoubtedly be the goal of a great deal of research in the near future. This section describes how many major tissue and organ systems work as they consider the many signals that play upon them.

As one reads entries in this section, it may be necessary for the uninitiated peruser to refer to earlier entries in other sections, which explain in detail how particular signal pathways function, as this section deals only with the identification of those pathways that are important in the control of organ and tissue function and not the iteration of how each pathway works. However, one concept not covered previously needs to be introduced, i.e. the idea that chemical signals may be provided locally or regionally in tissues by a group of mechanisms that have become known as paracrine, autocrine, intracrine and juxtacrine interactions. These constitute means for regulating tissue-specific signal responses–by providing the needed signals only on a local basis.

These concepts were born from the field of endocrinology. Traditionally this discipline held that certain specific organs produce and secrete particular signaling molecules into the blood stream, which delivered them elsewhere in the body to carry out their signaling activity. A classic example is the production of insulin by the beta cells of the pancreas, with the hormone transferred systemically to many other tissues, where it activates its receptors thus affecting glucose metabolism, among other responses. Over the last 10 to 20 years, it has become clear that, in addition to this classic **endocrine** notion, signaling molecules are also produced to function more locally. That is to say, although they may not enter the general circulation and consequently act only locally, they nevertheless work by binding receptors–either on the surface or within cells–and set off the same types of signal transduction pathways, as do traditional hormones. In fact, a general understanding has evolved that signal transduction pathways that usually have been addressed separately because of their inclusion in a particular discipline, e.g. endocrinology as contrasted with immunology, behave in much the same ways. A great unanimity in general mechanisms is seen as signals are transmitted between cells, whether they be signals from one immune cell to another or signals from a classic endocrine target organ to another tissue. In this sense, the same types of chemical and physical behaviors ultimately carry out cell signaling universally. The specific types of mechanisms mentioned above, in addition to endocrine, are defined according to the degree of localization of effect, but the signals generated carry out their functions by the same sorts of receptor-transduced pathways, as do the ubiquitous signaling molecules.

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In addition to the classical endocrine mechanism, localized signaling mechanisms can be conveniently sub-grouped into four types. **Paracrine** interactions induce signaling activities that occur from cell-to-cell within a given tissue or organ, rather than through the general circulation. This takes place as locally produced hormones or other small signaling molecules exit their cell of origin, and then by diffusion or local circulation act only regionally on other cells of a different type within that tissue. This has been found to be important in many organs and is a field of investigation that continues to develop rapidly. The local concentrations of paracrine signals can be quite high compared to the circulating levels, and thus can trigger effects by acting on low affinity receptors or by supplying sufficient local signal to bind to high affinity receptors even when the circulating level of a molecule that produces a similar signal is too low to do so. Paracrine signaling molecules sometimes are very rapidly metabolized locally to further limit the physical extent of their action. Examples are the prostaglandins and nitric oxide. Other, longer-acting signaling molecules also are employed in a paracrine fashion. Limiting their access to the blood supply and/or the total amounts produced within a tissue, so that local receptors can bind them before the general circulation is reached, keeps them acting in a paracrine fashion. Sometimes paracrine signaling molecules are moved by local physical means, so as to provide greater or lesser regional concentrations. An example is found in the development of the heart (see Chapter on Cardiogenesis). Many growth factors function by paracrine mechanisms.

The term **autocrine** refers to entities that are released from a cell and bind to receptors on that same cell, thereby activating it. This sort of self-stimulation occurs in carefully timed phases during normal embryonic development and tissue differentiation. It is also used in inflammation and wound healing. Such localized signals help direct the concentration of appropriate cells at the wound or inflamed tissue. In addition to their importance in normal tissues, these localized signaling systems have been discovered to be quite important in understanding the autonomy achieved by cancer cells. Quite often, one of the contributing mechanisms by which a malignant cell population escapes the normal control mechanisms for regulated growth is by producing autocrine, paracrine, and other localized types of signals that stimulate cell division or other activities that favor survival and expansion of the cancer cell population.

It is interesting to note that historically the underlying concepts inherent in these mechanisms were appreciated, although not at the molecular level, as early as 1775. De Bordeu, and later Brown-Sequard in 1891 [2], proposed that every cell, not just tissues and organs, actively secreted into the circulation substances that influenced other tissues. The focus on the role of endocrine glands (thyroid, pituitary, adrenal, pancreas, etc.) in providing these 'internal secretions' obscured the existence of the autocrine and paracrine messengers (as did the emphasis on the circulation) for some time, but the appreciation that all cells can and do actively secrete regulatory elements was an essential concept that was clearly recognized (but alas not considered to any great extent until relatively recently) over 200 years ago.

Both normal and pathological conditions can use the same hormone for autocrine and paracrine interactions. Autocrine regulation of a phase of keratinocyte development by NGF is well established [3], while the same factor generally acts as a paracrine regulator of sympathetic and selected sensory neurons [4]. At the same time, many tumors progress by autocrine stimulation by any one of several mitogenic factors, such EGF and FGF. These same substances also participate in numerous normal tissue situations using paracrine mechanisms (See entries on these factors in earlier sections).

Two additional mechanisms have been proposed that further extend cell signaling beyond the action of circulating messengers. These are **juxtacrine** signals, in which the signaling entity (receptor ligand) is not soluble but is membrane-bound on one cell and is delivered by cell-cell physical approximation to the cell bearing the receptor (usually but not necessarily different in type from the target cell) and **intracrine signals**, in which both receptor and ligand are expressed intracellularly and signals are generated without external stimuli. The former mechanism is now well established and is exemplified by a number of systems, such as the notch receptor [5] and the tyrosine kinase receptor family, Eph [6]. There is less compelling evidence for intracrine mechanisms, although there are a number of growth factors, e.g. FGF1 and 2 and interleukin 1, that are not exported in the usual manner via the endoplasmic reticulum and that clearly exhibit both extracellular and intracellular concentrations of factor (see chapter by Maciag). Thus the intracellular material could be appropriate for such signaling.

Throughout the chapters of this section, the reader will find multiple applications of these concepts as they are used in physiologically relevant systems.

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CHAPTER 334

Angiogenesis: Cellular and Molecular Aspects of Postnatal Vessel Formation

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Introduction

Angiogenesis is not the process by which vessels originate *de novo* in the embryo nor does it necessarily imply the use of stem or precursor cells in the adult. Those processes would be more typically referred to as vasculogenesis, and are not the subject of this review. In other words, angiogenesis does not mean the inceptive making of a vessel from the assembly of all its individual components. Rather, angiogenesis is the growth of a vessel from a preexisting one by mechanisms that include sprouting, branching, and intussusception. Historically, the term *angiogenesis* was used indiscriminately to describe the growth of both blood and lymph vessels. However, recent observations suggest that these two vessel types are formed and grow by different mechanisms. Indeed, hemangiogenesis (blood vessels) and lymphangiogenesis (lymphatics) are important multistep biological processes integral to both normal mammalian physiology (e.g., wound healing, adaptation of exercised muscles, development, maturation and endocrine function of the female reproductive tract) and disease (e.g., lymphedema, collateral vessel development in ischemic hearts and limbs, diabetic retinopathy, age-related macular degeneration, restenosis, rheumatoid arthritis, and tumor growth and metastasis).

The study of the angiogenic mechanisms can be divided into two disciplines: mechanisms responsible for the induction of vessel formation and mechanisms responsible for its inhibition. Because the latter have been quite controversial, our focus will be on the former. Here we present a basic analysis of the overriding principles of postnatal angiogenesis. Clearly, a comprehensive description of the method is outside the scope of this review. For a dissection of the intra- and intercellular signaling pathways involved in angiogenesis and vasculogenesis, the reader is referred to the accompanying work of Holash, Alitalo, and Donoghue (Part H) and various recent reviews [1–4].

Initiators of Angiogenesis: Cellular, Metabolic, and Mechanical

Apart from the self-directed and abnormal growth of vascular malformations and vascular tumors (e.g., hemangiomas and lymphangiomas) [5], postnatal angiogenesis and lymphangiogenesis occur only upon local tissue demands. For instance, blood vessels can either grow or degenerate according to tissue-specific needs for oxygen [6], glucose [7], and alterations in pH [8]. Blood rheology, as well as mechanical stress around a vessel, also controls angiogenesis [9]. Much less is known about the needs that lymph vessels fulfill and what specific tissue demands govern their morphogenesis [10,11]. However, the contribution of lymphatics to local fluid balance in tissues, to the drainage of metabolic products, as well as to immune surveillance suggests that parameters as diverse as hydrostatic pressure [12] and inflammatory stromal reactions [13] are likely triggers for

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lymphangiogenesis [11]. Other driving forces may include long-term alterations of extracellular pH and increases in cell death. Consequently, there are multiple factors that influence the growth of any particular vessel and perhaps an equally large set of mechanisms by which this growth proceeds. Notably, "angiogenesis is not angiogenesis is not angiogenesis" [14] because vessel growth occurs through different processes in tissue repair (wound healing [14], transplantation), chronic inflammation (psoriasis [15], rheumatoid arthritis [16], corneal neovascularization [17]), and tumorigenesis [4,18].

Vessel-Specific Requirements in Angiogenesis

Not only can different mechanisms be utilized in the making of a blood or lymph-carrying vessel, the complexity of those increases with the number of layers that comprise that vessel (intima, media, and adventitia). In vertebrates, functional vessels are more than endothelial-lined tubes. Vessels must adapt, accommodate, and respond to the temperature, pH, rheological, and mechanical parameters of both the surrounding tissue microenvironment and the fluid they carry [10,19]. That is how the blood vasculature most effectively utilizes its capillaries, sinusoids, venules, veins, and arteries [20] and how the lymph vasculature capitalizes on its network of initial lymphatics, collector lymphatics, ducts, and trunks [21]. Each branch serves a specific purpose, and each one of the latter is accomplished by a diverse set of cellular and extracellular interactions. Specific endothelial cell phenotypes (e.g., continuous, fenestrated, discontinuous, lymphatic valves, and anchoring filaments) are accompanied by the presence or absence of a particular mural cell [e.g., pericytes, smooth muscle cells (SMCs), podocytes, astrocytes] [22] and tailored perivascular matrices (e.g., variable collagen and laminin subtypes within the basement membrane). Certainly, *in vitro*, endothelial cells (ECs) appear to contain all of the necessary ingredients for forming a tubelike structure within a given provisional matrix [23,24]. But how functional are these tubes? In other words, how effective would these vessel-like structures be with regard to fluid uptake and transport *in vivo*? Recent studies have suggested that further components need to be incorporated when these vessel-like tubes are implanted *in vivo* [25]. Thus, while some of these questions are only now beginning to be elucidated, the answers will be critical to the development of successful cell-based strategies [26] and better biomaterials [27] for tissue repair.

Cellular and Soluble Regulators

The complete set of the cellular and molecular players involved in the production of each layer of a functional vessel is unknown, even for the most rudimentary of the capillaries. In hemangiogenesis, the preexisting vessel itself provides some but not all components and instructions for the formation of its new sprouts and branches (Table I). For instance, on one hand are the endothelial and mural cells (e.g., pericytes and SMCs) that migrate and multiply on extravasated clotted plasma (provisional matrix of fibrin and fibronectin) [24]. However, also frequently utilized are fibroblasts (which release angiogenic cytokines, as well as chemokines that attract inflammatory cells) [28] and a variety of subtypes of leukocytes [29]. The latter are important sources of other chemokines and proangiogenic factors [matrix metalloproteinases (MMPs), interleukin 1 (IL-1), IL-8, fibroblast growth factors (FGFs), interferons (IFNs), and others], in particular for angiogenesis occurring during wound healing and repair [3,30]. It is likely that the growth of blood and lymph vessels utilizes some common principles, but distinct players and mechanisms probably exist as well. For instance, lymphatic endothelial cells (LECs) seem to prefer VEGF-C to VEGF-A and indeed preferably express its major receptor Flt4/VEGFR3, a member of the VEGFR family [31]. However, although VEGF-C overexpression can lead to lymphatic hyperplasia [32], the factors involved in the promotion of lymphatic sprouting remain unknown. Furthermore, another member of the VEGFR family, neuropilin 2 (NRP-2), is expressed in visceral but not cutaneous lymphatics in the adult mouse [33]. A similar organ-specific modulation of lymphatic formation has been recently reported for angiopoietin 2 (Ang2) [34]. These observations further support the premise that there will be tissue-specific regulators of lymphangiogenesis, much like there are tissue-specific mitogens for blood vessel endothelial cells such as EG-VEGF [35]. Unfortunately, much less is known about embryonic and adult lymph vessel formation than about hemangiogenesis. Thus, we are limited in our description to some instances in which clear differences between these processes have been observed.

Coordination of Angiogenesis by Cellular and Molecular Interactions

During angiogenesis, vascular cells break up and establish new contacts (gap, tight, and adherens junctions), including contact with the surrounding matrix [36–38]. Moreover, angiogenic cells continuously lay down and degrade the extracellular matrix (ECM) to facilitate their migration and proliferation. Thus, angiogenesis likely results from the simultaneous and harmonized occurrence of multiple cellular and molecular processes. Altogether, a functional vessel may be obtained through the following temporal sequence of events: An initial angiogenic stimulus triggers cell activation, which is followed by cell migration, division, and alignment, vessel pruning and maturation, and phenotypic and organotypic differentiation [19] (Fig. 1). According to this process, the onset of hemangiogenesis is the opening of intercellular junctions in the endothelial lining, allowing the leakage of plasma into the subendothelial space. Thus, one of its possibly

Table I Cell–Cell, Cell–Matrix Interactions and Their Molecular Mediators in Angiogenesis

	Cell activation & local remodeling	Cell migration & vessel sprouting	Cell division	Lumen formation & maturation	Differentiation (Phenotypic & organotypic)
Adventitial cells	Protease production ECM degradation Release of growth factors (VEGF, FGF1 and 2, IGF1)	Migration stimuli S1P from aggregated platelets and mast cells is chemotactic for EC and inhibits PDGF-induced migration of SMC	Mitogen Production VEGFs, FGFs, TGFβ, IL1s, PDGF, and IGF1	Cell differentiation/survival S1P regulates cell differentiation via cadherins/adherens junction assembly; it can promote cell survival from apoptosis due to anoikis	EC-Inflammatory cells Different vascular beds acquire specific subsets of homing receptors for blood cells (selectins)
Leukocytes fibroblasts	TSP-1 stimulates the release of growth factors by fibroblasts				
Mural cells	Disruption of EC-mural cell interactions and underlying matrix	Cell-Matrix remodeling Integrin interaction with FN, laminin, vitronectin, osteopontin, HA, and others facilitates vessel sprouting	SMC proliferation regulated by ECM components (elastin and others)	Investment Ang1 produced by mesenchymal cells tightens EC investment by mural cells, which then inhibit EC migration and proliferation via TGF-β	Vascular-bed-specific mural cells brain astrocytes kidney podocytes venular pericytes arterial SMC Provide survival and vasomotor agents, growth factors and cytokines
Pericytes smooth muscle cells	Ang2	PDGF-BB induces migration and proliferation on pericytes	FGFs and PDGFs recruit mural cells to nascent vessel sprouts		Mesenchymal cell differentiation and ECM deposition TF, TGFβ endoglin, dHAND
Lumenal cells	Adhesion molecule redistribution PECAM, VE-Cadherin, endoglin	Cell-Matrix remodeling Disruption of EC-EC contacts (PECAM-1 VE-cadherin, ephrins) VEGF-B, PlGF, modulate degradation of ECM by MMPs, uPA	Mitogens FGFs TNF VEGFs TGF PDGF-BB (mural cell growth)	Lumen Formation VEGF121 & 165 combined with Ang1 (↑) but VEGF189, TSP-1 (↓)	Junctional communication Increase in tight, gap, adherens junctions (cadherins, connexins) Individual Ephrin bHLH factors determine vessel specification
Endothelial cells	Cell invasion Degradation of ECM by uPA, tPA. Activation of plasmin and MMPs TNFα, LPS, and IL1s induce EphA1 in EC	Cell Movement, Sprouting Facilitated by EphA1 activation, adhesion to FN and Tiam1 redistribution in adherens junctions	Tissue-specific mitogens for EC (e.g. EG-VEGF)	Branching, Tube Formation Integrins, Notch:Delta/Jagged FGFs, signals influence branching, S1P:Edg system is involved in tube-formation	

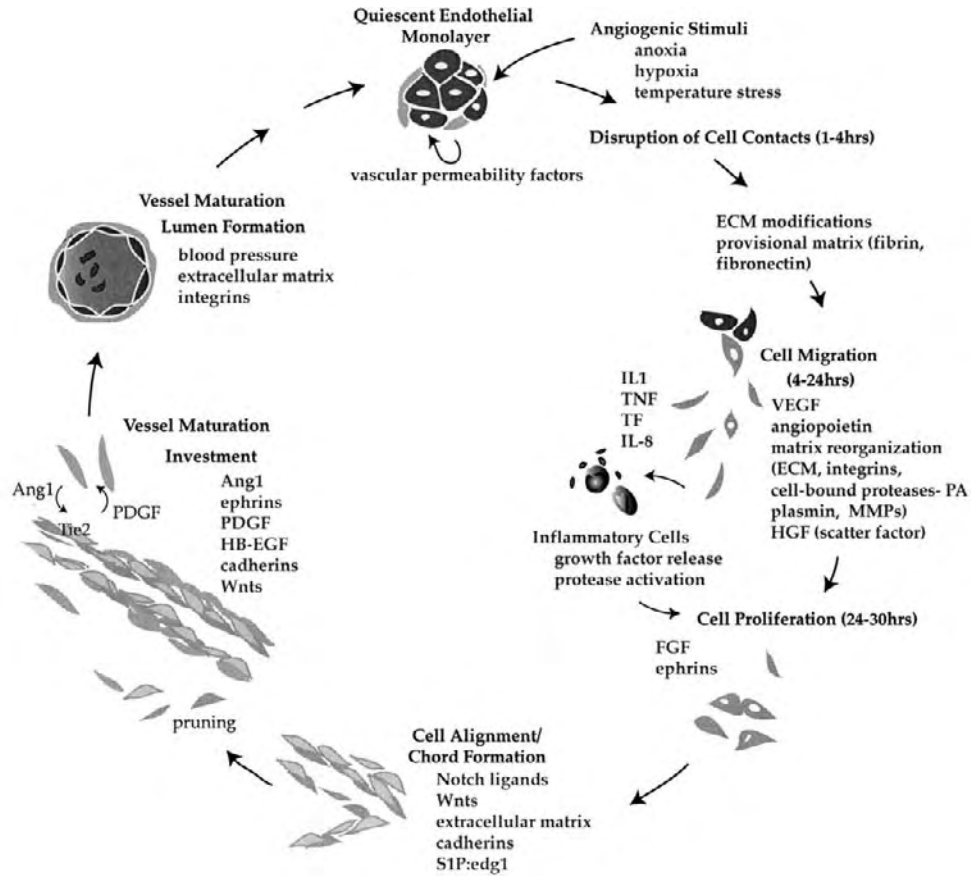


Figure 1 Regulation of the angiogenic cycle. The major steps involved in angiogenesis are receipt of an angiogenic stimulus, disruption of endothelial cell contacts and cell migration, cell proliferation, cell alignment and tubule formation, and the maturation of vascular structures into vessels by investment with mural cells and formation of a lumen continuous with the circulation. Factors that control the various steps are listed.

earliest modulators is vascular permeability factor (VPF) [39,40], the prototype member of the VEGF family. VPF, which may have variable cell sources including fibroblasts [41] and platelets [42], mediates EC dilation [43], permeability, and migration [44] both through the stimulation of nitric oxide (NO) production and as a result of NO accumulation [45]. Alternative vasodilators useful in angiogenesis are prostaglandins, lipid products of arachidonic acid metabolism by cyclooxygenases (COX-1 and COX-2) [46,47] that regulate multiple steps in angiogenesis.

Resident macrophages, polymorphonuclear leukocytes (PMNs), and mast cells can be attracted to sites in need of new vessels by proinflammatory growth factors and by cytokines and chemokines such as IP-10 and PF4 [29,30]. Once stimulated under angiogenesis-promoting conditions such as hypoxia and high lactate concentrations, these cells release IL-1s, TNFs, FGFs, and proteolytic enzymes (elastase, cathepsins, and proteinase-3) that facilitate ECM degradation and activation of MMPs [48]. In addition, they also induce the ECs, fibroblasts, and keratinocytes to release another set of proteases that further degrade the basal lamina, facilitating EC migration and growth toward the chemotactic source [49]. Consequently, there is an overall loosening of the original cell–cell and cell–matrix contacts during the earliest angiogenic events. These processes involve a large group of proteins of the integrin, selectin, and cadherin families, as well as members of the immunoglobin-like gene family (ICAM, VCAM, PECAM) [50]. For instance, the loosening affects PECAM-1 in that it becomes redistributed out of EC–EC junctions in response to VEGF and TNF-α [51]. As more PECAM-1 becomes available for interactions with integrins, EC migration is triggered. Notwithstanding, proteoglycans [52], as well as CD44 [53], LYVE-1 [11,54], and other receptors for hyaluronan, which is the most abundant sugar responsible for hydration of the ECM, may also facilitate cell migration. One of these molecules, LYVE-1, is concentrated in lymphatics and a subset of blood vessels (liver sinusoids) [11]. Thus, LYVE-1 may participate in hemangiogenesis and lymphangiogenesis as well [54]. Finally, proinflammatory molecules such as TNF, IL-1, and LPS as well as VEGF induce the expression of certain ephrins. It is thought that the interaction of ephrins with specific Eph receptors [3,38] on adjacent ECs promotes their sprouting, migration, and capillary tube formation [3,38]. Similar events may also be mediated by the Notch ligands, Jagged and Delta [55].

An adult human has approximately two pounds of ECs [56], primarily in a resting or quiescent state (G_0 phase,

"outside" of the cell cycle). Notably, the turnover rates of the endothelia are of the order of hundreds of days (5000 days for the retinal blood vessels), and during normal conditions less than 0.01% of the ECs are "in cycle" [4]. Thus, after their initial activation and migration, EC proliferation upon exposure to mitogens (such as FGFs and PDGFs) begins by reentry into the G_1 phase of the cell cycle [57,58]. However, commitment to division may actually require the continuous presence of potent mitogenic factors such as FGFs throughout most of the remaining phases [59]. EC mitogens activate the cell cycle via Rb phosphorylation as well as cyclin production and activation, but progression is also influenced by cues from EC contacts with the ECM. For instance, integrin-mediated signals from the matrix regulate the levels of p21, p27, and p53 [60]. It is anticipated that the recent development of methods allowing LEC identification *in vivo* [61], in addition to permitting establishment of LEC cultures *in vitro* [62], will provide a more clear insight into the mechanisms of LEC quiescence and proliferation. Indirectly, the inflammatory cytokines IL-1 and TNF may also induce LEC proliferation by promoting the expression of VEGF-C by fibroblasts and ECs [63]. As such, it has been proposed that lymphatics are activated in inflammation [11,63] and that the resulting lymphangiogenic response plays a role in this process by controlling the composition and interstitial fluid pressure, as well as by facilitating trafficking of immune cells [13,63].

For sprouting of new vessels, contacts between ECs (tight- and adherens-type junctions) have to be loosened, and new contacts established later [64,65]. During these processes, ECM deposition and production by the tissue stroma change remarkably, resulting in a transient provisional matrix. In blood vessels, this matrix serves both as a signal for EC adhesion, survival, and migration and as a regulator of growth factor activity. An early event in the establishment of the provisional matrix is the deposition of fibrin/fibrinogen, fibronectin (FN), and vitronectin (VN) partly due to increased vascular leakage [66]. Proteins including thrombospondin 1 (TSP-1), TSP-2, osteopontin (OPN), SPARC, tenascin-C [67], and del1 [68] are components of this provisional matrix and are postulated to participate in regulating EC behavior and hemangiogenic events. Recent *in vivo* genetic approaches using transgenes or null mutant animals have demonstrated that TSP-1 and -2 are context-dependent natural inhibitors of angiogenesis [69,70], whereas OPN promotes angiogenesis [71]. The combination of adhesive glycoproteins and those with antiadhesive properties illustrates that cells need to constantly adapt when undergoing remodeling events [72]. The observation that many of the components of the "angiogenic matrix" interact with alpha v-containing integrins is consistent with the importance of $\alpha v\beta 3$ and $\alpha v\beta 5$ in the angiogenic process [73]. Indeed, although many integrins are expressed in quiescent endothelium *in vivo* and interact with normal collagens and laminin in the basement membrane, EC integrin profiles change during angiogenesis. In particular, both $\alpha v\beta 3$ and $\alpha 5\beta 1$ are increased, and that correlates to their activities as receptors for multiple components of the provisional matrix [65]. Finally, continued remodeling of the cell environment is accomplished by modification/refinement of the provisional matrix by proteases. For instance, MMPs not only degrade proteins, but also modify the activity of matrix proteins as well as cellular receptors to provide a matrix suitable for cellular migration and proliferation [74].

Furthermore, most but not all vessels are invested with a SMC-like layer that provides them contractility, stability, and survival factors [75,76]. Notable exceptions are some blood sinusoids and the initial lymphatics [77]. The latter nevertheless appear to have evolved anchoring filaments that protrude from the LEC and establish close contacts with ECM that also involve integrins localized to focal adhesions [78]. These structures are not apparent in lymphatic ducts and trunks, which in turn are invested by mural cells. Consequently, in most situations, as an EC begins to migrate and divide, it does loosen its association with the eventual pericyte or SMC involving it. The investment of the endothelium by mural cells is largely regulated by Ang-1 and Ang-2/Tie receptor complexes at the EC surface and involves both tight and gap junctions, as well as adhesion plaques [64]. These interactions influence the EC proliferative state, and later help in EC survival during vessel pruning and maturation at a time in which the levels of proangiogenic growth factors (VEGFs, FGFs) and other molecules (Ang2) become lower than those of endogenous antiangiogenic molecules [34] (e.g., TIMPs, fragments of several ECM molecules, TSP-1, IL-4). SMC and pericytes also provide several useful proteases (e.g., plasmin, urokinase, tPA, MMPs) that may release ECM-stored growth factors (FGF and VEGF) and other molecules that participate in the angiogenesis process [22].

A primitive vessel thus assembles along the concentration gradient of angiogenic factors (mitogens, proteases, and others), laying the foundation for a new branch of the vasculature that is still only barely functional. As VEGF, FGFs, and Ang2 levels begin to fall (for instance, as a result of improved tissue oxygenation), Ang1 produced by mesenchymal cells activates the Tie2 receptor on EC, and this in turn leads to the production and release of a recruitment signal for pericytes (PDGF-BB) and SMCs (PDFG-AA, HB-EGF) [1]. Once these cells arrive and contact the endothelium, TGF-ß may be activated, inhibiting EC proliferation, altering integrin expression profiles, and stimulating matrix deposition [22]. Similarly, it is likely that ECs either secrete or express surface molecules that contribute to pericyte and SMC quiescence [79]. Vessel integrity is thus strengthened; on the other hand, some vessels get pruned, as is the case of premature blood vessels that fail to become properly invested by mural cells [76]. Thus, an intriguing question is how do initial lymphatics manage to survive and function without mural cells? Is their role somehow served by the anchoring filaments instead? As blood vessels mature, they also establish a different set of interactions with the newly deposited ECM (e.g., a continuous layer of laminin and type IV collagen surrounded by the interstitial collagens type I and III) that replaces the provisional

matrix (e.g., a fibrillar network of fibronectin and type V collagen with patchy deposits of laminin and type IV collagen). These EC–ECM contacts are important in lumen formation, vessel elongation, and acquisition of a vessel-specific EC differentiated phenotype. Furthermore, proper sprouting, branching, and lumen formation of vessels seem also to utilize helix–loop–helix (HLH)-containing Id (inhibitors of differentiation) proteins. This is partly possible because Id proteins can regulate the function of various transcription factors in EC, thus altering their production of integrins, MMPs, and other proteins utilized for EC–ECM interactions and remodeling [80].

Finally, the EC–mural cell interaction is important for effective function of the new vessel—and not just to its growth control and maintenance [22,81]. For instance, vessel tone is controlled by the action of EC-derived factors (NO, endothelin-1) on adjacent SMCs. Astrocytes contribute to the establishment of the blood–brain barrier and blood–retinal barrier in neuronal vessels by fostering the formation of tight junctions between ECs. Finally, type II alveolar epithelial cells play a critical role in the development of a correct vasculature in pulmonary alveoli, probably through VEGF-A [88]. The progression from an actively migratory and proliferative phenotype to quiescence is further accompanied by the acquisition of a more differentiated phenotype for each of the cellular vessel components. For instance, the endothelium itself can have microvalves such as in the lymphatics [82] and can be continuous, discontinuous, fenestrated, or a combination of the above according to the specific needs of each vascular bed [20]. Constitutive expression of VEGF/VEGFR complexes in adult tissues (choroid plexus and kidney glomeruli) suggests that this system can promote organotypic EC differentiation and permeability of fenestrated endothelium [20]. Thus, the variability of local organ and tissue microenvironments (e.g., biochemical, mechanical, and biophysical environments, presence or absence of inflammatory stimuli) expectedly requires a large array of subtypes of EC, each one with its unique differentiated phenotype to serve specific cellular and matrix environments. In other words, the exact properties of an EC are not easily predicted from the vessel from which it derives nor are they immutable. Some of the molecules that determine the fate and identity of specific EC have been recently discovered (Notch/Jagged and Delta [55,83] ephrins/EphRs [38], Frizzleds/Wnts [84], and Prox 1 [10,85]), but most are likely still to be determined. Whether these novel ligand-receptor signaling pathways integrate into the activated receptor tyrosine kinase-mediated signaling pathways is also currently not known. However, it is anticipated that they will indeed do so to direct the fate of an individual vessel in response to microenvironmental cues.

Therapeutic Implications

It is becoming increasingly evident that inflammation may be the common denominator to most post-natal and pathological microenvironments in which blood or lymph vessel growth occurs. Less clear, however, is how the multitude of cellular and molecular players that are present in inflammatory settings act in concert to induce the timely proliferation and regression of these vessels. Because of their multiple sources and target cells, the IL-1 prototypes, their structurally-related cytokines, the FGF1 prototypes (86), and the VEGFs are possibly some of the most crucial elements of the pro-inflammatory angiogenic response (Figure 2). Indeed, their relevance as therapeutic targets is highlighted by the recent observation that TTM, a specific chelator of physiological copper, can exert potent anti-angiogenic effects largely by complex biochemical mechanisms that involve sequestration of intracellular IL-1α and FGF1 thereby effectively reducing the extracellular pool of these proteins (87). Moreover, the very recent discovery that mice which are genetically null for the IL1 prototypes exhibit significantly impaired tumor invasiveness and angiogenesis (88) reinforces the role of these pro-inflammatory and angiogenic signals in the regulation of the microvasculature in response to inflammatory challenges. These results offer new and exciting venues for the treatment of cancer, atherosclerosis, lupus, psoriasis and a plethora of other autoimmune disorders associated with abnormal vessel proliferation.

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CHAPTER 335

Signaling Pathways Involved in Cardiogenesis

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Introduction

The heart is a complex organ that is derived from multiple cell types and requires extensive cell–cell signaling events that are often guided by specialized forms of extracellular matrices. As the earliest organ to form in an embryo, the heart must be functional well before it has the opportunity to shape itself into a mature organ. The combination of multifarious morphogenetic events necessary for cardiogenesis and the superimposed hemodynamic influences may contribute to the exquisite sensitivity of the heart to perturbations. This phenomenon is reflected in the estimated 10% incidence of severe cardiac malformations observed in early miscarriages and in the nearly 1% of live births affected by cardiac developmental defects.

The recognition that cardiac genetic pathways and morphogenetic steps are highly conserved across vastly diverse species from flies to man has resulted in a rapid growth of knowledge from studies in more tractable and accessible biological models than *Homo sapiens*. The fruit fly (*Drosophila*) has been a source of discovery for genes involved in early cardiac determination events in spite of its relatively simple cardiovascular system composed of a linear heart tube that pumps hemolymph through an open circulatory system. The reverse genetics available in flies are also possible in zebrafish, which have several distinct advantages. Zebrafish are vertebrates with a more complex two-chambered heart that is not necessary for survival during the period of cardiac development. Chick and mouse embryos have four-chambered hearts similar to humans, but the elegant genetics available in the latter have made it a particularly rich system. In a simplified view, it appears that higher organisms have retained the morphologic steps utilized by lower organisms and have built complexity into the heart in a modular fashion [1].

Here, some of the critical signaling events necessary for normal cardiogenesis are reviewed to provide a framework for considering the intricate interactions necessary between distinct cell types. Multiple signaling pathways often converge on individual events to establish domains or cell fates within the embryo in a combinatorial fashion. In addition, common pathways are often reemployed in various regions of the heart, although their ultimate morphogenetic effects are vastly different based on activation of unique transcriptional programs. Therefore, we consider the combined signaling events that contribute to specific steps of cardiogenesis using knowledge gained from multiple species.

Cardiomyocyte and Heart Tube Formation

Soon after gastrulation (about embryonic day 20 in humans), progenitor cells within the anterior lateral plate mesoderm become committed to a cardiogenic fate in response to an inducing signal thought to emanate from the adjacent endoderm [2,3]. The complete set of signaling molecule(s) responsible for cardiogenic commitment remains to be identified, although several pathways have now been implicated in defining the cardiogenic domain and in cardiac cell fate determination. In flies, a member of the transforming growth factor ß (TGF-ß) family, *dpp*, is essential for the initial determination of a cardioblast [4]. In chick and frog,

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dpp-like members of the TGF-ß family, including bone morphogenetic proteins (Bmps) -2, -4, and -7 are secreted from the endoderm in the anterior half of the embryo and act through Bmp receptors in the adjacent mesoderm to activate cardiac gene expression [3,5]. In flies or vertebrates, dpp or Bmp signaling is mediated by transcription factors of the Smad family that directly interact with cis elements in the enhancer of *tinman* or *Nkx2.5*, respectively [6,7]. Mutation of *tinman*, a homeodomain-containing transcription factor, in flies results in an organism similar to the *Wizard of Oz* character who is lacking a heart [8]. In contrast to the requirement of *tinman* for heart formation in flies, its mammalian ortholog, *Nkx2.5*, is not essential for specification of the cardiac lineage in mice, suggesting that other genes may share related functions with *Nkx2.5* or that cardiogenesis in flies and vertebrates differs with respect to its dependence on this family of homeobox genes [9,10]. Tinman and Nkx2.5 cooperate with zinc-finger transcription factors of the GATA family to activate cardiac gene expression [11].

Only certain spatial domains of the embryo are competent to respond to Bmp signals in a cardiogenic fashion, suggesting that other factors are also important. The expression of fibroblast growth factors such as Fgf4 and Fgf8 overlaps with Bmp in the cardiogenic field and the two appear to cooperate to promote cardiac cell fate determination [12]. The recent identification of Wnt signals emanating from the midline of the embryo provides insight into the cues that pattern the anterior domain, which is competent to respond to Bmp and Fgf signaling. Wnt molecules diffuse laterally from the midline notochord and inhibit cardiac gene expression [13,14]. An inhibitor of the Wnt receptor, frizzled, is expressed specifically in the precardiac mesoderm and is capable of promoting cardiac cell fate in frogs and chicks. Interestingly, the fly ortholog of Wnt, *wingless*, activates *dpp* signaling and heart formation, thus functioning opposite of Wnt signaling in vertebrates [15]. Nevertheless, a combination of Bmp, Fgf, and Wnt signaling is important in establishing the initial cardiac precursors, although it is clear that yet unknown factors are also involved.

Soon after their specification, cardiac muscle cells converge along the ventral midline of the embryo to form a beating linear heart tube composed of distinct myocardial and endocardial layers separated by a rich extracellular matrix. Mutations of GATA proteins in mice and zebrafish have demonstrated a critical role for this family of transcription factors in midline fusion of the heart tube [16–18]. In an example of the power of zebrafish genetics, a gene responsible for a cardiac bifida phenotype, *miles apart*, has recently been identified [19]. This gene encodes a novel sphingosine 1-phosphate receptor that may be mediating a midline signal to attract cardiomyocytes from the lateral aspect of the early embryo; however, the mechanisms of cell movement and fusion are yet to be determined.

As the straight heart tube takes shape, four distinct tubular segments form in a temporal sequence along the anterior-posterior (AP) axis. The primitive right and left ventricles are the first to be distinguished, followed by the atrioventricular canal segment. The sinoatrial segment forms most caudally and has distinct left–right asymmetry, with the right and left limbs of this segment later contributing to the right and left atria, respectively. The conotruncus is the last segment to form and lies in the most anterior portion of the heart tube. This segment appears to arise from cells that respond to Fgf10 signals and migrate from the pharyngeal mesoderm to populate the heart tube after its initial formation [20]. As the heart tube loops to the right, the cardiac chambers begin to become distinguished morphologically and adopt their left–right orientation (Fig. 1).

Cardiac Looping and Left–Right Asymmetry

The pathways that control the direction of cardiac looping along the left–right axis have recently been elucidated (reviewed in [21]) (Fig. 2). The heart is the first organ to break the bilateral symmetry present in the early embryo and the rightward direction of its looping reflects a more global establishment of left–right (LR) asymmetry that affects the lungs, liver, spleen, and gut. Defects in establishment of LR asymmetry in humans are associated with a wide range of cardiac alignment defects, suggesting that pathways regulating LR asymmetry dramatically affect cardiac development.

A cascade of signaling molecules regulating the establishment of embryonic LR asymmetry has been revealed from recent studies of chick embryonic development. Before the formation of organs in the developing embryo, asymmetric expression of the morphogen Sonic hedgehog (Shh) on the left side of Hensen's node leads to left lateral mesoderm expression of nodal and lefty, members of the transforming growth factor ß (TGF-ß) family [22]. Transfer of this signal from the node to the lateral mesoderm is mediated by the secreted molecule, caronte. Caronte inhibits Bmp on the left side, relieving Bmp-mediated repression of nodal in the left lateral plate mesoderm [23]. Left-sided expression of nodal induces rightward looping of the midline heart tube. Fibroblast growth factor and activin receptor-mediated pathways suppress caronte expression on the right side and the resulting activity of Bmp signaling results in suppression of right-sided nodal expression. Conversely, the snail-related (cSnR-1) zinc finger transcription factor is expressed in the right lateral mesoderm and is repressed by Shh on the left [24]. The above signaling pathways are active in the lateral plate mesoderm, but not in the heart or other organs that actually display LR asymmetry. Ultimately, the nodal-dependent pathways result in expression of a homoedomain protein, Pitx2, on the left side of visceral organs and repression of Pitx2 on the right [25]. Pitx2 appears to be the major factor that interprets the LR signaling cascade at the organ level. Asymmetric expression of Pitx2 is sufficient for establishing the LR asymmetry of the heart, lungs, and gut [26].

The mechanisms that control directionality of cardiac looping have also been explored by genetic analysis of mouse mutants with abnormalities in left–right asymmetry.

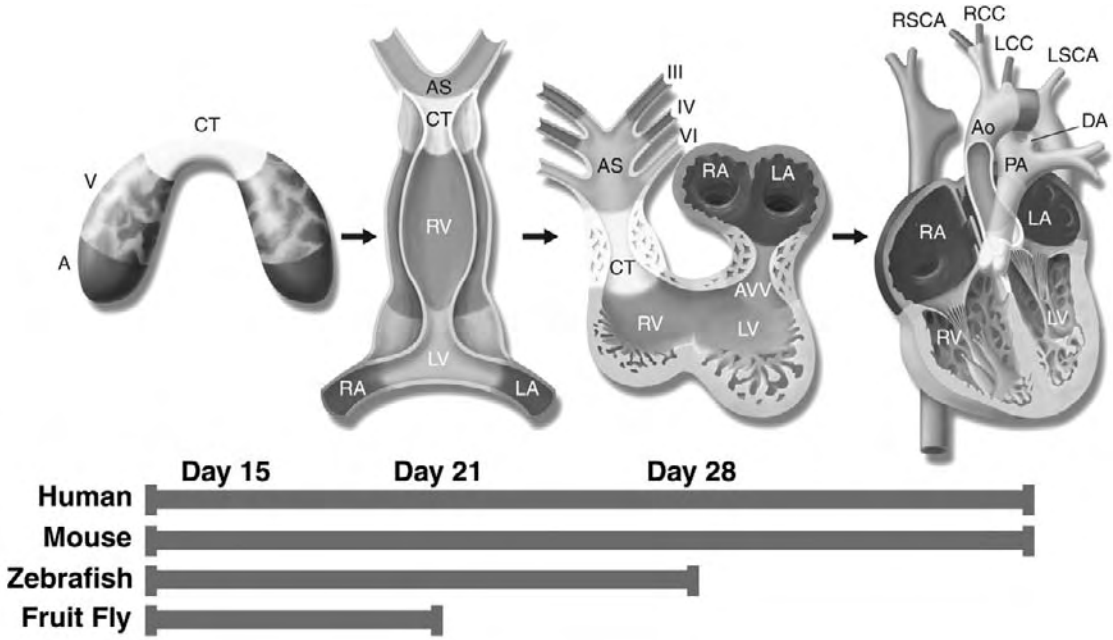


Figure 1 Schematic of cardiac morphogenesis. Illustrations depict cardiac development of morphologically related regions, seen from a ventral view. Cardiogenic precursors form a crescent (leftmost panel) that is specified to form specific segments of the linear heart tube, which is patterned along the AP axis to form the various regions and chambers of the looped and mature heart. Each cardiac chamber balloons from the outer curvature of the looped heart tube in a segmental fashion. Neural crest cells populate the bilaterally symmetric aortic arch arteries (III, IV, and VI) and aortic sac (AS) that together contribute to specific segments of the mature aortic arch. Mesenchymal cells form the cardiac valves from the conotruncal (CT) and atrioventricular valve (AVV) segments. Corresponding days of human embryonic development are indicated. RV, right ventricle; LV, left ventricle; RA, right atrium; LA, left atrium; PA, pulmonary artery; Ao, aorta; DA, ductus arteriosus; RSCA, right subclavian artery; RCC, right common carotid; LCC, left common carotid; LSCA, left subclavian artery. [Reproduced with permission from Srivastava and Olson, *Nature*, **407**, 221–226 (2000)].

Mice homozygous for mutation in the *left–right dynein* gene (*iv/iv*) display randomization of left–right orientation of the heart and viscera and have bilaterally symmetric, absent, or randomized expression of nodal and Pitx2 [27,28]. Nodal and Pitx2 are expressed along the right lateral mesoderm rather than the left, displaying complete reversal of the LR signals and have bilaterally symmetric, absent, or randomized nodal and Pitx2 expression. In contrast, in the situs inversus (*inv*) mouse [29], which has nearly 100% reversal of left–right asymmetry, nodal and Pitx2 are expressed along the right lateral mesoderm rather than the left, displaying complete reversal of the LR signals. *Pitx2* mutant mice have abnormal LR asymmetry of the lungs and a low penetrance of reversed cardiac looping, similar to *Shh* and *Fgf8* mutant mice [30,31]. Oddly, the initial LR asymmetry and roles of Fgf and Shh are opposite in mice and chicks, however the left–right sidedness of later events involving nodal and Pitx2 are conserved [32].

While the necessity of LR asymmetric gene expression is intuitive, how the initial asymmetry of molecules is established remains in question. Initial clues came from studies of immotile cilia syndrome, also known as Kartagener's syndrome, in which individuals had situs inversus totalis, with mirror-image reversal of all organs. It was recently found that, prior to organ formation, Hensen's node contains ciliary processes that beat in a vortical fashion, pushing morphogens to the left side of the embryo [33]; concurrent establishment of a midline barrier, possibly by *lefty* gene expression along the left midline, may be responsible for subsequent asymmetric gene expression. Mice lacking ciliary movement in the node display abnormal LR patterning, consistent with this model.

Patterning of the Developing Heart Tube

Numerous transcription factors are expressed in a chamber-specific fashion, providing a possible mechanism to explain how distinct segments of the heart adopt their respective fates. Two related basic helix–loop–helix (bHLH) transcription factors, *dHAND* and eHAND, are expressed predominantly in the primitive right and left ventricle segments, respectively, during mouse heart development [34,35]. Deletion of *dHAND* in mice results in hypoplasia of the right ventricular segment from a cell survival defect. Mice lacking eHAND die early from placental defects precluding detailed analysis of its role in left ventricular development [36]. eHAND is down-regulated in Nkx2.5-deficient mice, which fail to precisely segment the heart tube and die around the stage of cardiac looping [37]. Disruption of both *dHAND* and *Nkx2.5* in combination results in the absence of the right and left ventricle, suggesting that the combined function of dHAND and Nkx2.5, possibly through their regulation

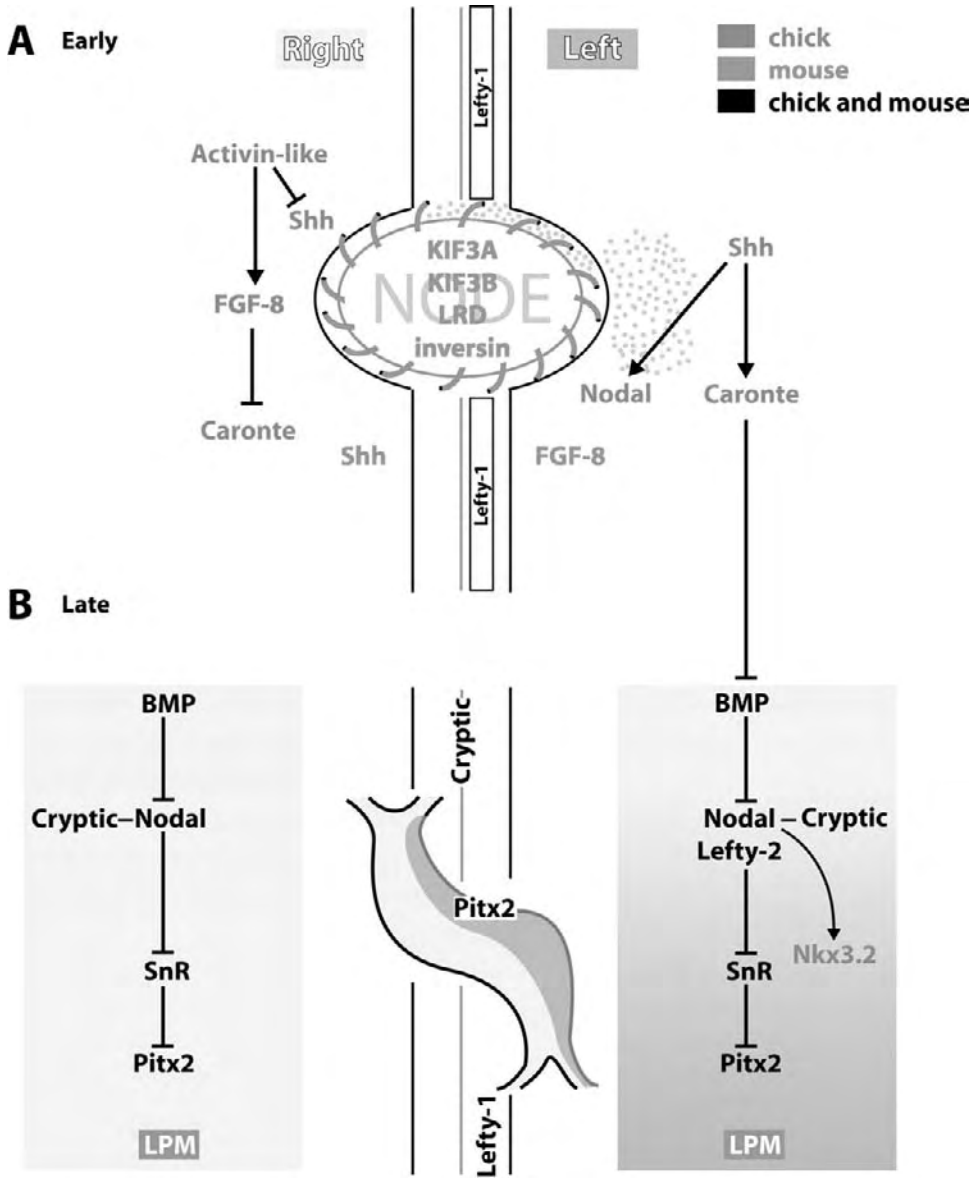


Figure 2 Cascades regulating LR asymmetry. Early asymmetrical gene expression around the node (A) results in activation or repression of sonic hedgehog (Shh) or fibroblast growth factor (Fgf)-8 dependent pathways on the right or left (ventral view). Early roles of Shh and Fgf8 are reversed in mouse and chick. Leftward flow of morphogens by nodal cilia establishes the asymmetric gradient around the node in mice. Expression of Lefty-1 near the midline may serve as a barrier to maintain left-sided asymmetry of morphogens. At later stages of organogenesis, LR asymmetric information at the node is transferred to the lateral plate mesoderm (LPM) by Caronte. Caronte relieves Bmp inhibition on the left, initiating a cascade of events culminating in expression of Pitx2 in the left LPM and in the left side of the heart tube (B). Consequently, a "leftness" signal appears to be actively propagated to overcome a default "rightness" program. [Reproduced with permission from Kathiriya and Srivastava, *Am. J. Med. Genet.* 97:271–279 (2001).]

of eHAND, is necessary for ventricular formation [38]. In zebrafish, which have a single ventricle, only one *HAND* gene has been identified (*dHAND*), disruption of which results in lack of a ventricular segment of the heart, similar to that seen in the absence of *dHAND* and *Nkx2.5* [39]. Expression of the ventricular-specific homeobox gene of the Iroquois family, *Irx4*, is dependent on both dHAND and Nkx2.5 [38,40], and misexpression of Irx4 in the atria is sufficient to activate ventricle-specific gene expression [41]. These findings suggest that HAND and Nkx2.5 proteins may cooperate in early ventricle-specific decisions. A cardiac-specific chromatin remodeling protein, Bop, is also essential for normal ventricular development and is required for activation of *dHAND* gene expression [42]. Deletion of one of the four MEF2 factors in mice, MEF2C, which also may be involved in chromatin reorganizing events, results in hypoplasia of the right and left ventricles as well, but not of the atria [43]. The chamber-specific role of MEF2C, in spite

of its homogenous expression in the heart, suggests that MEF2C might be a necessary cofactor for one or more of the other ventricular-restricted regulatory proteins.

How the segmental pattern of gene expression is established remains unclear; however, retinoid signaling has been implicated in atrial specification and positioning of the atrioventricular (AV) border along the AP axis of the heart tube [44]. The recent discovery of a novel class of hairy-related transcription factors (HRT1, HRT2, HRT3) may also provide some insight [45]. Hairy proteins often function downstream of the transmembrane receptor Notch in establishing boundaries of gene expression. Interestingly, HRT1 and HRT2 are expressed in a complementary fashion in the atria and ventricles, respectively. How the many transcription factors function in a coordinated manner to regulate chamber specification and differentiation remains to be determined.

Myocardial Growth

Mutations of a wide variety of genes in mice result in hypoplasia of the muscular wall of the heart. Mice homozygous for a null mutation in the *retinoid X receptor-alpha* (*RXRα*) gene display ventricular chamber hypoplasia and have a defect in compaction of the myocardium, although this may not be a cell autonomous effect [46]. Signaling between the endocardium and the myocardium also appears to be important for ventricular growth. Neuregulin growth factors are expressed in the endocardium and are required for the development of trabeculae, the finger-like projections of the ventricular myocardium [47]. In mice deficient in neuregulin or its receptors, erbB2 and erbB4, the ventricular trabeculae fail to form, possibly as a result of decreased endocardial signals. Similar defects in ventricular trabeculation have been observed in mice lacking angiogenic factors that are also expressed in the endocardium [48,49].

Cardiac Valve Formation

Appropriate placement and function of cardiac valves is essential for chamber septation and for unidirectional flow of blood through the heart. During early heart tube formation, "cushions" of extracellular matrix between the endocardium and myocardium presage valve formation at each end of the heart tube. Reciprocal signaling, mediated in part by TGF-ß family members, between the myocardium and endocardium in the cushion region induces a transformation of endocardial cells into mesenchymal cells that migrate into the cushion extracellular matrix (ECM). These mesenchymal cells differentiate into the fibrous tissue of the valves and are involved in septation of the common atrioventricular canal into right and left sided orifices.

The Smad proteins are intracellular transcriptional mediators of signaling initiated by TGF-β ligands. Smad6 is specifically expressed in the atrioventricular cushions and outflow tract during cardiogenesis and is a negative regulator of TGF-β signaling. Targeted disruption of *Smad6* in mice results in thickened and gelatinous atrioventricular and semilunar valves, similar to those observed in human disease [50]. In addition to *Smad6*, there are likely other genes in the TGF-β signaling pathway that, when mutated, result in the formation of hyperplastic valves that may be a result of excessive transformation of endocardial cells.

In mouse models, the absence of *PTPN11*, which encodes the protein tyrosine phosphatase Shp-2, has been shown to result in dysplastic cardiac valves by its involvement in a signaling pathway mediated by epidermal growth factor receptor [51]. The importance of *PTPN11* in congenital heart disease was shown by the identification of point mutations in *PTPN11* in patients with Noonan syndrome, whose phenotype commonly includes pulmonic valve thickening [52].

Nuclear factor of activated T cells-c (NF-ATc) is a transcription factor that is needed for cytokine gene expression in activated lymphocytes. It is controlled by a calcium-regulated phosphatase, calcineurin. In the heart, NF-ATc expression is restricted to the endocardium. Unlike the factors described earlier, mice lacking NF-ATc fail to form aortic or pulmonary valves, suggesting that this factor is necessary for formation of the semilunar valves [53,54]. The mechanisms through which NF-Atc regulates valve formation remain unknown.

Cardiac Outflow Tract and Aortic Arch Development

The cardiac outflow tract (conotruncus) and aortic arch undergo extensive and rather complex morphogenetic changes. The cardiac outflow is initially a single vessel, the truncus arteriosus, that becomes septated by mesenchymal cells into the aorta and pulmonary arteries. Six bilaterally symmetric vessels known as aortic arch arteries arise sequentially along the AP axis and undergo extensive remodeling to ultimately form distinct regions of the mature aortic arch and proximal pulmonary arteries (Fig. 3). In particular, the left fourth aortic arch artery forms the transverse aortic arch between the left common carotid and left subclavian arteries, while the sixth arch artery contributes to the proximal pulmonary artery and the ductus arteriosus.

A unique population of cells along the crest of the neural folds (neural crest cells) migrates away from the neural

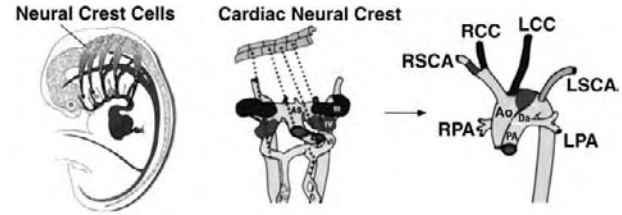


Figure 3 Migration and contribution of the cardiac neural crest. Neural crest cells arise from the crest of the neural folds between the otic placode and the third somite. Subsequent migration of the neural crest into the aortic arch arteries and cardiac outflow tract is required for arch and conotruncal development.

folds and retains the ability to differentiate into multiple cell types; therefore, these cells are pluripotent. Their migratory path and ultimate cell fates are dependent on their relative position of origin along the AP axis and their interactions with nearby matrices and adjacent epithelial cells. Neural crest cells differentiate and contribute to diverse embryonic structures, including the cranial ganglia, peripheral nervous system, adrenal glands, and melanocytes. Cranial neural crest cells migrate through the developing pharyngeal arches and populate the mesenchyme of each of the pharyngeal and aortic arch arteries, the conotruncus, and the conotruncal septum [55]. Because of their migratory path, this segment of the neural crest is often referred to as the cardiac neural crest.

Insight into the genes that regulate cardiac neural crest development has come from studies in other vertebrate models. Mice lacking the 21-amino-acid signaling peptide, endothelin-1 (ET-1), or its G-protein-coupled receptor, ET_A, show postmigratory cardiac neural crest defects, cleft palate, and other craniofacial anomalies reminiscent of DiGeorge syndrome in humans [56,57]. dHAND and eHAND, normally expressed in the neural crest-derived pharyngeal and aortic arches, are down-regulated in these structures in *ET-1*- and ET_A-deficient mice, suggesting that they are regulated by ET-1 signaling [58]. Analysis of the upstream regulatory region of *dHAND* revealed that ET-1 signals are mediated by D1x6, which directly activates dHAND expression [59]. This pathway has been developed further by the observation that neuropilin-1, a semaphorin and VEGF receptor, is down-regulated in *dHAND* mutants [60]. Targeted mutation of *neuropilin-1* or its ligand, semaphorinC, results in a phenotype similar to that of *ET-1* mutants, suggesting that ET-1, dHAND, and neuropilin-1 may function in a common pathway regulating neural crest development [61,62].

The Notch signaling pathway is involved in cell fate and differentiation decisions throughout the embryo, but has only recently been implicated in cardiovascular development. Alagille syndrome is an autosomal dominant disorder characterized by biliary atresia and defects involving the cardiac outflow tract. This syndrome is caused by mutations in *JAGGED-1*, a ligand for the Notch receptor [63,64]. Isolated outflow tract defects have also been associated with *JAGGED-1* mutations [65]. The intracellular events downstream of Notch signaling are mediated by a family of bHLH transcriptional repressors known as Hairy proteins. In mammals, the hairy-related transcription factors, HRT1 and HRT2, are expressed in the cardiac outflow tract and are activated by Notch signaling [45], raising the possibility that they may mediate the effects of JAGGED-1. The zebrafish ortholog of HRT2, *gridlock*, is activated by Notch signaling, similar to its mammalian counterpart, and is necessary and sufficient for the initial parsing of hemangioblasts into arterial rather than venous endothelial cells in the fish [66]. Hypomorphic mutations of *gridlock* result in narrowing of a specific region of the aorta, a defect commonly observed in humans, possibly because of an insufficient number of arterial endothelial cells in specific vascular locations [67].

Conclusions

The steps of cardiogenesis described here illustrate some of the signaling networks necessary for multiple cell types to communicate with one another in order to form a functioning organ. Reciprocal interactions between cell layers function to guide cells in the correct temporospatial pattern and ultimately to adopt specific cell fates and achieve terminal differentiation. Disruption of such signaling events often underlies pathologic development of the heart that manifests as congenital heart disease. Because fetal gene programs are often reactivated in the adult diseased heart with negative consequences, it is possible that inhibition or activation of specific signaling pathways involved in cardiogenesis may prove to have therapeutic value, even in late-onset heart disease.

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CHAPTER 336

Development and Regulatory Signaling in the Pancreas

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Introduction

The word *pancreas* derives from the Greek words *pan* (meaning "all") and *kreos* (meaning "flesh"), because the ancients believed that the pancreas was a pillow of flesh that was destined to provide the stomach with a comfortable cushion. In fact, the mammalian pancreas is a complex organ that consists of acinar cells that synthesize and secrete digestive enzymes, duct cells that produce bicarbonate rich fluid, and islets of hormone-secreting cells that are dispersed throughout the exocrine tissue. These endocrine islets were first described by Langerhans in 1869, who initially believed them to be intrapancreatic lymph nodes [1]. The concept that these islets may produce insulin arose following the discovery that pancreatectomized dogs develop diabetes mellitus [2–4]. Eventually, an active pancreatic extract that was rich in islet-cell-derived insulin was used to treat Leonard Thompson, the first human to receive live-saving insulin therapy [5]. This chapter delineates some of the aspects of pancreatic ontogeny and describes unique anatomic and cellular interactions that allow the pancreas to perform its complex functions.

Ontogeny of the Pancreas

The embryonic pancreas arises from endodermal cells in the duodenal region of the gut [6]. Commitment to pancreatic fate occurs at embryonic day 8.5 (E8.5) in the mouse, and dorsal and ventral pancreatic buds become evident on day E9 [7]. Pancreatic fate is dictated by the expression in these cells of the HOX-like homeoprotein pancreas duodenum homeobox-1 (PDX-1), a well-characterized transcription factor [8]. During organogenesis, PDX-1 is expressed in both the exocrine and endocrine components of the pancreas [8,9]. However, in the adult pancreas its expression is restricted to the insulin-containing β cells and the glucagon-containing α cells [9,10]. The hedgehog family of proteins also modulates pancreatic development, but in a negative manner. Thus, expression of *Sonic hedgehog* (*shh*) dictates intestinal development, and prevention of Shh expression, effected in part by fibroblast growth factor 2 (FGF-2), is absolutely essential for pancreatic development [11].

Studies of pancreas development in the mouse using classical approaches and gene knock-out strategies have revealed that there is a network of signaling molecules and transcription factors that together dictate the ultimate fate of pancreatic progenitor cells. For example, mice deficient for the type IIB activin receptor (ActRIIB) exhibit pancreatic hypoplasia and marked hyposplenism [12]. The pancreatic hypoplasia is due to loss of activin signaling, which acts like FGF-2 to suppress Shh expression [12]. Mice that are heterozygous for Smad2 deficiency also exhibit pancreatic endocrine defects [7], indicating that both activin and transforming growth factor β (TGF-β) pathways are important in islet cell development. Conversely, in mice lacking the transcription factor p48, there is a complete absence of exocrine pancreas development, whereas hormones expressing islet cells survive and are found in the spleen [13].

Inhibition of Notch signaling also blocks acinar cell formation and promotes islet cell formation [14]. Activation of the Notch receptor leads to enhanced transcription of

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hairy/enhancer-of-split genes (*Hes*), which encodes basic helix–loop–helix (bHLH) transcription factors that repress neurogenin expression [7,15]. *Ngn3*, a member of the neurogenin family, is expressed at high levels in both α and β cells, and *Hes1*-deficient mice exhibit acinar cell apoptosis and hypoplasia as well as precocious development of endocrine cells [16]. These observations point to a common precursor cell that gives rise to mature pancreatic acinar and endocrine cells. The hypothesis of a common progenitor cell in the endocrine pancreas is supported by the observation that islet cell tumors may give rise to cloned cell lines that express several islet cell hormones [17].

Pancreatic Islet–Acinar Interactions

The pancreatic endocrine cells are dispersed throughout the exocrine pancreas, which makes up approximately 95% of the total pancreatic tissue mass. Of the four major types of pancreatic endocrine cells, the β cells are the most important. In these cells, insulin is packaged into heterogeneous granules with electron-dense cores [18]. In contrast, the pancreatic polypeptide (PP)-containing cells have oblong granules that are markedly electron dense; the α cells exhibit uniform electron-dense granules that contain glucagon; the δ cells are small, somatostatin-containing cells that have a dendritic shape [18]; and the acinar cells store their digestive proenzymes in zymogen granules. Islet cells may modulate each other's actions as a result of intra-islet cell-to-cell communications through gap junctions [19]. However, the α, δ, and PP-containing cells are located at the periphery of the islets, and they release their hormones into venules that are located on the islet surface, thereby mostly avoiding the centrally located β cells [20]. In contrast, within the islet microcirculation some of the insulin is carried to the α, δ, and PP cells, thereby allowing for direct effects by this hormone on the islet cells [21]. Thus, the network of signaling molecules and transcription factors that dictates pancreatic development gives rise to cells that exhibit a high degree of specialization and an anatomic organization that allows for complex and highly regulated cell-to-cell interactions.

Although the endocrine islets constitute approximately 2% of the pancreatic volume, they receive approximately 20% of the intrapancreatic blood flow [22,23]. The venous effluent from the larger islets appears to bypass the surrounding exocrine tissue, whereas the effluent from smaller but more numerous islets forms an intrapancreatic portal circulation that passes through the exocrine tissue [20]. This anatomic arrangement has given rise to the hypothesis that islet cell hormones may participate in the regulation of pancreatic exocrine function [24]. Indeed, the pancreatic acinar cell has specific high-affinity receptors for insulin and somatostatin [25,26], and insulin deficiency is associated with enhanced cholecystokinin (CCK) binding and decreased epidermal growth factor (EGF) binding [27,28].

Several additional lines of evidence support a role for insulin in regulating pancreatic exocrine function. Insulin directly enhances pancreatic acinar cell glucose oxidation and transport, protein synthesis, and the secretory effects of the gastrointestinal hormone CCK, a calcium-mobilizing secretagogue [29–35]. Insulin markedly increases pancreatic amylase mRNA levels in insulin-deficient rats, but only slightly alters parotid amylase mRNA levels [36]. Pancreatic acini isolated from insulin-deficient rats exhibit attenuated increases in cytosolic free calcium and inositol phosphate levels [37,38]. Pancreatic duct cell function is also altered in states of insulin deficiency, as evidenced by the observations that patients with poorly controlled, long-standing type I diabetes mellitus exhibit a decreased ability to secrete bicarbonate-rich fluid and pancreatic digestive enzymes in response to pancreatic secretagogues [39–41], and that insulin increases the stimulatory effects of secretin on pancreatic juice secretion in the perfused rat pancreas [42]. Thus, the propinquity of the islet and exocrine cells in the pancreas allows for unique cellular interactions.

Cell–Cell and Matrix Interactions in the Endocrine Pancreas

Several growth factors have an important role in epithelial mesenchymal interactions during pancreatic development and may also exert a role in the adult pancreas. In addition to FGF-2 and activin, keratinocyte growth factor, or FGF-7, has been implicated in this process. FGFs act by binding to the protein products of four distinct genes encoding high-affinity FGF receptors designated as FGFR-1, FGFR-2, FGFR-3, and FGFR-4 [43]. These receptors possess an extracellular cytoplasmic ligand-binding domain that has three immunoglobulin-like (Ig-like) regions, a hydrophobic transmembrane domain, and a discontinuous intracellular tyrosine kinase domain exhibiting a short intervening sequence [43]. The presence of an intron–exon boundary in the third Ig-like loop (domain III) allows for the generation of two alternative carboxyl-terminal domains (IIIb or IIIc) of FGFR-1, -2, and -3. Expression of domain III isoforms is often restricted to specific cell types. For example, the IIIb splice form of FGFR-1 is generally found in epithelial cell types, whereas the IIIc splice form is often restricted to mesenchymal cell types [43]. This domain confers ligand-binding specificity, as underscored by the observation that the IIIb splice form of FGFR-2 is activated by FGF-7 but not by FGF-2 [43]. FGF-7 stimulates the proliferation of pancreatic epithelial cells that are the precursors of endocrine cells, and its removal leads to their differentiation into endocrine cells [44]. Furthermore, mice deficient for the FGFR-2 IIIb receptor exhibit abnormal pancreatic development [44], indicating that this receptor isoform and FGF-7 participate in important epithelial–mesenchymal interactions in the pancreas. Similarly, FGF-10 is expressed in the mesenchyme that is adjacent to the early pancreatic buds, and in mice that are deficient for FGF-10 there is an arrest in branching of the PDX-1 positive epithelium that is destined to become the pancreas [45]. PDX-1 positive cells

reappear in culture in the presence of exogenous FGF-10, confirming the importance of this growth factor in pancreatic organogenesis and in dictating the size of the pool of cells that are destined to become the pancreas [45]. EGF also enhances the proliferation of these cells [46], and all of these growth factors may, under certain circumstances, have the potential to expand the pool of endocrine cells in the adult pancreas.

Another important component of cell–matrix interactions in the pancreas is dependent on signaling pathways activated by the integrin family of receptors and their ligands. Integrins αvβ3 and αvβ5, and their major ligands vitronectin and fibronectin, are expressed in islet progenitor cells and have been implicated in islet cell migration and morphogenesis [47]. Both αvβ3 and αvβ5 mediate islet cell migration in culture, and both appear to contribute to the structural integrity of mature islets [47]. Furthermore, the survival and function of islets in culture is enhanced in the presence of extracellular matrix (ECM) [48,49]. These observations indicate that both islet differentiation and the maintenance of islet cell-differentiated functions are dependent on interactions with various components of the ECM.

Although exocrine and endocrine cells arise from a common population of progenitor endoderm cells [50], the endocrine cells are able to migrate toward the mesenchyme, attract endothelial cells, and eventually form the highly vascularized islets [6]. Integrins αvβ3, which are expressed in invasive and proliferating endothelial cells [51], may have a role in islet vascularization. In addition, rodent and human islets express relatively high levels of vascular endothelial growth factor (VEGF), and pancreatic endothelial cells express the two high-affinity receptors VEGFR1 and VEGFR2 [52–54]. Inasmuch as VEGF is mitogenic toward endothelial cells [55], it is possible that islet-derived VEGF may recruit endothelial cells and induce their proliferation during development. Furthermore, the appearance of insulin-expressing cells during islet development requires contact with blood vessel endothelium [56] and both VEGF and its receptors in the islet cells in adult humans [54]. Together, these observations point to the existence of complex regulatory interactions between pancreatic endocrine and vascular cells during organogenesis and in the mature pancreas, and they raise the possibility that VEGF-dependent pathways participate in the regulation of differentiated islet cell function.

Matrix and Cell–Cell Interactions in the Exocrine Pancreas

The preponderant cell type in the exocrine pancreas is the acinar cell. It has specific, high-affinity receptors for numerous agonists, including CCK, which acts via calcium, and vasoactive intestinal polypeptide (VIP), which acts via cAMP [57–59]. CCK mobilizes calcium from intracellular stores, and the resulting calcium waves appear to spread from acinar cell to acinar cell, partly as a consequence of open gap junctions that allow for this type of cell-to-cell communication [57].

Two lines of evidence indicate that pancreatic acinar cell functions can be modulated by interactions with the ECM. First, when pancreatic acini are placed in culture, they dedifferentiate into ductal-like cells [60], implying that maintenance of a differentiated acinar-like state is dependent on interactions with the pancreatic ECM. Second, the pancreatic acinar cell possesses a β1 integrin receptor, and ligation of this receptor with an anti-β1 integrin antibody leads to tyrosine phosphorylation phospholipase C-γ1, translocation of protein kinase Cα to the cell membrane, and a rise in cytosolic free calcium levels, pointing to a direct effect by the ECM on acinar cell differentiated functions [61,62].

The exocrine pancreas also has a defined but small stromal compartment that consists of connective tissue and fibroblasts surrounding the interlobular ducts, a small number of pancreatic stellate cells, vascular endothelial cells, and thin stromal partitions that separate adjacent pancreatic lobules [63,64]. In pathological conditions such as chronic pancreatitis, there is a marked expansion of this stromal compartment, with extensive deposition of fibronectin, laminin, and collagen types I, III, and IV [65]. This fibrotic reaction occurs, in part, as a consequence of excessive production of growth factors such as TGF-α, TGF-β, connective tissue growth factor, FGFs, and platelet-derived growth factor [66–70]. In addition, enhanced fibroblast proliferation and activation of pancreatic stellate cells are seen [64]. As a consequence, the pancreas exhibits regions of acinar cell degeneration and apoptosis and ductal cell proliferation, in association with pancreatic exocrine and endocrine dysfunctions [71,72], underscoring the importance of maintaining normal epithelial mesenchymal interactions in the adult pancreas.

Conclusions

Pancreatic function is regulated by complex interactions that include a multiplicity of extracellular signals from hormones, neurotransmitters, and nutrients. Proper coordination of the exocrine and endocrine components of the pancreas ensures the timely digestion of nutrients in the gut lumen and the subsequent assimilation of the absorbed components by target tissues through the actions of islet hormones. Multiple endocrine–exocrine–ECM interactions are necessary to maintain these differentiated functions. In addition, the dispersal of the islets throughout the exocrine tissue, the rich vascularization of the islets, and the existence of an intrapancreatic portal circulation translate into unique cell–cell interactions that are yet to be fully understood.

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CHAPTER 337

Tropic Effects of Gut Hormones in the Gastrointestinal Tract

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Introduction

The mucosa of the gastrointestinal (GI) tract is a complex and constantly renewing tissue that is characterized by rapid proliferation, differentiation, and subsequent apoptosis, followed by extrusion into the GI lumen. These events occur as GI luminal epithelial cells ascend the vertical axis of the microfolded crypts lining the GI tract [1,2]. This process normally takes 3–8 days, depending on the species and the location along the GI tract [3,4]. Numerous factors can contribute to growth of the GI mucosa. This chapter focuses specifically on the effects of GI hormones on the proliferation and repair of non-neoplastic tissues and on the receptors and signaling pathways that transmit signals from the cell surface to the nucleus.

By definition, any agent that stimulates growth can be considered a growth factor; however, these growth-stimulating agents are usually divided into those produced by normal cells and thought to act locally to control proliferation and hormones that are thought to act at a distance. Peptide growth factors that act locally include members of the epidermal growth factor (EGF) family, the transforming growth factor β (TGF-β) family, the insulin-like growth factor (IGF) family, the fibroblast growth factor (FGF) family, the trefoil factor (TFF) family, the colony-stimulating factor (CSF) family, and a few other unrelated regulatory peptides, such as hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), various interleukins, interferons, and tumor necrosis factor-related proteins [5].

Various gut hormones can regulate growth of the GI mucosa, usually through an endocrine effect although, on occasion, an autocrine or paracrine mechanism has been postulated for the proliferative effects of these trophic peptides [6,7]. The gut peptides that have been best described in their role as stimulating mucosal proliferation of the stomach, small bowel, or colon include gastrin, bombesin (BBS)/gastrin-releasing peptide (GRP), neurotensin (NT), glucagon-like peptide-2 (GLP-2), and peptide YY (PYY) [1]. These hormones are secreted by endocrine cells that are widely distributed throughout the GI mucosa and pancreas. In addition to mucosal proliferation, these gut peptides control many other functions in the GI tract, including regulation of secretion, motility, absorption, and digestion (Table I).

Tropic Effects of Gut Peptides in the Stomach, Small Bowel, and Colon

Stomach

Gastrin is the GI hormone that has been best characterized for its trophic effects in the stomach. Gastrin stimulates acid secretion from gastric parietal cells and is the single most important trophic hormone for the gastric mucosa. The trophic effect of gastrin on gastric mucosa was initially demonstrated with the synthetic gastrin analog, pentagastrin, which, when given to rats, stimulated protein synthesis and parietal cell mass [8,9]. These results were further confirmed using the natural amidated gastrins, G17 and G34, with most pronounced effects noted in the oxyntic acid-secreting mucosa and enterochromaffin-like cells. Resection of the gastric antrum, which removes endogenous gastrin, results in gastric mucosal atrophy; this atrophy can be prevented by administration of exogenous gastrin [10].

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Table I Gut Hormones Contributing to GI Mucosal Growth

Hormone	Location	Primary effects
Gastrin	Antrum, duodenum (G cells)	- Stimulates gastric acid and pepsinogen secretion - Stimulates gastric mucosal growth
Gastrin releasing peptide (GRP) (mammalian equivalent of bombesin [BBS])	Small bowel	- Stimulates release of all GI hormones - Stimulates GI secretion and motility - Stimulates gastric acid secretion and release of antral gastrin - Stimulates growth of intestinal mucosa and pancreas
Neurotensin (NT)	Small bowel (N cells)	- Stimulates pancreatic water and bicarbonate secretion - Inhibits gastric secretion - Stimulates growth of small and large bowel mucosa
Glucagon-like peptide-2 (GLP-2)	Small bowel (L cells)	- Potent enterotrophic factor
Peptide YY (PYY)	Distal small bowel, colon	- Inhibits gastric and pancreatic secretion - Inhibits gallbladder contraction - Stimulates intestinal growth?

Further confirmation of the effects of gastrin on gastric mucosal growth is provided by transgenic mice that either overexpress gastrin or are gastrin deficient. In mice overexpressing either unprocessed gastrin or the amidated gastrins (G17 and G34), a marked thickening is seen in the oxyntic mucosa with increased BrdU labeling, representing an 85% increase in cells undergoing proliferation [11,12]. In gastrin-deficient mice, a 35% decrease in parietal cell mass with no decrease in basal fundic proliferation rate is noted when compared with wild-type control animals [13,14].

Another peptide that has been shown to stimulate gastric mucosal proliferation is BBS/GRP, which stimulates pancreatic, gastric, and intestinal secretion, gut motility, smooth muscle contraction, and release of all gut hormones [15]. In addition, these peptides can stimulate growth of GI mucosa and pancreas. BBS stimulates gastric weight, fundic and antral mucosal height, and density of parietal cells in neonatal rats compared with saline-treated controls [16,17]. These results were confirmed in adult rats given BBS for 7 days, demonstrating increased weight, increased RNA, and DNA content of the oxyntic mucosa of the stomach and the duodenal mucosa; the inhibitory hormone, somatostatin, attenuated the proliferative effect of BBS [18]. In another study, the BBS receptor antagonist, RC-3095, prevented the proliferative effect of BBS on the gastric mucosa, thus providing evidence that BBS/GRP stimulates growth of stomach and duodenum, predominantly due to a direct effect of hormone stimulation and not secondary to release of other gut hormones [19].

Small Intestine

The intestinal hormones that have been shown to stimulate growth of small intestine mucosa include NT, BBS/GRP, PYY, and GLP-2. Wood *et al.* [20] first noted that NT stimulated the small bowel mucosa of rats fed a normal diet. Investigators in our laboratory have shown that administration of NT prevents gut mucosal atrophy induced by feeding rats an elemental diet and stimulates mucosal growth in defunctionalized, self-emptying jejunoileal loops or isolated small bowel loops termed *Thiry-Vella fistulas* (TVFs), thus supporting a direct role for NT in the stimulation of gut mucosal growth [21,22]. Vagianos *et al.* [23] reported that NT restores gut mucosal integrity in rats and prevents the translocation of indigenous bacteria after radiation-induced mucosal injury. Furthermore, Izukura *et al.* [24] and de Miguel *et al.* [25] demonstrated, in separate studies, that administration of NT can augment the normal adaptive hyperplasia of gut mucosa that is associated with a massive small bowel resection.

BBS also stimulates growth of the small bowel mucosa. Administration of BBS effectively prevented mucosal atrophy associated with feeding rats a liquid elemental diet [26]. Furthermore, BBS was noted to increase mucosal weight, DNA, and protein content in both jejunal and ileal TVFs compared to control animals, suggesting that the effects of BBS were directly mediated as opposed to indirect effects of stimulation of luminal pancreatic or biliary secretion [27]. In addition to its effects on gut mucosal growth, BBS exhibits protective effects on the gut after injury [28]. Using a lethal enterocolitis model in rats induced by the chemotherapeutic agent methotrexate (MTX), BBS enhanced gut mucosal growth and significantly inhibited mortality. A beneficial effect of BBS on survival was noted when BBS was given prior to or at the same time as MTX, which suggested that BBS may act through additional mechanisms other than gut mucosal growth alone. One possibility is that BBS may produce its beneficial effects through enhancement of the immune system, a known action of BBS.

Although the data are somewhat controversial, the gut peptide PYY has likewise been shown to produce a trophic effect in small bowel mucosa of both rat and mouse [29]. These effects were noted at relatively high dosages. Similarly, Chance *et al.* [30] found that PYY treatment in Sprague-Dawley rats given total parenteral nutrition (TPN)

produced significant increases in jejunal, ileal, and colonic protein contents.

A trophic effect for glucagon-derived peptides in the intestinal mucosa has been postulated since the description of a glucagon-secreting tumor of the kidney associated with small bowel mucosal hypertrophy. Drucker *et al.* [31] was the first to demonstrate that the intestinal trophic factor was GLP-2, which produced a 50% increase in small bowel weight and a significant increase in mucosal thickness. Similarly, Ghatei *et al.* [32] demonstrated prominent trophic effects of GLP-2 in Wistar rats, and Litvak *et al.* [33] demonstrated that GLP-2 significantly increased the weight of jejunum, ileum, and colon of athymic nude mice compared to control mice. In addition to the effects of GLP-2 on normal mucosa, the effects of this agent during periods of gut injury or atrophy have also been assessed. Mice treated with indomethacin developed small bowel enteritis associated with significant mortality at 48–72 hr after administration; treatment with human [Gly^2]-GLP-2, either before, during, or after indomethacin administration, resulted in reduced mortality and decreased mucosal injury [34]. The protective effects were attributed to the significantly increased crypt cell proliferation and decreased crypt compartment apoptosis. The effect of GLP-2 on chemotherapy-induced intestinal mucositis has also been assessed. Pretreatment of mice with human [Gly^2]-GLP-2 before administration of the topoisomerase inhibitor, irinotecan, resulted in reduced bacterial translocation, intestinal damage, and mortality [35]. Histological and biochemical analyses revealed significant reductions in crypt compartment apoptosis and reduced caspase-8 activation. Consistent with these reports, Tavakkolizadeh *et al.* [36] noted decreased intestinal damage in rats given GLP-2 in combination with the chemotherapeutic agent 5-fluorouracil. Finally, repeated cyclical administration of human [Gly^2]-GLP-2 resulted in significantly decreased mortality in groups of Balb/c mice given irinotecan.

Colon

Colonic mucosal growth may be affected by the gut peptides gastrin, BBS/GRP, NT, and GLP-2. Earlier reports suggested a role for amidated gastrin (i.e., G17 and G34) as trophic factors in the colon [37]. Recent studies now suggest that glycine-extended progastrin (G-Gly) may be the responsible agent producing the effects noted with gastrin administration. These findings have sparked renewed interest in a role for gastrin precursor products in colonic growth. Koh *et al.* [38] generated mice that overexpressed progastrin truncated at glycine-72. These mice demonstrate elevated serum and mucosal levels of G-Gly compared with wild-type mice. Mice overexpressing G-Gly displayed a 43% increase in colonic mucosal thickness and a 41% increase in the percentage of goblet cells per crypt. Furthermore, administration of G-Gly to gastrin-deficient mice resulted in a 10% increase in colonic mucosal thickness and an 81% increase in colonic proliferation as measured by BrdU incorporation.

Although the small bowel is significantly more sensitive to the effects of GLP-2, studies have shown that GLP-2 and analogs can stimulate the growth of colonic mucosa. Litvak *et al.* [33] demonstrated the trophic effect of GLP-2 on the colonic mucosa of athymic nude mice. Drucker *et al.* [39] demonstrated an increase in colonic growth using dipeptidyl peptidase IV-resistant GLP-2 analog, human [Gly^2]-GLP-2, in 6-week-old female mice. A significant increase in large bowel mass was detected in mice treated with this analog for 10 days. Furthermore, the combination of this agent with either IGF-1 or an IGF-1 analog produced a greater increase in large bowel mass than mice treated with [Gly^2]-GLP-2 alone. Administration of GLP-2 increased colonic weight in Wistar rats with atrophic colonic mucosa induced by TPN administration and reduced colonic mucosal injury in a dextran sulfate-induced colitis model [32].

Other intestinal hormones may play a contributory role in colonic proliferation; however, the effects are relatively minimal. For example, BBS administered three times a day for 7 days stimulated rat colonic mucosal growth [40] and, moreover, administration of BBS orally during the neonatal period stimulated colonic growth [17]. Investigators in our laboratory have shown that, in rats given an elemental diet, the proliferative effect of BBS was confined to the proximal colon [41]. Colonic proliferation is likewise noted with NT administration; however, the effects of NT on the colon are much less pronounced than in the small bowel [42]. NT-induced colonic proliferation appears to be dependent on age, with hyperplasia noted in the colon of young rats given NT, whereas NT significantly increased hypertrophy in aged rats. Similarly, PYY has been shown to have a modest effect on growth of the colonic mucosa [29].

GI Hormone Receptors and Signal Transduction Pathways

GI hormone-stimulated signal transduction occurs with the binding of hormones to their cognate cell surface receptors, which are G-protein-coupled receptors (GPCRs) [43]. These receptors have the typical structural features of G-protein-binding seven-transmembrane receptors, which can regulate a number of physiological processes, including proliferation, growth, and development. It was originally thought that in order for GPCR signaling to occur, specific interactions between the GI hormone and the receptor were necessary to produce conformational changes in the receptor and stimulate intercellular signal transduction pathways. However, recent studies suggest a more complex regulation of the GPCRs through (1) dimerization with themselves and other receptors, (2) activation of differing G-proteins, (3) internalization and desensitization, and (4) ability to change in conformation and interactions with empty or inactive receptors [44].

The seven-transmembrane-spanning α-helical domains function as ligand-regulated, guanine nucleotide exchange factors for the intercellular heterotrimeric G-proteins [43]. Heterotrimeric G-proteins are composed of the products of three gene families encoding α, β, and γ subunits.

The agonist-activated GPCR catalyzes the exchange of GTP for GDP bound to the Gα subunit, as well as the dissociation of GTP-Gα from its cognate Gβγ dimer. The activated GTP-Gα and Gβγ subunits, in turn, regulate the activity of various intercellular effector proteins, such as phospholipases, adenyl cyclases, protein kinases, membrane ion channels, and members of the Ras family of GTP-binding proteins. In addition, based on structural similarities, the 20 identified Gα subunits have been divided into four subfamilies: (1) the cholera toxin-sensitive (α) subunits that stimulate adenyl cyclase and increase cyclic AMP levels, (2) the pertussin toxin-sensitive ($\alpha_{i/o}$) subunits that inhibit adenyl cyclase activity, (3) the pertussin toxin-sensitive ($\alpha_{q/11/14}$) subunits that stimulate membrane phospholipases, and (4) the ($\alpha_{12/13}$) subfamily that links GPCR to the Ras-related GTP binding protein, Rho [43]. Additionally, twelve Gγ and six Gβ subunits have been identified. These βγ dimers have been linked to the signaling molecules, phosphatidylinositol 3-kinase (PI3K), and select forms of adenyl cyclase and receptor kinases.

Among the multiple intercellular signaling pathways that mediate the proliferative effects of GPCRs, a family of related serine-threonine kinases, collectively known as the mitogen-activated protein kinases (MAPKs), appear to play a central role [45,46]. Hormones act as ligands to eventually activate p42 and p44 ERKs, which occurs through the involvement of a complex interplay of several known nonreceptor kinases and receptor kinases. The ability of tyrosine kinase inhibitors to reduce the activation of MAPK by GPCR and the rapid tyrosine phosphorylation of Shc (*src* homology and collagen) following GPCR stimulation with the consequent formation of Shc-Grb2 (growth factor receptor bound 2) complexes provides evidence that tyrosine kinases link GPCRs to the Ras-MAPK pathway [47,48]. Additionally, GPCRs link to the Jun-N terminal kinase (JNK), p38, MAPK, and the big mitogen-activated kinase-1 (BMK-1) or ERK5 pathways [43].

The molecular mechanisms though which GPCRs transduce signals are complex and likely involve multiple signaling pathways. In addition, the signaling pathways are likely cell specific, which may explain the diverse physiological functions controlled by gut hormones, ranging from regulation of secretion, motility, and, in some instances, growth, depending on the target tissue.

Signaling Pathways Mediating the Effects of Intestinal Peptides

Figure 1 summarizes how signaling pathways mediate the effects of intestinal peptides. Once a trophic GI peptide binds its seven-transmembrane GPCR, signal transduction pathways are activated that ultimately can lead to cell proliferation depending on cell type [43,49–52]. A number of pathways and proteins have been identified that are stimulated by the trophic gut peptides. For the most part, these pathways have been identified using neoplastic cells that possess the receptor for the trophic gut hormone.

Pathways Involving Phospholipase C, Phosphatidylinositol Activation, Calcium Mobilization, and Protein Kinase C

An early event associated with binding of trophic peptides to its receptor is activation of the phospholipase C (PLC) signal transduction pathway. For example, gastrin stimulates PLC in a number of cell types, including gastric parietal

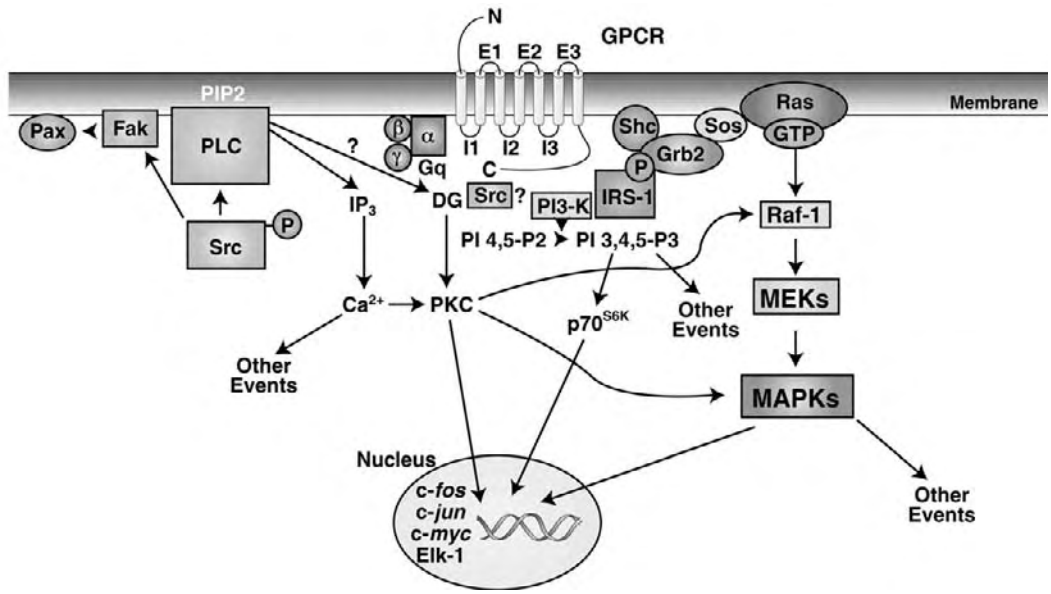


Figure 1 Summary of pathways that can mediate the trophic effect of gastrin. Although these pathways have been best described for gastrin, other gut peptides can interact with their specific G-protein-coupled receptor (GPCR) to stimulate similar pathways in receptor-positive cells. See text for details and abbreviations. [Adapted from Yassin, R. R., *et al.* (1999). Signaling pathways mediating gastrin's growth-promoting effects. *Peptides* **20**, 885–898.]

cells and colonic epithelial cells, NIH-3T3 fibroblasts that express the gastrin receptor (CCK-B/gastrin), and various neoplastic cell lines (reviewed in [53,54]). The activation of PLC, which may involve coupling of the CCK-B/gastrin receptor with selected members of the G-protein superfamily, induces the breakdown of membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) leading to the formation of two second messengers, IP_3 and 1,2-diacylglycerol (DAG). IP_3 binds to its intracellular receptor and triggers the release of calcium from internal stores. Gastrin stimulates inositol phosphate production in a variety of cell types, leading to IP_3 and DAG formation, protein kinase C (PKC) activation, and intracellular calcium mobilization [53,54]. These effects can be blocked by CCK-B/gastrin receptor antagonists. In addition to gastrin, other peptides, such as NT, have been shown to stimulate the PKC pathway, IP_3 turnover, and calcium mobilization in a number of cell types, including the colon cancer cell lines, HT29 and KM20, as well as the pancreatic cancer cell line, MIA PaCa-2 [55–58].

The trophic GI hormones also activate downstream PKC isoenzymes. Gastrin activates classic calcium- and phospholipid-dependent PKCs as demonstrated by translocation of the cytosolic activity of PKC to the membrane compartment of rat colonic epithelial cells [59]. The isoforms, PKC-α and -β, are responsive to gastrin treatment and mobilize from the cytosol with treatment [60]. In addition, recent results demonstrate that gastrin induces the novel protein kinase D (PKD, also known as PKC-μ), which has distinct structural and enzymological properties from the PKCs [61], in Rat-1 cells transfected with the human CCK-B/gastrin receptor [62]. Similarly, Guha *et al.* [63] recently reported that NT induced a rapid activation of PKD, which was linked to the mitogenic effect of NT in pancreatic cancer cells.

Tyrosine Kinases, Tyrosine Phosphorylation of Focal Adhesion Kinase, Paxillin, and CRK-Associated Substrate

Through pairing with nonreceptor tyrosine kinases, GPCRs utilize the tyrosine kinase pathway to stimulate cell growth [64]. Gastrin stimulates tyrosine kinase activity and tyrosine phosphorylation of a 57-kDa membrane protein in rat colonic mucosal cells and also membrane-associated protein tyrosine kinase and tyrosine phosphorylation of endogenous proteins in the IEC-6 intestinal cell line [65–67]. In addition, gastrin results in tyrosine phosphorylation of 62- and 54-kDa Src-like proteins in IEC-6 cells and $pp60^{c\text{-}src}$ kinase in rat colonic epithelial cells, which leads to tyrosine phosphorylation and activation of PLCγ1 [67,68].

Growth factor receptors with intrinsic kinase activity and those that signal through G-proteins can promote tyrosine phosphorylation of the adaptor protein Shc, which links activated growth factor receptors to the Ras signaling pathway, and its subsequent association with the Grb2-SOS (growth factor receptor binding protein-2/SON of sevenless) complex [69]. Gastrin promotes a rapid and transient increase in tyrosine phosphorylation of the Shc proteins and association with Grb2-SOS, leading to activation of MAPKs in the human gastric cancer cell line, AGS-B, which expresses the human CCK-B/gastrin receptor [70].

The involvement of PI3K, as well as other SH2 anchoring proteins, in gastrin's mitogenic pathway has been reported. Gastrin induces tyrosine phosphorylation of the insulin receptor substrate 1 (IRS-1) and association with the 85-kDa subunit of PI3K [71]. Gastrin also stimulates the association of phosphorylated IRS-1 with the adaptor Grb2, indicating that tyrosine phosphorylation of IRS-1 may be a mechanism whereby gastrin activates PI3K and other adapters [71]. Therefore, mobilization of the adapter proteins IRS-1, Shc, Grb2, and SOS could serve to link the CCK-B/gastrin receptor to the Ras-MAPK cascade, ultimately leading to transcriptional regulation [53].

Gastrin treatment results in the tyrosine phosphorylation of various proteins [67,72]. For example, gastrin promotes tyrosine phosphorylation of focal adhesion kinase ($p125^{fak}$), a tyrosine kinase that localizes at focal adhesions and is important for cell adhesion and transformation [72]. The focal adhesion proteins paxillin and CAS are potential downstream targets for $p125^{fak}$ and function as adaptor proteins. Gastrin induces phosphorylation of $p125^{fak}$, CAS, and paxillin in Rat-1 and NIH-3T3 cells transfected with the CCK-B/gastrin receptor [72,73].

MAPK Pathway

The MAPKs are a family of highly conserved serine-threonine kinases that are activated by a variety of extracellular signals and relay mitogenic signals to the nucleus [74]. The activation of the MAPK pathway by gastrin has been demonstrated in a variety of cell types, both containing the endogenous CCK-B/gastrin receptor and cells stably transfected with this receptor [69,70,72]. Gastrin also stimulates the serine-threonine kinase Raf-1, the cellular homolog of the Raf oncogene and an upstream modulator of the MAPKs [70,72]. The mechanism for the effect of these hormones on MAPK activation is dependent on cell type. For example, in the AR4-2J pancreatic cell line, gastrin activated ERK, which was attenuated by treatment with agents that interfere with calcium mobilization or PKC activation [75]. In other cells, the activation of MAPK by gastrin does not involve PKC. In Chinese hamster ovary (CHO) cells transfected with the CCK-B/gastrin receptor, gastrin induces ERK activation partly through the Src and PI3K pathways and partly through PKC [76]. Conversely, gastrin-stimulated Raf-1 and ERK activation in Rat-1 fibroblasts stably transfected with the CCK-B/gastrin receptor occurs independent of PKC [72]. Likewise, PYY stimulates MAPKs through binding of the Y1 receptor [77]. The stimulation of MAPK can occur via multiple and diverse pathways, but in the case of PYY, it has been shown that PKC plays a major role in the signaling pathway between the Y1 receptor and MAPK, acting between the EGFR and MAPK. In CHO cells transfected with the human Y1 receptor, both PKC and Ras are needed for activation of MAPK [78]. Specifically, only PKC-ε, an

isoform that has been specifically linked to mitogenic effects in gut epithelium [79], was activated by PYY in the IEC-6 intestinal cell line. NT has been shown to stimulate ERK and JNK activity in various neoplastic cell types containing endogenous NT receptors, ultimately leading to transcription factor activation [57,58]. Therefore, activation of MAPKs appears to be an important mechanism for the mitogenic effects of intestinal hormones; this activation can occur by a variety of signal transduction mechanisms and depends on cell context as to which pathway is active in which cell type.

Downstream Transcription Factors

Ultimately, stimulation of various signaling pathways, such as PKC, the MAPKs, or ribosomal S6 kinase ($p70^{S6K}$), can lead to activation of downstream transcription factors. Gastrin stimulates the expression of early response genes, including c-*fos* and c-*jun* in AR4-2J cells, and c-*fos* and c-*myc* in NIH-3T3 cells transfected with the CCK-B/gastrin receptor [73,80]. Gastrin induced ERK-mediated phosphorylation and activation of these transcription factors was prevented by pharmacologic inhibition of PKC [81], suggesting an important role for the PKCs and ERKs in the activation of the AP-1 transcription factors. In addition, NT and BBS have been shown to stimulate expression of c-*jun* and c-*fos* in cell types possessing endogenous receptors for these peptides [57,58,82]. It is likely that the activation of these, as well as other transcription factors, such as Elk-1, ultimately plays a major role in the mitogenic response of these hormones.

Conclusions

The growth of GI mucosa is modulated by multiple factors, including intraluminal nutrients and the local release of growth factors and various GI hormones. These hormones bind to their specific receptors, stimulating many signal transduction pathways, which ultimately lead to the mitogenic effects of these gut peptides. In this regard, the GI hormones have been suggested as potential therapeutic agents in disease states in the non-neoplastic GI tract related to gut disuse or atrophy, mucosal ulcers, or inflammatory conditions. For example, the trophic peptides, NT, BBS/GRP, and GLP-2, can augment or maintain GI mucosal growth during periods of gut disuse or atrophy [21,22,27,34]. In addition, these peptides enhance adaptive hyperplasia associated with massive intestinal resection [24,25]. In a limited clinical trial, administration of GLP-2 improved intestinal energy absorption, decreased energy excretion, increased body weight and lean body mass, and enhanced urinary creatinine excretion in patients with short bowel syndrome [83]. In addition, GI hormones may play a role in preventing the severe sequelae of chemotherapeutic agents on the intestinal mucosa. Both BBS and GLP-2 have been shown to prevent the severe mucosal inflammation associated with various chemotherapeutic agents [28,35,36]. In the future, it will be important to further define the signaling pathways regulated by the trophic intestinal peptides so that more effective agents can be developed that can take advantage of the gut-specific effects of these hormones on the proliferation and maintenance of the GI mucosa.

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CHAPTER 338

Integrated Response to Neurotrophic Factors

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Introduction

Neurotrophic growth factors are proteins responsible for cell survival, proliferation, migration, neurite elongation, neurotransmitter expression, and synaptic maturation in the nervous system. The cellular targets of neurotrophic factors are not restricted to neurons because they act on both neurons and glia (for example, astrocytes, oligodendroglia, microglia, and Schwann cells). The mechanisms of action of neurotrophic factors are probably best understood as they apply to effects on cell survival, the focus of this chapter.

Neural Cell Death

Programmed cell death during development of the nervous system, also called *apoptosis*, is a tightly controlled, highly ordered, physiological form of cell death wherein a cell actively commits to apoptosis if it fails to garner sufficient trophic factor activation of its endogenous programs regulating metabolism [1]. The distinction is rarely made between the original term of apoptosis and the mechanism of programmed or delayed cell death, and these terms are used interchangeably. Apoptosis can be triggered at the level of the individual neuron or glia by a wide variety of ligand/receptor-mediated stimuli during embryonic development [2]. Thus, various ligands, or a combination of ligands, can set off signals that culminate in apoptotic cell death, appropriate ongoing physiological function, or proliferative expansion. In addition, neural cells (neurons or glia) can also commit to cell death, exhibiting apoptotic features as part of a spectrum of responses to trauma and disease [3]. These responses have a common component of oxidative stress, and the resultant cell death is also referred to as delayed cell death [4]. Although these forms of cell death can have different properties, they all commit a neural cell to death with some common cellular and molecular mechanisms. Here, we refer to these forms of cell death by the name of *apoptosis* in contrast to another form of cell death, *necrosis*, which results from the abrupt impairment of energy metabolism associated with sudden trauma. While the morphological hallmarks of apoptosis include cell shrinkage and membrane blebbing, nuclear condensation and DNA fragmentation with a ladder-like electrophoretic appearance [4], necrosis is a pathological form of cell death that results from massive cellular injury and a resultant prompt and robust energy depletion that is characterized by swelling of the cytoplasm, organelle dissolution, and rupture of the plasma membrane followed by inflammation [2–4]. Apoptosis also displays characteristic molecular changes that include increases in intracellular calcium ion fluxes, increased activation of caspase enzymes, and shifts in the levels and the intracellular occupancy of the Bcl-2 family of proteins [5–8].

During the last two decades, the morphologic appearances of dying cells and their relationship to specific metabolic pathways responsible for cell death have been the focus of extensive studies and discussions. The literature regarding the roles of apoptosis and necrosis in brain trauma is complex and sometimes contradictory, with morphological studies often giving results that differ from biochemical studies (see [3] for a review). Furthermore, when the adult nervous system undergoes injury, the dying neural cells typically first display necrosis and only later display versions of apoptosis with properties that differ from that observed in either

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undisturbed developmental cell death or the cell death that results from injury to a developing nervous system [3]. For example, in adult animal models of injury, both necrosis and apoptosis have been reported following focal cerebral ischemia in rats and mice [9–13] or after global cerebral ischemia [14,15], and in both instances significant delayed neuronal death occurs in the same regions of the hippocampus. Others have stated that, based on electron microscopic morphological criteria, no neuronal apoptosis occurs after global cerebral ischemia in adult animals [16–18]. Recently, it has been suggested that neurons exhibit a spectrum of morphological and biochemical changes indicative of cell death, with apoptosis and necrosis at opposite ends of the spectrum of observed changes [3,8,17,19–21]. Thus, the specific morphological features seen in a given cell may reflect a variety of factors that influence the cellular events that follow injury, including cellular phenotype and developmental stage, the severity of the insult, the survival time after insult, and the history and aftermath of previous insults.

In immature animals experiencing trauma early in development, the light microscopic appearance of dying neurons is different from that observed in adults. For example, the term *karyorrhexis* has been used in immature brains to describe a nucleus that in the early stages after ischemic injury breaks up into relatively large, darkly basophilic, rounded clumps of chromatin. In the human fetal and neonatal brain, Scott and Hegyi [22] found the presence of karyorrhexic neurons in the basis pontis, an area known to be susceptible to selective neuronal death (*pontosubicular necrosis*) and suggested that karyorrhexis has features common to both necrosis and apoptosis. Other investigators have suggested that both necrosis and apoptosis occur after perinatal ischemia, or any form of excitotoxic injury, in the immature rat [23,24] and that the type of cell death observed varies among brain regions [17,25]; the severity of injury [26,27], the specific stage of development of the animal, and the individual cell phenotype studied [28]. Other factors that may determine whether a necrotic or apoptotic outcome results from trauma to the nervous system are interactions among different neuronal phenotypes—oligodendrocytes, astrocytes, and microglia—together with the different trophic and hormonal activity levels present at the time of injury.

In summary, responses to trauma display a spectrum of responses ranging from cellular commitment to necrosis to apoptosis, or, of more significance, to recovery events that result in a complete return to physiological function [8]. Furthermore, there is evidence for overlap in the features of these different end points that depends on neuronal development and the nature of injury. Here, we focus on apoptosis and apoptotic cell death to reflect the fact that the phenotype associated with cell death during development of the nervous system is the best understood in terms of its regulation by epigenetic factors (neurotrophic factors) whose activity is dependent on cell–cell interactions [29]. By contrast, trauma-induced delayed cell death typically displays morphological and molecular features in common with apoptosis and dissimilar from necrosis [2]; the broad spectrum of cell-type-specific features in both kinds of cell death outcomes has not been fully characterized [3].

The Neurotrophic Hypothesis

The strategy for establishing the final neural net that allows a nervous system to process sensory signals, establish memory engrams, and generate responses ranging from the endocrine and motor to the sensation of self-awareness depends in some fashion on the principle of the *neurotrophic hypothesis*, perhaps best illustrated during the early neuronal development of the sympathetic nervous system. Early in development there is an overproduction of precursor sympathetic neurons, sympathicoblasts, that when exposed to the nerve growth factor protein (NGF), in the absence of glucocorticoids, extend neurites oriented to the NGF source (Fig. 1A). Over time a decrease is seen in the ambient levels of NGF even as the extended growth cones contact target tissues that synthesize and release the reduced amounts of NGF. Synaptic contact

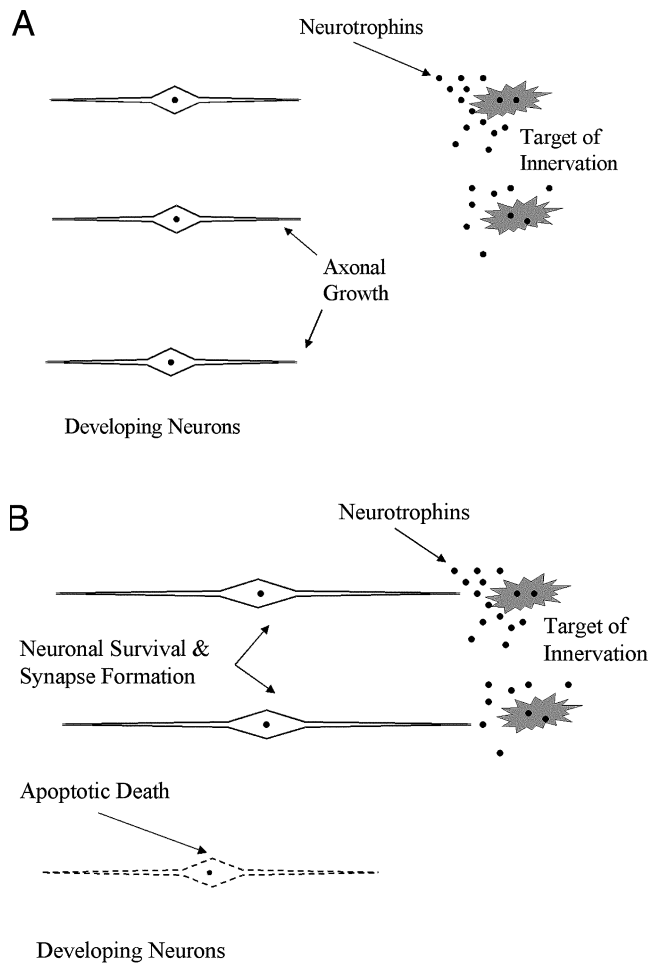


Figure 1 Neurotrophic hypothesis. (A) Target tissues release neurotrophic factors that guide axons. (B) Neurons that reach targets form synapses with targets and compete successfully for neurotrophic factors, whereas neurons that do not make successful contact experience neurite pruning and cell death with apoptotic properties.

between the neuronal growth cones that display NGF receptors takes place and binding of nearby NGF molecules to these receptors occurs, followed by internalization and the eventual retrograde transport to the neuronal soma of both encapsulated NGF-receptor complexes and activated NGF receptors [30]. Those sympathicoblasts whose axons fail to reach a target become growth factor deprived, experience neurite pruning, and perish via apoptosis, the fate of half of all sympathetic neurons during development (Fig. 1B). This is not a unique event because the fate of neuronal apoptosis during development is common to almost all neuronal populations [29].

Experiments that support the neurotrophic hypothesis have shown that an overproduction of neurons early in development is followed by a significant decrease in their number as maturity is reached; that the synthesis of neurotrophic substances takes place at tissues that are targets of innervation, but not the neurons that innervate them; and that cognate high-affinity neurotrophic receptors are present in the innervating neurons but not in their target tissues. Furthermore, reducing the levels of the neurotrophic factors or of their cognate receptors or interrupting the retrograde transport of neurotrophin results in the death of the innervating neurons, an event that can be abrogated by the external application of the appropriate growth factors to the innervating neurons [32–36].

Neurotrophins

Although a large number of growth factor proteins have been shown to display neurotrophic activity, the best characterized are the neurotrophins. During the last half-century four neurotrophins (NGF, BDNF, NT-3, and NT-4/5) were isolated [37–52] and shown to display a 50–60% sequence homology among themselves [52] and 25% sequence homology to proinsulin [42]. The neurotrophins are a family of related protein growth factors that share certain structural features with other signal proteins belonging to the cysteine knot superfamily [53]. The predominant feature of the cysteine knot family of proteins consists of ring structures made up of intracellular disulfide bridges that form a tightly packed "cysteine knot" that allows for homodimers with extensive surfaces in contact between antiparallel disposed monomers and a strong noncovalent bond between dimer members ($K_d \sim 10^{-13}$ *M* for NGF). Neurotrophins are typically very basic small proteins (pI ~ 9–10; MW ~ 12–14 kDa). For one such neurotrophin, NGF, the biologically active dimer (β-NGF) can be isolated as part of a hetero hexamer made up of the β-NGF dimer and two other protein dimer kallikreins, one active, γ-NGF, and one inactive, α-NGF, which together with two zinc atoms form a stable equilibrium complex ($\alpha_2\beta_2\gamma_2Zn_2$) called 7S NGF based on its equilibrium sedimentation constant [52].

Murine 7S NGF is a stable multimer within a pH range of $5 \leq pH \leq 9$ and at concentrations consistent with its dissociation equilibrium constant ($K_d = 10^{-9}$ *M*) [52]. Dissociation and association of the 7S complex serves regulatory functions given that, whereas cross-linked β-NGF is biologically active, the cross-linked 7S multimer is not active and the equilibrium dissociation constant for 7S NGF is in the same range as the binding equilibrium constant of neurotrophins for the $p75^{NTR}$ receptor [53]. The α-NGF subunit stabilizes the 7S NGF complex, but has no other known biological function [54] and the γ-NGF subunits are arginine-specific esteropeptidases of the serine family that process the pro-β-NGF precursor yielding the mature NGF form [55]. The significant levels of neurotrophin precursor forms present throughout adulthood may serve a complex regulatory function given the reported widespread presence of proneurotrophins and the selective cleavage of pro-NGF by γ-NGF and of pro-BDNF by the matrix metalloproteimerase MMP-7 but not MMP-2 or MMP-3 [56]. The nature and processing of NT-3 and NT-4/5 are not known.

It has been proposed that the relatively high ambient levels of proneurotrophins, compared to their mature forms, suggest a regulatory role for proneurotrophin [56]. For example, the NGF $p75^{NTR}$ receptor has been shown to exhibit preferential binding to proNGF as compared to the mature β-NGF dimer, with likely proapoptotic consequences [56]. Given that $p75^{NTR}$ itself enhances the affinity of the other NGF receptor, TrkA, for NGF and decreases the affinity of TrkA for NT-3, it is likely that proneurotrophin processing and binding of precursor forms to $p75^{NTR}$, together with environmental effects of pH and neurotrophin concentration on neurotrophin multimer dissociation, such as 7S NGF, provide a delicately balanced set of interactions that can selectively affect cell survival in response to both developmental cues and environmental changes during development or in the aftermath of stress-related events.

Although the large proportion of neurons affected by the neurotrophic hypotheses suggests that this is a dominant mechanism for the appropriate matching of neuronal numbers and connections and targets to be innervated, it also serves as a mechanism to correct inappropriate or aberrant connections and the culling of neuronal populations that serve transient functions during narrow developmental windows [57–60]. Given that regulation of the synthesis of neurotrophic factors and their receptors is itself regulated by steroids and cytokine and chemokine signaling molecules, it is not surprising that neurotrophic factors play a role in the establishment of sexual dimorphism in the central nervous system [61,62]. Thus coexpression of steroids and neurotrophins in the central nervous system is consistent with the regulation of neurotrophin synthesis by steroids both *in vitro* and *in vivo* [61,62]. For example, estrogen stimulates BDNF and TrkA synthesis and dexamethasone treatment inhibits $p75^{NTR}$ expression [63]. Cytokines have also been shown to stimulate NGF synthesis by astroglial cells in the central nervous system [64]. Given the role of steroids and cytokines/chemokines in the stress response signaling pathways, it is not surprising that the neurotrophin trophic factors attenuate oxidative-stress-induced cell death via stimulation of antioxidant enzymatic conversion of ROS to nontoxic molecules, inhibition of DNA repair energy depletion,

and stimulation of anti-apoptotic activity such as the synthesis and translocation of members of the Bcl-2 family of proteins [65–70].

The neurotrophic hypothesis is dependent on the retrograde transport of neurotrophic factors from nerve terminals with access to growth factor secreting tissues. There is also evidence for the anterograde transport of growth factors (principally NT-3 and BDNF; [71–73]) in the avian and mammalian nervous system. While retrograde transport of neurotrophins, a basic element of the trophic hypothesis, has been best documented for NGF, the evidence for anterograde transport is best demonstrated for BDNF, in the context of a response to increased neurotransmitter signaling [72,73]. It is tempting to suggest that although NGF is principally involved in cell survival outcomes, BDNF plays a role in synaptic plasticity in the adult nervous system. Such speculation would be consistent with the temporal pattern of expression of the neurotrophins and neuronal dependence on the different neurotrophin molecules during development with NT-3 and NT-4/5 playing a major role earlier in development at a time when cellular proliferation, migration, and differentiation are central, and BDNF and NGF appearing later in development as both neurite growth and synaptic connectivity patterns are established.

The evidence for an interaction between neuronal activity and neurotrophin action is not limited to BDNF. Both limbic seizures and physical exercise have been shown to stimulate cortical and hippocampal NGF levels [74–76]. Whether the mechanisms linking physiological activity and neurotrophin levels and effects are the same as those triggered by injury is not known. In part, this follows from the ubiquitous nature of the glutamate-ROS-cytokine inflammatory cascade triggered by all trauma and the close linkage between neurotransmitter action (glutaminergic, cholinergic, dopaminergic and serotonergic) and oxidative stress in the nervous system. For example, this is especially true of brain regions involved in memory and cognition where synaptic and functional plasticity occur via neurotransmitter systems whose robust action is associated with significant oxidative stress. Thus, exaggerated plasticity results in toxicity; although the quantitative determination of the correlation between neurotransmitter action and toxicity has not been determined in terms of specific molecular pathways.

Neurotrophin Receptors

The initial event in the regulation of cell death by neurotrophins during development is the binding of a neurotrophin ligand to a neurotrophin receptor, and for the neurotrophin factors (NGF, BDNF, NT-3, NT-4/5; [77]) there are two categories of receptors: $p75^{NTR}$ and the Trk receptors. The structure–function features of the actions of these ligands with their cognate receptors ($p75^{NTR}$, TrkA, TrkB, TrkC) is fairly well understood, although the details of the machinery activated may be less so [77,78]. The $p75^{NTR}$ receptor belongs to the tumor necrosis factor (TNF), fas antigen receptor (Fas) family of receptors, all of which contain an intracellular death domain. The $p75^{NTR}$ is a relatively small receptor that is able to trigger ceramide signaling and NFκB activation [79–82]. The ability of $p75^{NTR}$ to mediate both the cell survival and cell death of neurons and glia depends on its interaction with other neurotrophin receptors [53,83–90]. This is because $p75^{NTR}$ is a relatively low-affinity ($K_d \sim 10^{-9} M$) receptor typically present at 25- to 40-fold higher levels than the Trk high-affinity ($K_d \sim 10^{-11} M$) neurotrophin receptors. The three Trk receptors ($p140^{TrkA}$, $p145^{TrkB}$ and $p145^{TrkC}$) belong to the tyrosine kinase family of receptors that share an amino acid sequence homology with the tropomyosin receptor kinase. They are commonly called TrkA, TrkB, and TrkC. The Trk receptors display differential binding to the different neurotrophins [53,89]. Whereas the $p75^{NTR}$ receptors appear to bind equally to any of the neurotrophins, the Trk receptors do show some specificity with TrkA preferentially binding to NGF, TrkB preferentially binding to BDNF and NT-4/5, and TrkC preferentially binding to NT-3 (Fig. 2) [89]. As with other tyrosine kinase receptors, there are nonfunctional truncated TrkB and TrkC receptors, which may serve to modulate neurotrophin levels extracellularly, although this has not been unequivocally demonstrated [90]. In addition, although the $p75^{NTR}$ receptor is supposed to bind any of the neurotrophin ligands, there are reports of selective activation of ceramide signaling only in the presence of NGF, and not the other neurotrophins, which suggests that NGF signaling is the more selective and specific of the Trk neurotrophin interactions. Not surprisingly, BDNF and NT-3 Trk receptors are widely distributed in the nervous system compared to the more restricted distribution of TrkA receptors.

Studies using neurotrophin and neurotrophin receptor null mice would suggest that abolition of the TrkA gene disturbs sensory neurons in the periphery to a greater extent than the TrkA bearing neurons in the central nervous system (basal forebrain cholinergic neurons and striatal cholinergic interneurons) [91,92]. It is also interesting that both TrkB and TrkC are widely distributed in the central and peripheral nervous system and that null mice lacking individual neurotrophin or neurotrophin receptor genes have phenotypes in

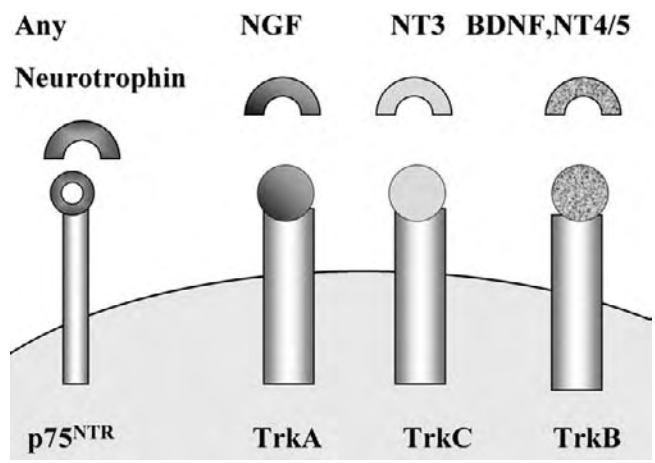


Figure 2 Neurotrophins and neurotrophin receptors.

which it appears that the absence of one growth factor or its receptor is compensated by the expression of others [93–96]. Thus, although there are specific developmental stages for the maximal expression of the different neurotrophins, null mice studies would suggest that there is also a great deal of compensation for the absence of a neurotrophin at a specific developmental stage. This is in agreement with more recent gene network descriptions that would suggest that specific physiological functions dependent on epigenetic growth factors are dependent on the actions of clusters of growth factors working in a complementary, but also redundant, fashion [91,92].

Nevertheless, cell death regulation by neurotrophins can be very selective. For example, in the central nervous system, NGF is synthesized and released by glutaminergic neurons in the hippocampus, where it is taken up by axons projecting from the basal forebrain cholinergic neurons, which express $p75^{NTR}$ receptors [53,83–90,98–104]. TrkA is not exclusively expressed by the cholinergic neurons of the basal forebrain; TrkB is also expressed there in association with minor responses to BDNF [97]. The basal forebrain cholinergic neuron $p75^{NTR}$ receptors can also bind and internalize the well-characterized monoclonal antibody to $p75^{NTR}$, 192 IgG. When 192 IgG is cross-linked via a disulfide bond to the ribosomal inactivating protein, saporin, the immunotoxin 192 IgG–saporin complex is itself internalized into cholinergic basal forebrain neurons [99–104]. Thus, after an i.c.v. injection of 192 IgG–saporin, the immunotoxins are internalized at the terminals of $p75^{NTR}$-bearing cholinergic basal forebrain neurons, retrogradely transported, and accumulated in the cell bodies of cholinergic basal forebrain neurons where they cause cell death. Treatment with intraventricular administration of immunotoxin produces selective and dose-dependent cell death of up to 90% of all $p75^{NTR}$-bearing cholinergic basal forebrain neurons with substantial reductions in the activities of the acetylcholine degrading and synthesizing enzymes, acetyl cholinesterase and choline acetyltransferase, in the rat basal forebrain and its neocortical and hippocampal afferents [99–104]. These cell losses and cholinergic deficits accompany cognitive deficits.

Neurotrophin Signaling Pathways

The neurotrophic hypothesis states that the competition for target-derived trophic factors provides a mechanism for the innervation of target tissues during the development of the nervous system. The properties of neurons that perish apoptotically during development, the transient or permanent nature of their dependence on those trophic growth factors at the termination of the developmental sequelae, or the role of these growth factors in adult neuronal function or responses to injury can vary greatly. However, in spite of these variations, certain common features are worth examination.

Two triggers bring about the death of a neuron. First, we have the intrinsic signaling pathways, which act via binding of neurotrophins or cytokines (for example, NGF or TNF-α) to cognate receptors, such as $p75^{NTR}$, that can irreversibly commit a cell to apoptosis [84,98,105–110]. Second, we have the signaling pathways, which result from changes in membrane transport, energy metabolism, and cellular redox and pH that can directly activate intracellular signaling cascades committing a neuron to apoptosis.

The nature and magnitude of Trk and $p75^{NTR}$ receptor activity are modulated by interactions between these two receptor families [84]. For example, although $p75^{NTR}$ can act as an apoptotic stimulus and Trks typically mediate anti-apoptotic outcomes, $p75^{NTR}$ can also enhance neurotrophin binding to Trk receptors by increasing the local neurotrophin concentration in the environment of a Trk receptor, a function that is dependent on the ratio of $p75^{NTR}$ to Trk receptors present on a particular cellular membrane. There is also evidence for a direct $p75^{NTR}$-mediated conformational change in TrkA that augments binding and activity. The antagonistic actions of $p75^{NTR}$ and the Trk can serve two functions: (1) In the absence of growth factor in the environment of a neural cell early in development, cells die promptly and when neurotrophins become available, high-affinity binding to Trk receptors abrogates the $p75^{NTR}$ apoptotic effect; and (2) once a neuron extends neurites, if it reaches an inappropriate target that releases the "wrong" neurotrophin, its weak binding to the inappropriate Trk will not able to overcome $p75^{NTR}$ proapoptotic signaling. In this way wrong "connections" are abolished. Thus, having two antagonistic signaling systems for the regulation of cell survival allows for developmental adjustments of neuronal number to target size and the elimination of errors in innervation. Experimental evidence for these two developmental regulatory phenomena are present for both peripheral and central neurons [106–108] and glial cells [109,110] that bind NGF, BDNF, and NT-3 [105].

Although the intracellular signaling pathways triggered by neurotrophin binding to $p75^{NTR}$ and Trk receptors differ significantly, consistent with the different outcomes of their activity, there are also common signaling elements acting in both instances (Figs. 3 and 4). The signal transduction pathway activated by $p75^{NTR}$ consists of three major interactive pathways. $p75^{NTR}$ stimulates sphingolipase-mediated hydrolysis of sphingomyelin to ceramide, which will alter membrane fluidity and hence Trk-Trk association, known to markedly stimulate Trk autophosphorylation [111,112]. Increased ceramide levels can also directly stimulate activation of the transcription factor NFκB proapoptotic pathways directly [80,109,110]. Neurotrophin binding to $p75^{NTR}$ can also stimulate NFκB via TNFR-associated factor (TRAF) 6, as part of a complex pathway regulating cell cycle progression with anti-apoptotic properties that is likely to stimulate transcription of Bcl-2 and Bcl-x in early development and Bcl-x in the adult central nervous system [70,80–82,113,114]. A third apoptotic pathway that is stimulated by $p75^{NTR}$ is the mitogen-activated protein kinase (MAPK) kinase (MEKK) $\rightarrow$ Jun amino-terminal kinase (JNK) $\rightarrow$ c-Jun $\rightarrow$ p53 $\rightarrow$ Bax pathway [115–118].

The Trk receptors mediate a number of other neural functions in addition to their well-known survival effects.

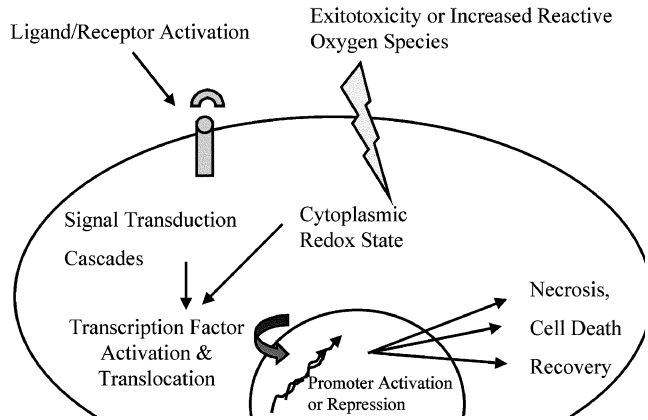


Figure 3 Schematic of activation of stress response signaling mechanisms that determine cellular outcomes: necrosis, apoptosis, recovery.

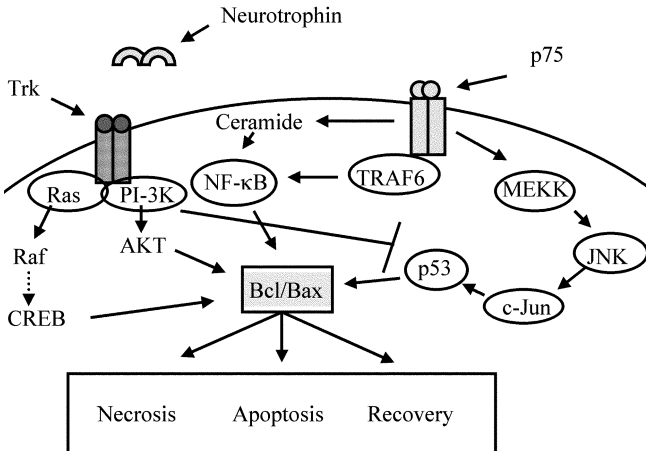


Figure 4 Regulation of cellular commitment to cell death or recovery by neurotrophin receptor activation.

These include regulation of neurite elongation, synapse maturation, neurotransmitter phenotype, and activity levels. Although the specific pathways involved in some of these developmental and plasticity-related functions are not known, it is known that the Ras → Raf → MEK → MAPK pathway, which also is a determinant of cellular commitment to apoptosis, is also likely to be involved. The Ras pathway, together with the PI3K → AKT pathway also promote cell survival by stimulating Bcl-2, Bcl-x while inhibiting p53 effects on Bax expression [116–124]. Furthermore, synergistic and antagonistic effects are acting on Bcl/Bax expression via phosphorylation cascades involving several of the pathways affected by neurotrophin occupancy of $p75^{NTR}$ and Trks in addition to transcriptional regulation via the AP-1 (Fos/Jun family) and NFκB transcription factors. To summarize, there are both antagonistic and mutually interactive effects at the level of proneurotrophin to mature neurotrophin processing, generation of truncated versus full-length Trk receptors, differential triggering of receptor-associated phosphorylation cascades, and AP-1 and NFκB differential transcription factor activation and repression of specific genes that orchestrate the balance of apoptotic versus anti-apoptotic determinants and shift the cell from a necrotic to an apoptotic outcome or from an apoptotic outcome to unimpeded survival and function.

The common signaling elements underlying the events that can bring about apoptosis act via reactive oxygen and nitrogen molecular species and the phosphorylation of proteins as activating or deactivating events. For example, NO generation in the glutaminergic system, or oxygen radical generation in the dopaminergic system, are triggers for cascades whose end point can be cell death and neurological dysfunction after injury or ischemia [4,6]. Furthermore, both NO and H_2O_2 participate in cross-reacting signaling pathways that yield the very reactive hydroxyl and peroxynitrite radicals that, while required for learning and memory processing in brain, are also toxic to neurons and oligodendroglia and over time, or when exacerbated after acute trauma, interfere with proper signal processing by neurons. Downstream from the cytoplasmic signaling cascades responsible for cellular regulation of apoptosis [105], the AP-1 and NFκB transcription factors determine neuronal death outcomes during development or after trauma.

Transcriptional Regulation

The final outcomes of neurotrophin action in terms of cell death versus survival, or aspects of neuronal growth or regeneration, and synaptic plasticity result from the balanced integration of signaling cascades triggered by ligand binding to cell-surface receptors and the subsequent signal transfer to transcription factor assemblies, themselves responsive to the cytoplasmic redox milieu [5,105]. In turn, the selective activation of transcription factors determines the activation or repression of hosts of genes that determine physiological outcomes. Although the number of transcription factor families that participate in these orchestrated events is both large and ever growing [125], we discuss here just two transcription factors that regulate cell death commitment: activator protein-1 (AP-1) and nuclear factor kappa B (NFκB). The first step in AP-1 activation is via prompt *de novo* synthesis of AP-1 proteins and their nuclear translocation and phosphorylation in response to redox changes that activate or repress transcription. Direct activation of NFκB results from the phosphorylation of inactive cytoplasmic protein complexes that permit nuclear translocation and binding to DNA consensus sequences on gene promoters.

AP-1

Members of the AP-1 transcription factor family have been shown to play an important role in cell proliferation, differentiation, and survival [126]. The AP-1 complex recognizes a set of specific DNA sequences (TREs) present in many enhancer or promoter regions of genes [126]. The AP-1 Jun proteins have two functional domains that bind to a DNA consensus

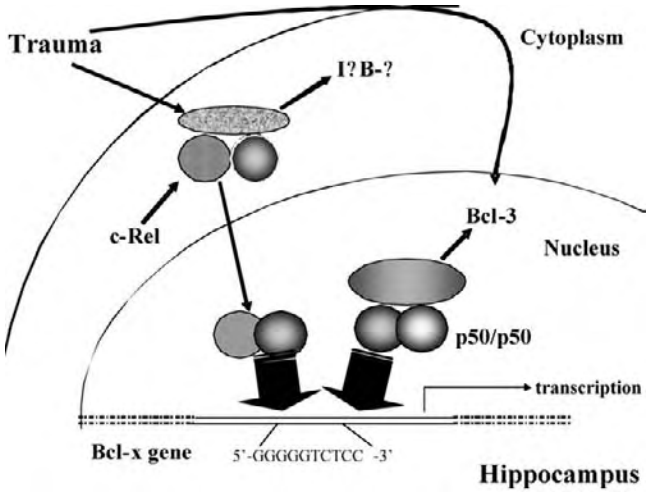


Figure 5 The AP-1 proteins.

binding sequence CCCAGGC and to Fos proteins, respectively. The AP-1 contains a leucine zipper. Cellular AP-1 typically consists of dimeric complexes made up of different Fos and Jun protein family members (Fig. 5) made up of Jun protein homodimers or Fos/Jun heterodimers. The Fos family of proteins consists of c-Fos, Fos B, Fra 1, and Fra 2. The Jun family of proteins consists of c-Jun, Jun B, and Jun D.

Changes in the relative levels of the different members of the AP-1 family or their state of phosphorylation serve to provide specific outcomes in terms of cellular commitment to modes of cell death versus cellular proliferation, for example. In some cell types, repression of AP-1 activity has been shown to trigger apoptosis. For example, both dexamethasone and retinoic acid repress AP-1 function and induce apoptosis in lymphocytes, and E1A, which represses AP-1 transaction activity in transfected cells, also induces apoptosis in several cell types [127]. In other instances, induction of the AP-1 c-Jun protein appears to cause apoptosis. It has been shown that overexpression of c-fos and c-jun is required for apoptosis in a murine cytokine-dependent lymphoblastoid cell line after growth factor deprivation [128]. Of relevance here is that it has been shown that the c-Jun protein plays a necessary role in the death of sympathetic neurons after NGF withdrawal and that AP-1 activity is necessary for apoptosis under these conditions [129]. However, in a rat pheochromocytoma cell line with some sympathetic cell properties (PC12), treatment with NGF induces differentiation into sympathetic-like neurons and prompt and transient expression of c-fos and c-jun mRNA [130] with increased AP-1 DNA binding activity [131]. A more thorough examination of AP-1 DNA binding activity, and the composition of AP-1 and AP-1-mediated transcription during NGF-mediated rescue in serum-deprived PC12 cells [132] and also the hippocampi of rats exposed to oxidative stress [133], showed that although there is no significant change in the overall composition of AP-1 in PC12 cells after serum deprivation, protection with NGF treatment significantly increased the activity levels of participation of Fra 2 and Jun B in AP-1 DNA binding, as determined by immunodepletion/supershift assays [132]. Thus, although transcription of some common genes may take place under both serumless and NGF treatment conditions, a unique set of genes is transcribed after exposure to NGF via increased levels of Fra 2 and Jun B that contribute to PC12 cell differentiation and to NGF-mediated anti-apoptotic outcomes [134,135]. Thus, the differences in the AP-1 dimer proteins induced or activated by phosphorylation of c-Jun can alter cell death outcomes.

NFκB Transcription Factor

The NFκB family of transcription factors regulates genes mediating inflammation, responses to infection, oxidative stress, and the aftermath of ambient necrotic events [136]. NFκB is made up of five structurally related protein subunits: p50, p52, p65/RelA, c-Rel, and RelB, which form a variety of homo- and heterodimers in multiple tissues [70]. Unlike p65, RelB, and c-Rel, the p50 and p52 subunits result from proteolytic cleavage of their p105 and p100 precursor molecules, respectively. The precursor forms include in their structure C-terminal IκB-like ankyrin repeats that inhibit p50 and p52 activity by virtue of their high affinity for these two proteins. The Bcl-3 proteins (not related to the Bcl-2 family) are nuclear proteins with sequence homology to the IκB inhibitor proteins that can "bind away" p50 or p52 homodimers from promoter NFκB binding sites in nuclear DNA and allow p65/p50 or cRel/p50 activating heterodimers to bind the unoccupied NFκB binding sites and thus stimulate gene-specific transcription [137]. All five NFκB/Rel proteins share a 300-amino-acid region, the Rel homology domain, which is the structural basis for dimerization, DNA binding, and nuclear localization [138]. The NFκB proteins p65/RelA, c-Rel, and RelB subunits contain a C-terminal acidic activation domain not present in the p50 and p52 subunits. Thus, heterodimers containing p52 or p50 combined with p65, c-Rel, or RelB are capable of activating transcription [138], whereas p52/49 and p50/p50 are generally considered transcriptional repressors, although these homodimers can participate in promoter activation through interaction with other non-Rel proteins such as the IκB family member Bcl-3 [137]. Despite this diversity, the active heterodimer p50/p65 is generally referred to as NFκB. NFκB activation can be measured by determining levels of cytoplasmic IκB; nuclear levels of p65, c-Rel, or RelB; nuclear levels of Bcl-3; or binding of nuclear extracts to oligonucleotides bearing the different NFκB binding consensus DNA sequences present in specific gene promoters of interest identified by immunodepletion/supershift assays (Fig. 6).

Role of IκB

One major regulatory influence on NFκB activity is its intracellular localization. Inactive NFκB homo- and

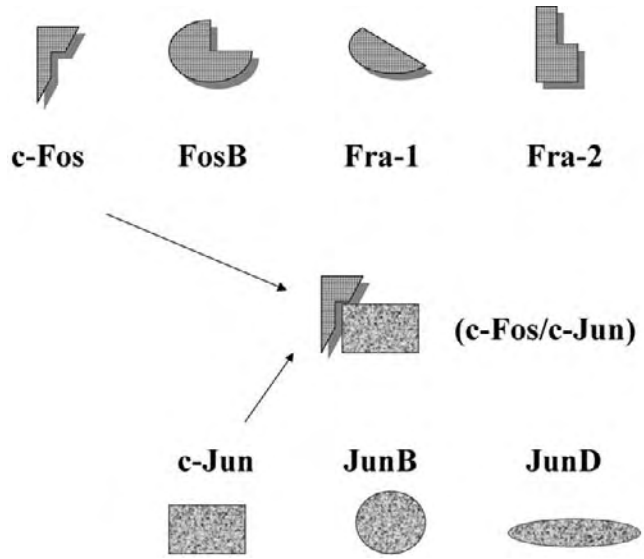


Figure 6 NFκB proteins modify the activate gene transcription (two separate functions). The IκB proteins modify the activity of the NFκB protein dimers. For example, Bcl-3 can bind to p50/p50 homodimers and temove then from promoter sites, facilitating the binding of activating NFκB heterodimers p65/p50 or c-Rel/p50.

heterodimers are sequestered in the cytoplasm of cells complexed with one of several distinct inhibitory subunits, including IκB-α [139]. IκBs contain six copies of an ankyrin repeat structural motif that are required for protein–protein interactions with NFκB dimers [140]. IκB-γ is the C-terminal domain of the 105-kDa precursor protein of the p50 subunit and binds p50 monomers via its seven ankyrin repeats [141]. IκB-γ can also exist as an independent 70-kDa form that results from transcripts using an alternate, internal promoter within the p50/p105 gene [142]. An additional level of regulation is imparted to the NFκB system since different IκB molecules show preference for inhibiting different subunit combinations, whereas IκB-α and IκB-β sequester p65/p50, p65/p52, c-Rel/p50, or c-Rel/p52 homodimers and heterodimers. IκB-ε complexes only with dimers containing p65 and c-Rel [143].

Stimuli that activate NFκB are generally either pathogens (bacterial lipopolysaccharide, viruses, hydrogen peroxide, UV light), or cytokines (tumor necrosis factor, interleukin 1 and 2), and signals associated with these stimuli, which result in the activation of IκB kinase (IKK), a large, multisubunit complex containing two catalytic subunits (IKK-α and IKK-β) and a regulatory subunit (IKK-γ) [144]. IκB molecules are subject to phosphorylation by IKK at two serine residues (serines 32 and 36 in human IκB-α) in their N-terminal domain. These are critical for making the IκB molecule a substrate for ubiquitination at lysine residues [145]. Once ubiquitinated by an ubiquitin ligase [146], the IκB molecule is degraded by 26S proteasomes, releasing NFκB, which is active because the loss of the IκB molecule from the NFκB dimer exposes nuclear localization signals that allow NFκB translocation into the nucleus via interactions of Rel homology domain nuclear localization sequences with importin-α/importin-β heterodimers at the nuclear pore [147,148]. NFκB dimers bind target gene regulatory regions through a wide variety of binding sites that generally match a 5′-GGGRNTY(C/T)C-3′ consensus (R = A or G, Y = C or T, N = any nucleotide). NFκB achieves target gene specificity in part through preferential binding of different subunit combinations to the different DNA consensus binging sequences [70,113,149,150]. Although the inhibition of total NFκB binding to DNA consensus sequences present in the IgG-κB promoter has been shown to increase apoptotic cell death [81,82], the consequences of more selective interventions in the interactions between specific NFκB protein dimers and gene-specific DNA consensus sequences present in the promoters of genes whose expression is known to be altered by oxidative stress and to have significant effects on apoptotic outcomes have not been determined. Based on the outcomes observed for changes in $p75^{NTR}$ versus Trks or intracellular phosphorylation cascades (Figs. 2, 3, and 4), it is likely that balanced counteracting transcriptional events are regulated by a complex symphonic array of NFκB homo- and heterodimers with the eventual determination of life versus death at the cellular level as the outcome.

Conclusions

The aggregate of these observations is consistent with the hypotheses that cellular events that take place when cellular homeostatic set points are shifted as part of the developmental process or in response to abrupt trauma or disease trigger signaling pathways with quite opposite end points. Consequently, eventual physiological outcomes will depend on delicate interactions at the level of receptors, receptor-associated pathways of activation, and differential transcriptional regulation by transcription factor dimers binding to similar but different promoter sites.

Neurotrophic regulation of neuronal fate during phenotypic changes associated with the development of the nervous system or during neuronal recovery after injury or disease rely on a complex orchestration of signaling pathways and gene activating events balanced by virtue of shared molecular components and of binding events at the cell surface, in the cytoplasm, or at gene promoter sites that are similar but different enough to selectively promote or prevent cell death.

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CHAPTER 339

Cell–Cell and Cell–Matrix Interactions in Bone

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Introduction

Far from being the static, hard skeleton hanging in the anatomy classroom, the skeleton within the body is dynamic and constantly responding to internal and external forces. The internal forces include cytokines, growth factors, and hormones, and the external force is response to muscle and to strain placed on the skeleton. In fact, the adult skeleton undergoes greater remodeling than other organs in the body. Several hormones have been shown to play important roles in the skeleton such as the estrogens and androgens, parathyroid hormone (PTH), and 1,25-dihydroxyvitamin vitamin D_3 (1,25-D_3). The three major bone cell types, the osteoclasts, osteoblasts, and osteocytes, are in constant communication with each other and with cells of the immune and hemopoietic systems. Not only are bone cells in constant communication with cells of other systems but also with the extracellular matrix (ECM) composed of osteoid, nonmineralized bone tissue and the mineralized bone matrix. Although previously viewed as mainly a support structure for bone cells, it is now clear that the bone ECM controls and directs bone cell function.

Diseases of Bone

Manifestation of bone disease is usually later or slower than manifestation of disease in other organs. For example, bone cancer is usually discovered after manifestation in other tissues such as breast or lung. Bone cancer such as osteosarcoma usually does not present until fracture or pain occurs, often after the cancer is fully entranced and difficult, if not impossible, to cure. Another example is osteoporosis in which bone loss can occur over decades before being identified and treated. At present, the only hope of treatment for many bone malformations is surgery. Therefore, a greater understanding of normal bone function and pathology is required for the design of preventive therapy and for treatment of disease.

Osteoporosis

Osteoporosis has become a major medical problem as the world population ages. Bone strength is reduced in the postmenopausal female and in both sexes with aging. Bone strength is a function of size, connectivity of trabecular structures, level of remodeling, and the intrinsic strength of the bone itself. Osteoporosis is defined as "the condition of generalized skeletal fragility in which bone strength is sufficiently weak that fractures occur with minimal trauma, often no more than is applied by routine daily activity" [1]. *Primary osteoporosis* is a disorder of postmenopausal women and of older men and women. *Secondary osteoporosis* occurs due to clinical disorders such as endocrinopathies and genetic diseases or to drugs such as in glucocorticoid-induced bone loss. As the U.S. population ages, osteoporosis takes a greater and greater toll in terms of both suffering and economic cost. Each year osteoporosis is the underlying basis for 1.5 million fractures. These cause not only pain and morbidity, but also diminish the quality of life for these individuals, as they lose their independence. Hip fracture results in up to 24% mortality, 25% of hip fracture patients require long-term care, and only a third regain their prefracture level of independence [2]. Treatment for osteoporosis includes hormone therapy and the use of bone resorption inhibitors such as calcitonin and the bisphosphonates. First-, second-, and third-generation bisphosphonates have been developed based on when the drug became available, its structure, and potency. These are

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not only used for the treatment of osteoporosis but also to prevent bone loss due to glucocorticoid use and Paget's disease. These compounds are useful not only to treat bone loss but also to treat and reduce bone metastasis in metastatic breast cancer and multiple myeloma [3].

Skeletal Malignancies

It has become clear that tumor cells use disruption or enhancement of normal cell–cell and cell–ECM interactions to enhance their own growth and metastasis. A prime example is multiple myeloma in which the tumor cells express an integrin complex, VLA4, that allows it to home to bone marrow [4,5]. Myeloma is characterized by extensive bone destruction; therefore, efforts are under way to prevent metastasis to bone through the use of agents that block myeloma–bone ECM interactions. Breast, lung, and prostate cancer preferentially metastasize to bone [6]. Factors that may play a role in osteoblastic bone metastasis include fibroblast growth factors (FGFs), transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF), and, more recently, endothelin-1 [7]. Systemic syndromes can be associated with such cancers; these include leukocytosis and hypercalcemia. A well-studied factor clearly associated with hypercalcemia is parathyroid hormone-related peptide (PTHrp) that is normally produced by keratinocytes, uterus, placenta, and mammary tissue. This factor mimics PTH action by binding to the same receptor. Other factors implicated in osteolytic bone loss due to malignancy include interleukin (IL)-1, IL-6, tumor necrosis factor α (TNF-α), RANKL, and MIP-1α [8]. These factors are discussed in more detail later.

Bone Malformations and Genetic Defects

A baby with craniofacial defects is born every hour, and 75% of all malformations seen at birth fall under this category. Cleft lip/palate occurs in 1 out of 500 live births, and the cost of treating one of these children over a lifetime is more than $100,000. Osteogenesis imperfecta is a heritable disease of bone characterized by recurring bone fractures. It is caused by mutations affecting the structure of the type 1 collagen molecule and is the most common single gene defect causing bone disease [9]. Mutations in type 1 collagen can cause moderate disease or can be lethal during the perinatal period.

As bone growth proceeds through the growth plate in long bones, defects or mutations in a number of factors essential in this process lead to chondrogenic dysplasias. Jansen's metaphyseal chondrodysplasia and Blomstrand's lethal chondrodysplasia are due to mutations in the PTH receptor [10]. The mutations responsible for other genetic disorders have not yet been identified, such as those responsible for Paget's disease of bone, a common disorder with variable clinical presentations [11]. Complications of this disease include deformations of skull, face, and lower extremities, pain, degenerative arthritis, hearing loss, hypercalcemia, and hyperuricemia. It has been proposed that individuals with this disease are predisposed to the effects of Paramyxoviridae viruses on their osteoclasts, however, this is still controversial [12].

Bone Cells and Their Functions

Osteoclasts

The sole function of the osteoclast is to resorb bone. The mature osteoclast is described histologically as a multinucleated, tartrate-resistant acid phosphatase (TRAP) positive cell. However, macrophage polykaryons can have these same characteristics, so the "gold standard" for identifying an osteoclast is the formation of resorption lacunae or "pits" on a mineralized surface. Other characteristics of the osteoclast include the expression of calcitonin receptors, enzymes such as cathepsin K, and matrix metalloproteinase-9 (MMP-9), which play a role in matrix degradation, and the vacuolar proton pump for the transport of protons to the resorption lacunae. For the osteoclast to resorb, it must form a sealing zone around the periphery of its attached area to concentrate its secreted proteases and protons into a limited area. Underneath the cell, a ruffled border is formed and in this region the pH is reduced to approximately 2–3, which enhances the degradation of mineralized matrix. [13].

Osteoclast precursors are derived from hematopoietic precursors, the same stem cells that become granulocytes and monocytes/macrophages. Cell lines such as RAW 267.4 and MOPC-5 are available that represent osteoclast precursors, because these cells can form TRAP positive multinucleated cells that resorb bone [14]. (However, one must be careful when interpreting results from these cell lines because it appears that only a portion of these cells can fully differentiate into osteoclasts.) It has been well known for the last 10–15 years in the bone field that osteoclast precursors require supporting cells for osteoclast formation. The importance of macrophage colony-stimulating factor (M-CSF) as a supporter of proliferation of osteoclast precursors has been determined [15]. Critical factors and cell surface molecules involved in osteoclast formation have only recently been elucidated with the discovery of RANK ligand (RANKL) and osteoprotegerin (OPG) [16,17]. The osteoclast precursor expresses a receptor known as RANK (receptor activator of NFκB) that signals through the NFκB pathway. The binding of the cell membrane-bound ligand, RANKL, activates RANK receptor. However, a soluble factor, OPG, acting as a "decoy" receptor can bind to RANKL, preventing osteoclast formation. The expression of RANKL on the surface of supporting cells occurs when these cells are exposed to bone resorbing cytokines, hormones, and factors such as IL-1, IL-6, IL-11, PTH, PTHrp, oncostatin M, leukemia inhibitory factor, prostaglandin E_2, or 1,25-D_3 [18]. These factors up-regulate RANKL to a level capable of overcoming the effects of circulating OPG, thereby resulting in osteoclast formation. Efforts to generate osteoclasts without supporting cells have only recently been accomplished *in vitro* by using an artificial, soluble form of RANKL [19].

Osteoblasts

The formation of bone matrix on bone-forming surfaces has been well studied. The osteoblast is derived from an as yet to be identified precursor stem cell of mesenchymal origin and in cell culture behaves similarly to fibroblasts, except for its ability to form mineralized matrix. The osteoblast undergoes three major phenotypically identifiable stages of differentiation that Pockwinse and coworkers [20] have characterized as proliferation, matrix production, and maturation in which mineralization occurs. During the proliferation phase, high-level expression of c-fos and histone H4 occurs, and during matrix production, transforming growth factor β type 1 (TGF-β1) and type 1 collagen. During the maturation phase these proteins decrease and the expression of alkaline phosphatase, osteopontin, and core binding factor 1 (Cbfa1) increases. During the mineralization phase, these previous proteins decline in expression, whereas proteins such as osteocalcin increase with increases in mineralized bone formation. Osteoblast cells in each of these phases are often described as early preosteoblasts, proliferating osteoblasts, mature osteoblasts, and finally preosteocytes/osteocytes within the mineralized matrix.

Osteocytes

The osteocyte is the bone cell that we know least about and, therefore, the function of this cell is the focus of numerous hypotheses. These potential functions include the capacity to regulate calcium homeostasis, to respond to mechanical strain, and to send signals of bone formation or bone resorption to the bone surface. These functions are proposed to be accomplished through gap junctions, through the secretion of factors, through glutamate receptors and through the direct dendritic contact with cells on the bone surface [21].

Osteocytes are terminally differentiated osteoblasts, making up the majority, more than 90%, of all bone cells. During osteocyte ontogeny, the matrix producing osteoblast becomes either a lining cell or a preosteocyte embedded in the newly formed osteoid. These preosteocytes produce factors that locally inhibit mineralization (such as osteocalcin) and form a lacunae around the main body of the osteocyte and canaliculi around the dendritic processes [22,23]. A mature osteocyte is defined as a cell surrounded by mineralized bone and is described as a stellate or star-shaped cell with a large number of slender, cytoplasmic processes radiating in all directions but generally perpendicular to the bone surface. Osteocytes first attracted the attention of electron microscopists because of their extensive networks within the mineralized bone matrix that connect the embedded osteocytes to form an extensive syncytium that also connects with cells on the surface of bone. Mature osteocytes are most likely coupled by GAP junctions and appear to be linked to lining cells by the same connections [24]. Osteocytes are attached to mineralized matrix via integrins [25]. Osteocytes have been shown to die by apoptosis due to lack of estrogen or high levels of glucocorticoids [26,27], suggesting that osteocyte viability is important for bone integrity. These properties support the hypothesis that osteocytes function as a network of mechanosensory cells in bone that can mediate their effects through their extensive communication network.

Mechanical Strain

Julius Wolff in 1892 was the first to suggest that bone accommodates or responds to strain. To paraphrase Wolff's law, the law of bone remodeling, alteration of internal and external architecture occurs as a consequence of the stressing of bone. In general, athletes such a wrestlers and chronic exercisers such as tennis players have higher bone mineral density and mass than matched, nonexercising controls. Astronauts subjected to long periods of weightlessness during space flight lose bone. The cells of bone with the potential for sensing mechanical strain and translating these forces into biochemical signals include bone lining cells, osteoblasts, and osteocytes.

The skeleton adapts to mechanical usage. When the skeleton is not used, as in immobilization, bone is lost. During growth the skeleton is in "mild overload," which results in bone modeling and resulting new bone formation. When growth ceases, muscle strength is no longer in overload and bone strain is therefore reduced to the adapted level of strain. The estimated levels of microstrain for each "window" or level of strain have been determined by *in vivo* animal and human experiments. At less than 100 υE, only resorption occurs, and this has been called the "disuse window." The "adapted window" is where remodeling (resorption followed by formation) occurs, between 100 and 1000 υE. This adapted window can be raised or lowered depending on the hormonal environment. The "mild overload window" is where modeling (formation only) occurs between 1000 and 3000 υE and this only occurs in growing animals. The "pathologic overload window" is where microdamage (resorption followed by formation) occurs at strains greater than 3000 υE. Microdamage can occur in race horses and recruits during basic training. Microdamage due to pathological overload results in rapid resorption followed by formation at areas of microdamage. Fracture strain is approximately 25,000. [28].

It has been proposed that bone possesses a *mechanostat*, a mechanism whereby bone can reset its response to particular levels of strain [29,30]. Hormones have been proposed to lower the mechanostat, that is, addition of hormones such as estrogen, PTH, or 1,25-D_3 can lower the magnitude of strain necessary to induce a response [31–33]. For example, if bone normally responds to 2000 υE with an increase in modeling, in the presence of hormone, the same response would occur to lower strain levels, say, 1000 υE. However, it has been shown that the skeleton cannot respond optimally to mechanical strain without the presence of estrogen; therefore, estrogen is essential for the normal response of bone and not for just resetting the mechanostat [34]. Therefore, for normal skeletal growth and maintenance, both biochemical

signals and mechanical strain are essential. For the skeleton to optimally respond to mechanical strain, hormones must be present and, conversely, bone cannot develop normally in the absence of strain.

Hormones Responsible for Bone Development, Growth, and Maintenance

A number of hormones play a role in the maintenance of bone, including estrogen, progesterone, aldosterone, androgens, vitamin A, and glucocorticoids. These hormones' actions are mediated by hormone-activated transcription factors belonging to the superfamily of ligand-dependent nuclear receptors. These nuclear receptors, upon binding to ligand, can form homodimers or heterodimers at specific DNA-binding sites. They also interact with a wide array of other transcription factors, as well as general and specific coregulatory proteins. The complexity of these interactions leads to intrinsic specificity of gene regulation, but the exact determinants of specificity are still under study.

Estrogen

Estrogen is clearly the major sex hormone affecting growth, remodeling, and homeostasis of the skeleton. Reduction in estrogen levels that occurs with menopause or through ovariectomy can result in bone loss. However, estrogen also plays a role in skeletal integrity in the male. Strong support for this comes from the clinical reports of two human males, one with a mutation in the alpha isomer of the estrogen receptor (ER-α) gene, causing partial estrogen resistance, and another individual with aromatase p450 deficiency causing complete estrogen deficiency. They both showed continued longitudinal bone growth due to delayed epiphyseal growth plate ossification and osteopenia [35,36]. Therefore, there is increased clinical interest in the use of selective estrogen receptor modulators (SERMS) in the treatment of bone loss. These synthetic ligands appear to have greater specificity for estrogen receptor in bone and, therefore, the undesired side effects of estrogen in other tissues is avoided while bone mass is maintained.

Estrogen appears to have direct effects on osteoblasts, osteocytes, and osteoclasts. Estrogen is a viability factor for osteoblasts and osteocytes, but appears to induce apoptosis of osteoclasts [37,38]. Estrogen also appears to down-regulate the production of several factors that play a role in bone resorption such as IL-1, TNF, and IL-6 [39]. Therefore, considerable interest has been directed to inhibitors of these cytokines such as their receptor antagonists.

There are two forms of the ER, designated α and β, and both forms are found in bone. The phenotypes of mice lacking either or both of these receptors are complicated and show sexual dimorphism. One receptor isoform can partially compensate for the other. Also, it appears that estrogen can have opposite effects on mice compared to humans, especially on longitudinal bone growth where estrogen enhances long bone growth in rodents but causes epiphyseal closure in humans. Homozygous deletion of ER-α results in reduced cortical bone formation and density in both male and female mice [40]. Female mice still lose bone with ovariectomy and estrogen responsiveness is reduced. Studies with female estrogen receptor β (ER-β) knock-out (–/–) mice indicate that ER-β is involved in the regulation of trabecular bone during adulthood by suppressing bone resorption, whereas this is not the case for male mice where no effect is seen [41]. Both sexes exhibit delayed growth plate closure. Mice with both ER isoforms deleted generate a similar skeleton in the male as the ER-α knock-out, but in contrast, females exhibit a more pronounced phenotype with reduced cortical thickness and trabecular bone density [42]. It is clear that ER-α and ER-β perform different functions in cortical and trabecular bone and that these functions differ between the sexes.

1α,25-Dihydroxyvitamin D_3

Vitamin D is well known to prevent rickets; however, the compounds ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3), are really prohormones that are converted to the biologically active form, 1α,25$(OH)_2$vitamin D_3, which functions in a manner analogous to that of the steroid hormones [43]. Metabolism of the precursors occurs first in the liver and is completed in the kidney. The final active form then acts on the major target organs, bone, intestine and kidney. A vitamin D binding protein (DBP) transports the hormone in the circulation for delivery to cells, where the hormone is freed from its binding protein for binding to the vitamin D receptor (VDR), a canonical nuclear receptor. However, there is mounting evidence that 1,25-D_3 can also signal through membrane receptors [44, 45] and can have nongenomic effects [46]. These rapid responses, such as enhanced transport of Ca^{2+} and activation of protein kinase C and phospholipase A2, have been attributed to the interaction of the hormone with a membrane receptor and not with the canonical VDR. This membrane receptor remains to be completely characterized.

Hereditary vitamin D-resistant rickets is a rare autosomal recessive disease in which patients exhibit defective bone mineralization and hypocalcemia, due to decreased intestinal calcium absorption. These patients are unresponsive to 1,25-D_3 due to mutations in the VDR. Targeted disruption of this receptor in mice results in animals that appear normal at birth, but after weaning show growth retardation and alopecia [16,47]. These mice are also infertile, which suggests a role for vitamin D for gonadal function. Much attention has focused on 1α,25$(OH)_2$vitamin D_3, the major metabolite of vitamin D. The second metabolite 24R,25$(OH)_2D_3$, may also be important. Deletion of the hydroxylase necessary for the generation of this metabolite results in mice that show poor viability and bones with an accumulation of unmineralized matrix [48].

Parathyroid Hormone and PTH-Related Protein

PTH is responsible for calcium homeostasis in the body by its direct actions on bone, that of calcium release from

bone, and enhanced calcium reabsorption from kidney, and indirectly by actions on the gastrointestinal tract, that of conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$. The principal form is intact PTH (1-84) however, there are several circulating cleavage forms, the functions of which are not clear [49]. It is clear that both the intact and amino terminal forms of PTH bind to the PTH type 1 receptor, a G-protein-coupled, seven-transmembrane receptor that signals through cAMP and potentially also through PKC and calcium activation. Mutations in this receptor result in various forms of chondrodysplasias in humans that resemble the disorganized growth plate phenotype of mice lacking this receptor [50]. Whereas both PTH and PTHrp bind to the PTH type 1 receptor, a second receptor, PTH type 2, has been identified that responds to PTH, but not to PTHrp [51]. Recently, it has been clearly demonstrated that receptors also exist that are specific for carboxy terminal fragments of PTH [52].

PTH has been shown to have both anabolic and catabolic effects on bone *in vivo*, to stimulate activation of osteoclasts, and to either stimulate or inhibit osteoblast proliferation and matrix production *in vitro*. These effects of PTH have generated considerable debate about the role of PTH in bone remodeling. It appears that continuous PTH is responsible for resorption, whereas intermittent PTH can induce new bone formation [53]. Studies suggest that continuous exposure of cells results in down-regulation of PTH receptor. Injections of either PTH or PTHrp into humans and animals can result in new bone formation, and intermittent application can stimulate mineralization of osteoblasts *in vitro* [54,55].

PTHrp was first identified as the factor responsible for causing humoral hypercalcemia of malignancy, but soon afterward was identified in many normal tissues. Nonetheless, it was undetectable in the normal circulation [56]. This molecule shares homology with PTH at the amino acid level with 8 of the first 13 amino acids being identical and three-dimensional homology in regions 13–34. PTHrp mRNA is alternatively spliced to yield three isoforms of 139, 141, and 173 amino acids. PTHrp is required for development of cartilage, morphogenesis of mammary gland, and tooth eruption as demonstrated with tissue-specific rescue of in PTHrp null mice that normally die at birth [57]. PTHrp also plays a role in calcium transfer across the placenta and appears to play a role in smooth muscle contractility. Generally, bone cells appear to respond in a similar manner to PTHrp as to PTH.

Growth and Transcription Factors Responsible for Bone Development and Growth

Bone formation proceeds through two ossification processes: endochondral ossification and intramembranous ossification. The former process involves a cartilage intermediate and occurs during most of the skeletal ossification, postnatal growth, bone remodeling, and fracture repair processes. The second process, by which bones form from mesenchymal condensations without a cartilage intermediate, only occurs in some craniofacial bones. The process of chondrogenesis and osteogenesis is tightly regulated at specific times and sites. Several transcription factors are important in this process such as Indian hedgehog and Sonic hedgehog, and the FGFs and their receptors (see later discussion). Indian hedgehog, expressed in prehypertrophic and hypertrophic chondrocytes [58], couples chondrogenesis to osteogenesis through PTHrP-dependent and PTHrP-independent pathways [59,60]. The PTH signaling pathway also plays a critical role in growth plate development (see later discussion).

Core Binding Factor 1, a Master Gene for Bone

Cbfa1, also known as Pebp2a1, Aml3, and Runx2, was originally thought to be T-cell specific [61]. However, direct evidence that Cbfa1 is essential in bone and tooth development comes from Cbfa1 gene knock-out experiments [62]. In these mice, there is a total absence of bone, as well as arrested tooth development. The membranous bones of the skull are replaced by fibrous tissue and endochondral bone does not replace the cartilaginous skeleton. Heterozygous Cbfa1 mice express a phenotype that is similar to the clinical manifestations of cleidocranial dysplasia, in which functional mutations of the Cbfa1 gene have been identified. Cleidocranial dysplasia is characterized by hypoplasia/aplasia of the clavicles, patent fontanelles, supernumerary teeth, short stature, and changes in skeletal patterning and growth. Cbfa1 is the earliest and most specific marker of osteogenesis identified to date [63]. Several homeodomain transcription factors, such as Msx2, Dlx5, Bapx1, and Hoxa-2, have been suggested to regulate Cbfa1 expression.

A second transcription factor that is required for osteoblast differentiation during development that acts downstream of Cbfa1 is osterix (Osx) [64]. Mice lacking Osx have a similar phenotype to those lacking Cbfa1. Target genes for Cbfa1 and Osx include osteocalcin, collagen type 1, collagenase 3, TGF-β type 2 receptor, and other genes necessary for osteoblast function. Although Cbfa1 mRNA do not correlate with target gene regulation, phosphorylation of Cbfa1 protein does [65].

Transforming Growth Factor β

TGF-β1 is the prototype and the founding molecule for the TGF-β superfamily [21]. This family has grown to include more than 40 members including the TGF-β isoforms, the activins and inhibins, Mullerian inhibitory substance, growth differentiation factors (GDFs), and an ever-increasing number of bone morphogenetic proteins (BMPs). Members of this superfamily appear to mediate many key events in growth and development evolutionarily maintained from fruit flies to mammals. The actions of these proteins appear to be mediated through structurally similar serine/threonine kinase transmembrane receptors.

Members of the TGF-β superfamily bind to two distinct forms of serine/threonine kinase receptors, called type I and type II [66]. The constitutively active type II receptor

initially binds to active TGF-β and upon binding subsequently associates with the type I receptor and phosphorylates it. The direct substrates for the phosphorylated type I receptor appear to be Smad-2 and Smad-3, also known as receptor-activated Smads (R-Smads), whereas negative regulators of the type I receptor include Smad-6 and Smad-7, the inhibitory Smads (I-Smads) [67]. The discovery and naming of the Smads originated from studies of the *dpp* signaling pathway in *Drosophila* [68]. Smad-2 or Smad-3 heterozygous mutant mice are viable, but the compound heterozygous Smad-2/Smad-3 mutant is lethal, suggesting a gene dosage effect and that the relative expression level of Smad-2 and-3 in the cell may influence the nature of the TGF-β response. Loss of Smad-3 results in a lower bone formation rate and osteopenia in mice [69]. Smad-4, also called a common mediator (Co-Smad), appears to bring the cytoplasmic Smad-2 and Smad-3 into the nucleus where together they can regulate transcription of target genes. Smad-4 was found to be homologous to a gene deleted in pancreatic carcinomas called *deleted in pancreatic cancer-4* or DPC-4 [70]. Smad-4 is not always required for TGF-β signaling, since a number of Smad-4 independent TGF-β responses have been identified [67]. These include Jun N-terminal kinase (JNK) and extracellular signal-related kinases (ERK) mitogen-activated protein (MAP) kinase pathways. The potential exists for other Co-Smads to be identified.

The major function of TGF-β in bone is as an induction of matrix formation. Mice lacking specific isoforms of TGF-β have boney defects. Injections of TGF-β can induce new bone formation or prevent bone loss, but inappropriate expression of TGF-β or its receptor can lead to bone loss. Even though TGF-β stimulates osteoid production, it actually inhibits mineralization of osteoid. Therefore, this factor must be activated in a specific time and tissue and then inactivated for normal bone remodeling to occur. TGF-β can enhance either bone formation or bone resorption depending on the assay system and the presence of other factors. TGF-β was proposed to be a "coupling" factor, coupling bone resorption with bone formation as this factor is released from the bone matrix where it is stored by resorbing osteoclasts [21,71].

TGF-β is well known among growth factors for its potent and widespread actions. Almost every cell in the body has been shown to make some form of TGF-β and almost every cell expresses receptors for TGF-β. The largest source of TGF-β in the body is bone. This growth factor must be tightly regulated to prevent disease. Appropriately, the mechanisms of regulation of TGF-β are extensive and complex. One unique set of regulatory mechanisms centers around the fact that TGF-β is produced in a latent form that must be activated to produce biologically active TGF-β. The mechanisms of regulation not only include regulation of the latency of the molecule, but the production of different latent forms, such as the small and large latent complexes, TGF-β targeting to matrix for storage or to cells for activation, and the various means of activation. The extracellular matrix protein, latent TGF-β binding protein (LTBP-1), appears to play a major role in the regulation of TGF-β (see later discussion).

Bone Morphogenetic Factors

Unlike the TGF-βs that can only induce bone when injected in proximity to existing bone, the BMPs can induce new bone formation when injected into muscle. Urist [72] was the first to describe bone regenerative capacity of bone extracts, but Celeste and coworkers [73] were the first to identify the factors responsible through the use of peptide sequences from these mixtures and then cloning the resulting recombinant DNA for *in vivo* studies to provide the first identification of these factors. The BMPs are more closely related to proteins involved in differentiation during embryogenesis than they are to the TGF-βs. In fact, although it is clear that these factors are important or essential for development, it is not clear if these factors play an important role in the adult skeleton. This will not be known until time- and tissue-specific null mice are generated.

At present it is known that deletion of BMP2 or BMP4 is embryonic lethal, whereas deletion of other BMPs is not so dramatic. Deletion of BMP7 results in mice with mild limb skeleton abnormalities, $BMP6^{-/-}$ mice appear normal, and $BMP5^{-/-}$ mice exhibit the short ear phenotype. These results suggest that BMPs in some cases can compensate for deletion of one member. $BMP3^{-/-}$ mice are normal and, in fact, have increased bone density that may explain why injection of recombinant BMP3 has never induced bone formation. The growth and differentiation factors (GDFs) are also members of the BMP family. $GDF5^{-/-}$ mice exhibit brachypodism, reduction in number of digits, and misshapen bones. Deletion of GFD11 leads to defects in skeletal patterning and palate abnormalities. Even deletion of the some of the receptors for BMPs (see later discussion) do not result in severe or lethal phenotypes, suggesting that the receptors can compensate for one another. Deletion of either the BMPR1B receptor or the ActRIIA receptor is not severe, whereas the deletion of the BMPR1A is embryonic lethal [74]. Negative regulators of the BMPs include the following: noggin for BMPs 2, 4, 7 and GDF 5 and 6; chordin for BMPs 2, 4, and 7; follistatin for BMPs 2, 4, 7, 11; and gremlin for BMP 2, 4, and GDF 5.

Like the TGF-βs, the BMPs signal through type I and type II receptors, however, BMPs can signal through type II receptors alone, yet this signal is enhanced when both receptors are engaged [75]. Seven type I receptors have been identified, called *activin receptor-like kinases* (ALKs), and three type II receptors. Members of the BMP family bind with different affinities to the type I and type II receptors adding to redundancy and complexity in signaling. BMP receptors also signal through the Smads. The R-Smads for BMPs include Smad-1, -5, and -8, (for TGF-β they are 2 and 3 as described above). Smad-4 is the only ad that is shared by both the BMPs and TGF-β. I-Smads are Smad-6 and -7. Smad-5-deficient mice have defects in angiogenesis and Smad-6 mice exhibit cardiac defects. Transcriptional corepressors of the Smads include TGIF, c-Ski, and SnoN. Target genes of

the BMPs such as Tlx-2, a homeobox gene related to human HoxII, Dad (Daughters against Dpp), and Id gene products, are generally responsible for patterning and development.

Recombinant BMPs 2, 4, and 7 are being used for clinical studies to induce fracture repair, augmentation of alveolar bone, and for gene therapy. To date, none of these applications has been approved for general application, probably due to the difficulties in determining ideal doses, times for administration, and ideal carriers. However, the potential still exists for therapeutic treatment.

Insulin-Like Growth Factors

The IGFs were shown to be the mediators of the effects of growth hormone. IGF I and II are 7-kDa proteins that share homology with proinsulin. Originally it was found that IGF is made by the liver, but it has also been shown that osteoblasts produce this growth factor. Bone is the major storage organ for IGFs, and IGF II is the most abundant of all the growth factors stored in the skeleton [76]. Factors such as PTH, estrogen, prostaglandin E2, and BMP-2 will increase IGF expression in osteoblasts.

Animals with targeted overexpression of IGF in bone using the osteocalcin promoter have greater bone mineral density [77]. IGF-I-deficient neonates have a marked increase in death rate compared to IGF-II-deficient animals that have normal survival rates. Mice lacking the receptor die at birth. Regardless of whether ligand or receptor are deleted, the pups express normal morphogenesis [71]. This suggests that the major function of the IGFs are growth, not morphogenesis, as is true for other growth factors such as the BMPs and FGFs.

The actions of the IGFs appear to be tightly regulated by the IGF binding proteins. These are found in serum and in bone matrix. Six have been cloned and characterized. They bind with high affinity to the IGFs, preventing their interaction with the IGF receptor. IGFBP-1 can inhibit or enhance IGF action dependent on its phosphorylation state and may be responsible for suppression of bone formation in malnourished individuals. IGFPB-2 is the major binding protein secreted by osteoblasts. IGF-PB3 has both inhibitory and stimulatory activity depending on location within the cell. IGF-BP5 is not normally in the circulation, but is preferentially found in the bone matrix where it appears to be protected from proteases and appears to potentiate IGF activity. IGF-BP6 has a selective affinity for IGF II over IGF I. [71,78]. The binding proteins can be degraded by specific and nonspecific proteases, thereby adding another level of regulation of IGF activity. For example, cathepsin D will degrade IGFBPs 1–5, whereas pregnancy-associated plasma protein-A will specifically proteolyze IGFBP-4.

The IGFs appear to stimulate new bone formation *in vivo*, with little or no preliminary resorption phase. In animal models, IGF I can enhance longitudinal growth, bone formation, and bone mass in various but not all models. For example, growth can be restored in hypophysectomized rats, but little effect is seen in normal rats. It is clear that recombinant IGF I can enhance trabecular and cortical bone mineral density in humans with an impaired growth hormone–IGF axis, but such data are not available for normal or older adults. Therefore, at this time, use of IGF can only be justified in specific conditions such as growth hormone-resistant short stature [79].

Fibroblast Growth Factors

The FGFs were first so named for their ability to stimulate the growth of 3T3 fibroblasts. The FGF family has grown to include at least 23 genes [71,80]. The first two FGFs were called acidic and basic FGF, based on their isoelectric points, but have since been renamed FGF-1 and FGF-2. In addition to promoting cell growth, these factors can induce a mitogenic response, stimulate cell migration, angiogenesis, vasculogenesis, transformation, morphogenesis, wound healing, and tissue repair. FGF-2 and -3 are distinguished from all other growth factors by a novel translation initiation mechanism. Four high-molecular-weight isoforms of FGF-2 are initiated with an unconventional CUG translation codon, whereas a smaller 18-kDa isoform is initiated by the classical AUG codon. Another interesting feature of some of the FGFs, like IL-1, is their ability to be nonclassically secreted even though they do not contain hydrophobic signal peptide sequences. On the cell surface, the FGFs interact with at least three types of molecules, including four high-affinity signaling receptors (FGFRs 1–4), low-affinity receptors such as perlecan and syndecan that potentiate ligand/receptor interactions, and cysteine-rich nonsignaling receptors that may function to antagonize and remove ligand.

FGFs clearly stimulate new bone formation; however, injections of FGF cause serious side effects such as acute hypotension. Mutations in FGF receptors result in a number of human dysmorphic (dwarfism) syndromes such as achondroplasia, thanatophoric dysplasia, Jackson-Weiss syndrome, and Pfeiffer syndrome. The clearest indications that FGFs are important in bone development is revealed through the bone phenotypes of null mice lacking the FGF receptors, rather than mice lacking a particular FGF as compensation among this family of factors appears to occur. Disruption of FGF-2, however, results in decreased bone mass and bone formation [81]. Mice expressing activated *FGFR3* mutants reproduce the dwarfism phenotype of the chondroplasias and show a marked decrease in the proliferation rate of the columnar proliferating chondrocytes and a decrease in size of the zone of hypertrophic chondrocytes [82,83,84]. Thus, a normal function of FGF signaling in chondrocytes is to inhibit chondrocyte proliferation.

FGFs may have additional effects on the skeleton. The newest member of the family, FGF-23, appears to play a key role in hypophosphatemic disorders [85]. This FGF is produced by tumors that cause osteomalacia, and when injected into mice causes hypophosphatemic rickets. In all tumors causing hypophosphatemic osteomalacia, mutations

around Ser180 have been identified, resulting in a noncleaved 32-kDa protein. In a condition of autosomal dominant hypophosphatemic rickets, FGF-23 is also not cleaved. It is presumed that under normal conditions, FGF-23 is cleaved at residue Ser180 and that these mutations may cause a gain of function for FGF-23. The mechanisms for the role of this FGF in hypophosphatemia are under intense investigation.

Low-Density Lipoprotein Receptor-Related Protein 5 as a High Bone Mass Gene

Recently, a mutation in the extracellular domain of the low-density lipoprotein receptor-related protein 5 (Lrp5) gene was shown to result in extremely high bone mass in a human cohort. These individuals essentially never break their bones and have no other clinical features, suggesting solely positive effects of this mutation [86]. However, mutations in the intracellular domain of this receptor result in a condition called osteoporosis pseudoglioma syndrome of juvenile onset. These individuals have osteoporosis and exhibit progressive blindness [87]. The extracellular mutation appears to result in constitutive activation and the intracellular mutation results in a loss of function. Surprisingly, these studies show the importance of Lrp5 in regulation of bone mass. This was unexpected because this protein is ubiquitously expressed and had only been associated with lipoproteins and liver function.

Lrp5 is a coreceptor with the seven-transmembrane receptor, frizzled, in the canonical Wnt signaling pathway [88]. The potential role of Wnt signaling in maintenance of bone density will be an exciting area of study, sure to involve other members of this pathway such as β-catenin, glycogen synthase kinase 3B, dishevelled, dickkopt-1, axin, and targets of this pathway such as members of the t-cell factor/lymphocyte enhancer factor family such as Cox-2, c-jun, and connexin 43 [89,90]. This gene may regulate bone mass during development and also may be responsive to mechanical strain. This is a novel area of investigation, because the role of Lrp5 in regulation of bone remodeling and signaling pathways is speculative at this time.

Bone Extracellular Matrix

The ECM and the proteins it contains have not received the same attention as other areas of bone biology such as cytokines, receptors, cell signaling, and transcription factors. This is partially due to the difficulty in determining the potential functions of large, extensively modified ECM proteins. Recently, more attention has focused on the extracellular components of bone due to the advent of new technologies. Transgenic animals and null mice have greatly assisted in determining the functions of these proteins [91]. Knock-outs of the ECM proteins lead to various bone defects, such as thickened bones in osteocalcin null mice, and an osteoporosis-like phenotype in biglycan null mice. On the other hand, no bone phenotype was observed in the decorin or osteonectin null mice [91]. Occasionally deleting a specific ECM protein gene, such as that for fibronectin, can result in embryonic lethality that, unfortunately, does not give information concerning the specific function of the matrix protein. In these cases, the phenotype of a transgenic animal or a deletion heterozygote can be more informative.

Although the major ECM protein in bone is collagen type 1, there are numerous noncollagenous proteins. These include proteoglycans such as decorin and biglycan that are characterized by glycosaminoglycans attached to core protein, chondroitin sulfate proteoglycans such as aggrecan and versican, glycoproteins such as osteonectin, vitronectin and thrompospondins, proteins containing γ carboxy glutamic acid such as matrix Gla protein and osteocalcin, and a group of proteins known as the SIBLINGS for small integrin-binding ligands with *N*-linked glycosylation [92]. Members of the SIBLINGS include osteopontin, bone sialoprotein, dentin matrix protein-1 (DMP-1), dentin sialophosphoprotein, and MEPE. Bone proteins are proposed to have a role in the mineralization process. Whereas deletion of osteocalcin, osteonectin, and bone sialoprotein has not resulted in significant changes in the bone phenotype, deletion of DMP-1 has dramatic effects on the growth plate, and the mice display a chondrodysplastic phenotype and dwarfism [93]. DMP-1 may also be important in osteocyte function [94,95].

Latent TGF-β Binding Proteins

Latent TGF-β binding proteins (LTBPs) appear to be an important mechanism whereby TGF-β is controlled. To date, four LTBP genes have been isolated (LTBPs 1–4) containing cysteine and EGF-like repeating domains. LTBPs are highly homologous to fibrillins 1 and 2, major constituents of connective tissue microfibrils. LTBP 2 does not bind latent TGF-β and therefore may be more homologous than LTBP 1, 3, and 4 to the fibrillins. The third eight-cysteine repeat in LTBP-1 forms a covalent disulfide bond with the TGF-β1 precursor or "latency associated peptide." The major isoform of TGF-β stored in bone matrix is TGF-β1 (80–90%) as part of a latent complex containing LTBP-1.

LTBP-1 does not confer latency to the TGF-β complex, but has other unique functions. The latent TGF-β complex produced by matrix-forming osteoblasts is targeted by LTBP-1 to fibrillar structures known as microfibrils in bone ECM [92]. Although LTBP-1 covalently associates with small latent TGF-β1, it is also produced by osteoblasts in a free form (80% of total) not associated with latent TGF-β1. This molar excess suggests a function separate and distinct from its association with TGF-β. Many ECM proteins contain EGF-like repeats that mediate protein–protein interactions, suggesting that LTBP-1 may have similar functions [21].

Deletion of LTBP 2 is lethal, whereas deletion of LTBP 3 results in mice with ossification of synchondroses at 2 weeks that normally do not ossify [97]. At 6 and 9 months, these animals develop osteosclerosis and osteoarthritis. To date, the LTBP-1 gene has not been successfully deleted.

Microfibrils

Recently the role of components of microfibrils in bone development has attracted attention. The components of microfibrils include fibronectin, fibrillins 1 and 2, elastin, microfibril-associated glycoprotein (MAGPs) 1 and 2, fibulin, and others [98,99]. Mice lacking the genes for the microfibril proteins often have a more dramatic bone phenotype than mice lacking genes for many of the bone-specific matrix proteins. For example, deletion of the bone-specific gene osteocalcin results in a modest bone phenotype, whereas deletion of LTBP 3 appears to have a more dramatic effect on bone [100].

The deletion of fibronectin, a major component of ECM and microfibrils, is a neonatal lethal and there are no known human mutations of this protein [96,97]. The fibrillin 1 knock-out does not appear to have a bone phenotype, whereas fragments of overexpressed fibrillin 1 result in mice with overgrowth of ribs and long bones [98]. It was actually suggested that microfibrils control bone growth in a negative fashion based on these results. The fibrillin 2 null mouse has a bone phenotype with contracture at birth that resolves with age, rear joints that do not flex, and fusion of three toes in the hind limbs into one phalange. The elastin null mouse dies at 4.5 days postpartum due to arterial obstruction but the heterozygote shows many features of supervalvolar aortic stenosis in humans [99]. Proteins that are components of microfibrils could function by physical influence through alteration of the physical properties of matrix, through indirect signaling by way of retaining and releasing growth factors such as TGF-β, and through presentation and binding of protein to receptors or signaling molecules on cell surfaces.

Components of microfibrils have strong protein–protein interactions and protein–cell interactions. Cells have been shown to bind to fibrillin through integrins [100]. MAGP-2 has an RGD sequence motif that modulates cell-to-microfibril interactions and binds to αvβ3 integrins as does fibrillin-1 [101]. Fibulins all have long EGF repeats and bind to nidogen, aggrecan, versican, fibronectin, endostatin, collagen IV, laminin α2, and perlecan [102,103]. The matrix glycoproteins laminin, perlecan, tenascin, and nidogen all have calcium-binding EGF-like repeats. These calcium-binding repeats are necessary for stabilizing their tightly folded structures. Several growth factors have also been shown to bind to components of microfibrils, for example, connective tissue growth factor binds to fibrillin.

Matrix Metalloproteinases

An intricate balance between deposition and breakdown of ECM is critical for growth and development of bone, and significant progress has been made in understanding the roles of MMPs in the balance between osteoblasts and osteoclasts [104]. MMPs belong to a family of zinc- and calcium-dependent endopeptidases that catalyze the proteolysis of components of ECM at neutral pH. Each member has specificity for a particular subset of ECM components. The most important members are MMP-2 and MMP-9. Martignetti and colleagues [105] found that a human disease with an enhanced degradation of ECM, which features osteolytic lesions in facial bones, arthritis, and subcutaneous nodules, is due to the lack of a single proteolytic enzyme, MMP-2. Similarly, deficiency of mouse MT1-MMP, which activates MMP-2, results in a decrease of collagen breakdown by osteoblasts, a decrease in bone formation, and an increase in the number of osteoclasts [76,106]. MMP-13 is predominantly expressed in the skeleton, and null mice have elongated growth plates and reduced bone resorption, suggesting that MMP13 directly or indirectly inhibits chondrocyte growth and stimulates osteoclastogenesis [107] while overexpression of MMP-13 leads to osteoarthritis [108].

Conclusions

This chapter merely touches on many important areas in bone research, as room is not available for review of others topics such as the bone resorbing cytokines including IL-1, -6, and -11, tumor necrosis factors, and regulatory factors such as arachidonic acid metabolites including the prostaglandins, nitric oxide and its regulatory enzymes, and so on. Although many of the factors involved in regulation of bone function are similar to those in other organs, a level of complexity is added due to the mineralized nature of bone. Hematopoietic and immune cells have been well characterized because they are relatively easy to obtain, but this is not the case for bone cells, especially for osteocytes embedded in bone. Investigators in the bone field have often referred back to the areas of hematology, immunology, and development to understand the potential role of factors in bone. Determining the function of matrix proteins in bone has relied heavily on studies in other tissues, such as cartilage, skin, and other connective tissues. However, bone biologists are not able to rely on studies in other organs with regard to mineralization, the unique feature of the skeleton.

In summary, bone is a storehouse of factors ready to be released during resorption that can modify the bone coupling process or provide circulating growth factors. A number of transcription factors have been identified that are specific for bone induction and development. Clearly these growth factors and transcription factors are regulated by a number of circulating hormones such as parathyroid hormone, estrogen, and $1,25(OH)_2$ vitamin D_3. Another layer of complexity is added as bone is also regulated by mechanical strain. Understanding the normal physiology of bone and its diseases should lead to prevention and treatment of disease, acceleration and initiation of repair, and treatment or reversal of abnormal development.

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CHAPTER 340

Cell-to-Cell Interactions in Lung

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Introduction

The lung is a remarkably complex organ that requires an intricate interaction of the processes of branching morphogenesis and cell proliferation for organ development in an intrauterine environment and for the function of gaseous exchange upon transition to an extrauterine environment [1–3]. Once developed, the lung processes 10,000 liters of air per day, exposing it to numerous pathogenic and noxious environmental insults, thus requiring programs for repair responses to these insults. The complexity of the organ is demonstrated by the fact that more than 40 different cell types with respiratory and nonrespiratory functions are present in the lung; and these cell types can be divided into several classes: type I epithelial cells, type II epithelial cells, endothelial cells, interstitial or mesenchymal cells, neuroendocrine cells, and alveolar macrophages.

The number of different pulmonary cell types and their individual functions require precise and timely communication in order to embark on a developmental program in the fetus and to maintain the integrity of lung function in the face of damage and injury. Cell–cell communication and cell–matrix interactions play an important role in aspects of both lung development and repair [4]; it is believed that repair following lung injury recapitulates some of the pathways and processes mediating lung formation. This chapter deals with various soluble cell-to-cell signals that are involved in the processes of lung development and lung repair. However, due to the complexity of the lung and space limitations, the primary focus of this chapter is on cell-to-cell interactions that involve epithelial cells of the lung.

Lung Organogenesis and Development

Organogenesis and development of the lung begins at gestational age day 26 in the human embryo as a ventral out-pouching of the foregut that results in a tracheal bud. Lung development continues in distinct stages from this prenatal period until the postnatal period in early childhood [5]. During the embryonic period (1–7 weeks), lung lobe formation is established and subsegmental branching of the bronchial tree occurs, which seems to be determined by interactions with the mesenchyme. At this time, vascular connections to the pulmonary system are established. In the pseudoglandular stage (5–17 weeks), formation of the bronchiolar tree is completed. Formation of the upper (proximal) airway, development to the alveolar ducts, and innervation of vasculature takes place during this stage as well. The transition to the canalicular stage (16–26 weeks) marks the point at which the lung parenchyma is canalized by capillaries. The epithelial cells that line the air ducts start differentiating into type I and type II cells and formation of a thin air–blood barrier are other hallmarks of this stage.

The final prenatal stage, the saccular (24 weeks to term), encompasses the time in which the peripheral or distal airways form the typical terminal clusters of widened air spaces called *saccules.* During this time, the interstitial tissue between the air spaces is decreased and the capillary networks get closer together. Postnatally, lung development entails changes in which the size of the lung is increased and the ability to perform efficient gaseous exchange is enhanced. The alveolar stage (up to 18 months) is the time when the number of alveolar sacs is increased. While alveolarization proceeds, the stage of microvascular maturation (about 6 months to 3 years) becomes more pronounced. During this time, the capillary networks of the lung are established.

Soluble Factors of Cell-to-Cell Interactions Involved in Lung Cell Differentiation

During all stages, the various types of epithelial cells in the respiratory tract are derived from common progenitors and are generated through cell–cell, matrix–cell, and

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autocrine–paracrine pathways: The mesenchyme produces growth factors and patterning information that determines how epithelial cells will proliferate and differentiate. The soluble factors listed next are those believed to be involved in these interactions.

Fibroblast Growth Factor

Fibroblast growth factor (FGF) signaling is critical for the regulation of lung development and epithelial cell differentiation. FGF receptor-2 (FGFR-2) is preferentially expressed in epithelial cells during development, and ablation of FGFR-2 signaling results in a nearly complete lack of lung formation in the mouse due to the failure of branching morphogenesis [6]. FGF-3, FGF-7 (keratinocyte growth factor, KGF), and mesenchymal-derived FGF-10 bind to FGFR-2 [7,8]. A lack of lung development is observed in FGF-10-deficient mice [9]. In addition to being critical for lung formation, FGF is also involved in epithelial cell proliferation and differentiation in postnatal lung. KGF and FGF-10 increase epithelial cell proliferation [10]. KGF regulates alveolar type II cell differentiation in adult lung [11], and expression of KGF during the pseudoglandular stage of development disrupted normal pulmonary branching morphogenesis, demonstrating the importance of correct temporal expression of members of the FGF family in lung development [12].

Epidermal Growth Factor and Transforming Growth Factor α

Epidermal growth factor (EGF) has been shown to accelerate epithelial cell development when administered *in vivo* [13]. Both transforming growth factor α (TGF-α) and EGF act through the EGF receptor (EGFR), and TGF-α, EGF, and EGFR colocalize to epithelial and smooth muscle cells of bronchioles and bronchi and in epithelial cells of saccules of rat lung [14]. Epithelial cells cultured from late-gestation fetal rat lung express TGF-α and EGFR, whereas cultured fibroblasts express EGFR mRNA, but no detectable TGF-α mRNA. These results suggest that TGF-α produced by fetal lung epithelial cells might act through an autocrine or paracrine mechanism with epithelial and mesenchymal cells. In addition, colocalization of TGF-α and EGF suggests that these agents might act in parallel in lung development [15]. EGFR-deficient mice show evidence of lung immaturity due to impaired branching and deficient alveolization and septation as well as evidence of type II cell immaturity [14].

Platelet-Derived Growth Factor

The platelet-derived growth factor (PDGF) family acts through two tyrosine kinase receptors, PDGFR α and β, and three of the ligands (PDGF-A, -B, and -C) bind to PDGFR-α with high affinity. Although PDGF-A and PDGF-B can be found in the lung, expression of PDGFR-α and PDGFR-β are mainly restricted to lung mesenchyme and PDGF-A expression is restricted to developing lung epithelium [16]. Early data suggested that PDGF-BB stimulation of both receptors leads to lung growth, whereas PDGF-AA stimulation of PDGFR-α induces transduction pathways that lead lung branching [17]. However, mice deficient in the PDGF-A gene have evidence of branching morphogenesis but lack lung alveolar smooth muscle cells, exhibit reduced deposition of elastin fibers in the lung parenchyma, and develop lung emphysema due to complete failure of alveogenesis. Failure of postnatal alveogenesis in these mice is apparently due to a prenatal block in the distal spreading of PDGFR-α+ cells along the tubular lung epithelium during the canalicular stage of lung development [16].

Studies with mice deficient in the PDGFR-α gene have demonstrated its role in the recruitment of smooth muscle cells to the alveolar sacs and their further compartmentalization into alveoli [18]. These data suggest that although PDGF-AA signaling through PDGFR-α may have direct or indirect roles in overall lung growth, it does not specifically control early branching of the lung epithelium. Transgenic mice expressing PDGF-A in the distal lung epithelium were shown to have increased mesenchymal cells and acinar buds and decreased bronchioles and dilated air spaces. These results indicate that PDGF-A is a potent growth factor for mesenchymal cells in the developing lung and that the downregulation of PDGF-A expression that normally occurs in the lung during late gestation is required for transition from the canalicular to the saccular stage of lung development [19].

Insulin-Like Growth Factors

Insulin-like growth factors (IGFs) are mitogens that share structural homology with proinsulin and are involved in the growth and development of many organs, including lung. The regulation of IGF action depends not only on the expression of IGFs and IGF receptors, but also on the modulation of IGF activity by IGF-binding proteins (IGFBPs). In human lung, IGF I and predominantly IGF II mRNAs are expressed throughout gestation with decreasing expression of both IGFs after the canalicular phase of gestation. They are mainly detected in the mesodermal-derived components of the respiratory tract, especially in the undifferentiated mesenchyme of the lung buds before and during the canalicular phase of gestation. Expression of the IGFs is weak in the epithelium during the first stages of growth and progressively decreases on maturation. In the endothelium, expression of IGF II, but not IGF I, is consistent throughout gestation. The levels of expression and the cellular distribution of the IGF receptors, IGFR-1 and IGFR-2, are very complex and vary with gestational age [20,21]. IGF and IGFBP-2 mRNAs were expressed both in mesenchymal and epithelial cells. Type I IGF receptor transcripts are present throughout the developing lung, with the exception of the epithelial cells of the bronchi later in gestation. IGFBP-2, -4, and -5 genes are differentially regulated during embryonic development and suggest that each may have a discrete function, and a possible

role for these IGFBPs is to participate in the regulation of cell-specific IGF responses during mouse lung development [22]. The IGFs may act on lung epithelial cell proliferation in both autocrine and paracrine ways, and may also stimulate the maturation of the connective tissue [23].

Hepatocyte Growth Factor

Hepatocyte growth factor (HGF) is a mesenchyme-derived heparin-binding growth factor that has mitogenic, motogenic, and morphogenic activities on lung epithelial cells and may be a mediator of epithelial–mesenchymal interaction during lung organogenesis and regeneration following lung injury [24]. HGF is expressed in mesenchymal fibroblasts and the c-met/HGF receptor is expressed in epithelium in the developing lung. HGF apparently stimulates branching morphogenesis of the fetal lung, and appears to work in collaboration with the FGF family in epithelial branching [25]. On the other hand, mice deficient in the HGF gene have been shown to have lung development that is identical to wild-type mice, suggesting that HGF may not be of primary importance in lung development [26,27].

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen and is abundant in distal epithelium of midtrimester human fetal lung [28]. KDR, a major VEGF-specific receptor, can be localized to distal lung epithelial cells of human fetal lung tissue, suggesting a possible autocrine or paracrine regulatory role for VEGF in pulmonary epithelial cell growth and differentiation [28]. Expression of VEGF in the developing respiratory epithelium of transgenic mice increases growth of the pulmonary blood vessels, disrupts branching morphogenesis of the lung, and inhibits type I cell differentiation [29]. Addition of exogenous VEGF to human fetal lung explants causes proliferation of distal airway epithelial cells and expression of type II cell-specific markers [28], indicating that VEGF may be an important autocrine growth factor for distal airway epithelial type II cells in the developing human lung.

Fibroblast-Pneumonocyte Factor

Fibroblast-pneuomonocyte factor (FPF) was first discovered as an organ-specific differentiation factor produced by glucocorticoid-treated fetal lung fibroblasts that enhances the differentiation of the alveolar type II epithelial cells [30]. The identification and characterization of the agent has been elusive, but it is thought to be very stable with a molecular mass of 5–15 kDa [31]. FPF production is positively regulated by glucocorticoids and negatively regulated by dihydrotestosterone, which interferes with progression of lung development by delaying the appearance of FPF production by the fibroblast, and TGF-β, which appears to interfere with type II epithelial cell development such that the cell cannot respond to FPF [32].

Bombesin-Like Peptides

Bombesin-like peptide (BLP) or gastrin-releasing peptide (GRP) is produced by pulmonary neuroendocrine cells (PNEC). The function of GRP as a growth factor involves lung morphogenesis, but the precise mechanism and the target cells have not been defined. GRP-R mRNA was localized mainly in the distal airway epithelial tubes and surrounding mesenchyme, whereas proximal airways showed decreased signal. Postnatal lung, a strong signal for GRP-R mRNA, was localized in the airway epithelial cells. PNEC in lung expressed mRNA and peptide. The expression of GRP-R in mammalian lung is developmentally regulated, peaking both spatially and temporally during the phase of rapid airway growth and differentiation. Both epithelial and mesenchymal components express GRP-R consistent with paracrine mechanism for GRP activity during lung morphogenesis [33]. BLP stimulates type II cell differentiation, neuroendocrine differentiation, and cell proliferation in fetal lung [34,35].

Transforming Growth Factor β

Unlike the aforementioned factors, TGF-β has been shown to inhibit rather than activate branching morphogenesis fetal lung through its receptor: the TGF-β type II receptor. TGF-β is believed to promote expression of extracellular matrix components around the airways, prevent budding of branches, and suppress epithelial cell proliferation [1,3]. TGF-β mRNA is confined to fibroblasts around epithelial ducts and to smooth muscle cells in the lung [36]. It is not known whether the function of TGF-β resides in lung epithelium or mesenchyme, but it is believed that TGF-β signaling inhibits lung morphogenesis by hampering the inductive and permissive effects of other growth factors such as EGF, TGF-α, and PDGF-AA [37]. Addition of TGF-β to explant cultures of fetal mouse lungs inhibits branching morphogenesis, and its effect could be reversed by washing out the compound [38]. In the development of glandular organs, it has been suggested that activins, dimeric proteins that are members of the TGF-β superfamily, are key growth and differentiation factors in the process of branching morphogenesis [39]. Although activin A has been shown to be expressed by and stimulate pulmonary fibroblast proliferation and differentiation [40], it seems that TGF-β plays a more important role in branching morphogenesis.

Bone Morphogenic Protein

Bone morphogenic proteins (BMPs) are members of the TGF-β family that act to suppress the proliferative effects of the various growth factors in the lung. Distal expression of BMP4 has profound effects on cell proliferation and cell fate in the lung bud [41]. Interestingly, expression of BMP4 is controlled by FGF-10. BMP4 is expressed in the epithelial cells in the tips of growing lung buds. Inhibiting BMP4 signaling in the developing lung results in a severe reduction in distal epithelial cell types and a simultaneous increase in proximal cell types [42].

Soluble Factors of Cell-to-Cell Interactions Involved in Lung Injury

Hyperoxia or exposure to cytotoxic reactive oxygen species results in the death of endothelial cells followed by type I epithelial cells, then type II cells. Although we do not know how endothelial cells are replaced, it is clear that an important process in the repair of lung following damage is the proliferation and transdifferentiation of cuboidal alveolar type II cells into a flattened type I cell. Normal repair requires interaction between fibroblasts and epithelium in which there is balanced proliferation and differentiation of both epithelial and mesenchymal cells. To this end, the lung performs the processes of repair by the use of many of the signals that are involved in lung development and lung cell differentiation. It has been shown that fibroblasts exposed to 100% O_2 produce unidentified growth factors for type II cells [43]. Expression of KGF, FGF, and IGF-1 from the mesenchyme increases in lung on exposure to high levels of O_2 [44,45], while TGF-β, a growth inhibitory signal, and parathyroid hormone (PTrH), which inhibits type II cell proliferation, are decreased [46,47]. Recent studies have demonstrated a strong expression of activin in repair processes in the lung with roles in epithelial differentiation, fibroblast proliferation, and expression of matrix molecules by these cells [48]. BLP has been shown to stimulate type II cell differentiation and cell proliferation in response to lung injury [34,35]. Apart from its role in lung development, KGF has been implicated in protection from hyperoxic damage [49]. FGF-3 appears to have a role in repair following lung injury in adult lung [50]. Low levels of FGF-3 expression resulted in massive free alveolar macrophage infiltration, indicating that FGF may influence cytokine and chemokine levels involved in host defense mechanisms. On the other hand, high levels of FGF-3 expression resulted in diffuse alveolar type II cell hyperplasia. HGF is a potent mitogen for airway epithelial cells and alveolar epithelial cells, and may act as a pulmotrophic factor responsible for airway and alveolar regeneration during lung regeneration after acute lung injury. Expression of HGF is rapidly induced in the lung after acute lung injury in experimental animals and that HGF levels are elevated in blood of patients with lung diseases [51]. VEGF is a specific mitogen for endothelial cells that is expressed by alveolar epithelial cells. Hyperoxic injury decreases neonatal lung VEGF mRNA and protein, which may be a contributory mechanism of impaired postnatal microvascular development in oxygen injury [52].

Conclusions

The lung is a complex organ and the various forms of interaction between the 40 or so cell types cannot be described fully in the space allotted. The focus of this chapter has been on the soluble factors that play a role in cell-to-cell communication during development and repair of the lung, particularly as they pertain to interactions between epithelial cells and other cell types in the lung. It has often been said that a picture is worth a thousand words, so in the interest of space, Fig. 1 has been included to summarize the interactions between the signals produced and signals received by the various cells, resulting in the proliferation and differentiation of lung cells. Although the figure does not fully address all of the interactions in the lung, it should give the reader a sense of the balance that is required for the development of the lung and to maintain integrity of its function in the event of damage postnally.

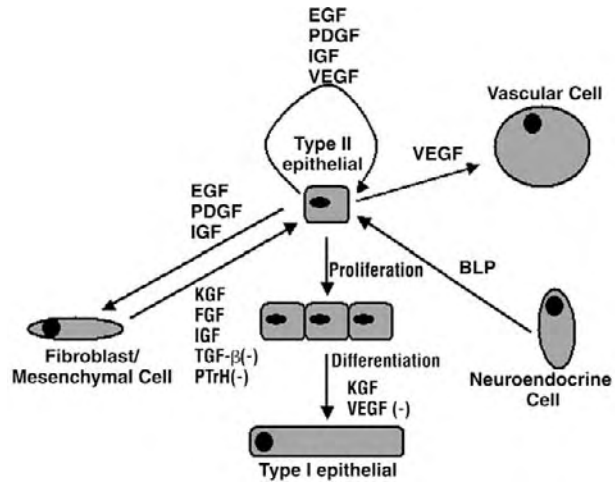


Figure 1 Summary of the cell-to-cell interactions involved in the processes of proliferation and differentiation of lung cells.

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CHAPTER 341

Mechanisms of Stress Response Signaling and Recovery in the Liver of Young versus Aged Mice: The p38 MAPK and SOCS Families of Regulatory Proteins

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Introduction

Biological signaling is a highly specific process that regulates, by activation or repression, gene expression in response to environmental factors, growth factors, and hormones. Signaling processes involve a series of protein–protein interactions that comprise pathways that target the activities of specific genes. Furthermore, biological signaling also involves protein–protein interactions that attenuate or turn off the signals in the process of recovery. This chapter discusses the molecular mechanisms of the protein–protein interactions of two major hepatic signaling pathways: (1) the p38 MAPK stress response pathway that involves the activation of stress response genes and (2) the mechanisms of turning off cytokine signaling pathway(s) by the SOCS family of negative regulators. The p38 MAPK proteins contain docking domains that facilitate (1) activation by upstream signaling proteins, (2) translocation to the nucleus, and (3) activation of stress response transcription factors that regulate the expression of stress response genes. The SOCS family of negative regulators interacts with various protein components of cytoplasmic domains of membrane receptors, thereby preventing the transduction of signals to downstream activator proteins. The characteristics of the proteins that interact to transduce and to turn off these signals as they pertain to the p38 MAPK and SOCS families of proteins are discussed.

The p38 MAPK Pathway in Stress Response Signaling

Biological signaling is a highly specific process that regulates gene expression in response to changes in intrinsic and extrinsic factors. Any alteration in these biological processes affects organismal function and survival. Thus, the status of biological signaling contributes to the overall biochemical phenotype of tissues at all stages of growth and development of aging. Hepatic signaling is, therefore, an important factor in this tissue-specific response to various

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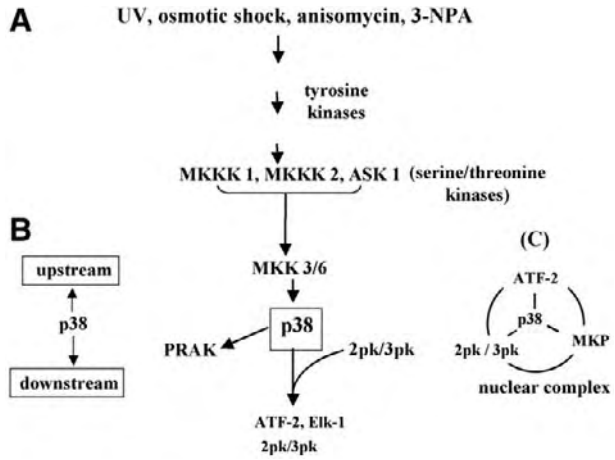


Figure 1 A map of the serine/threonine kinases of the p38 MAPK stress response pathway. The individual upstream tyrosine kinases are not included in this map. (A) Environmental factors (UV, osmotic shock, anisomycin, and 3-nitropropionic acid) activate stress response genes via the p38 MAPK signaling pathway. p38 is depicted as a central component of the pathway because of its role in the translocation of signals to the nucleus where transcription factors and gene activation are carried out. (B) The upstream activators of the p38 MAPK pathway are MAP kinase kinase kinases (MKKKs 1, 2, and ASK1) and the MAP kinase kinases (MKKs 3 and 6). (C) A nuclear complex of p38 MAPK–MAPKAPK-2/3–MAPK–phosphatase. The complex represents a potential mechanism for the activation of transcription factors (ATF-2) by p38 MAPK and 2pk/3pk. (See [105] for recent data on activation of ATF-2 by p38 MAPK pathway.)

factors including growth factors, hormones, intermediates of metabolism, and environmental factors. In particular, members of the mitogen-activated protein kinase (MAPK)/stress-activated protein kinase (SAPK/JNK) families of signaling proteins are activated by a wide variety of extracellular signals (Fig. 1).

The family has three major subgroups: ERKs (extracellular regulators of kinases), p38, and SAPK/JNK. ERKs are activated by growth factors (extracellular matrix interactions with growth factors) and phorbol esters, whereas p38 and SAPK/JNK are activated by such extracellular stresses as UV irradiation, osmotic stress, and inflammatory cytokines. Activation of MAPK signaling leads to the regulation of gene clusters that mediate complex biological responses such as inflammation, cell proliferation, differentiation, and apoptosis [1–3]. The proteins in the MAPK pathways comprise a cascade of multiple tyrosine and serine/threonine kinases and phosphatases; the signals are transduced by protein kinase phosphorylation/dephosphorylation events that mediate docking and enhance specific serine/threonine kinase/phosphatase activities that attenuate the signaling. Although these molecular interactions are both efficient and specific, they also exhibit cross-talk among the MAPK cascades or with other signaling pathways, thereby mediating complex biological processes. Numerous reviews have been published on the structure and function of these signaling proteins [1–3]. In this chapter, we focus first on the serine/threonine kinase components of the p38 MAPK stress response signaling pathway, its mechanisms of signal transduction, and its relevance to liver function and, secondly, the attenuation of hepatic stress signaling pathways.

The p38 MAPK Stress Response Pathway

p38 MAPK is a focal point of interactions of the serine/threonine kinases that transduce stress signals via upstream cytoplasmic activator proteins to their downstream substrates or nuclear targets, that is, the stress response genes (Fig. 1). The pathway in Fig. 1 shows that the transduction of biological signals involves interactions of upstream tyrosine kinases with downstream serine/threonine kinases thereby linking external environmental signals to gene targets. Furthermore, the p38 MAPK isoforms, and the SAPK/JNK isoforms are major nucleocytoplasmic trafficking proteins of these pathways and are, therefore, the crossroads where the interactions of these isoforms with upstream activators and downstream targets (genes) deliver the biological responses to environmental challenges (Fig. 1). Thus, the protein–protein interactions of the MAPK pathways are the major mechanism for controlling the activation of transcription factors and their targeted genes in response to extrinsic (environmental) and intrinsic (biological) factors.

The p38 MAPK Stress Signaling Pathway Specificities and Mechanisms of Protein–Protein Interactions

p38α Was first isolated as a 38-kDa protein rapidly phosphorylated on Tyr182 in response to the inflammatory stress caused by bacterial lipopolysaccharide (LPS). Treatment of mammalian cells in culture [4–6] in mammals indicates that the p38 MAPK family consists of α, β, γ, and δ isoforms: p38α and p38β MAPK are 60% identical to p38γ and p38δ, indicating that they represent related but distinct MAPK subgroups [7]. p38α and p38β Are ubiquitously expressed but at significantly different levels in each tissue. Both p38α and p38β are abundant in the liver. Both p38γ and p38δ are also differentially expressed; p38γ is predominant in skeletal muscle [8,9] and p38δ is enriched in lung, kidney, testis, pancreas, and small intestine [10]. The isoforms have distinct biological functions *in vivo* due, in part, to their ability to target (phosphorylate and so activate) specific downstream transcription factors and to be selectively activated by extracellular stimuli via the upstream tyrosine kinases. Importantly, the p38 MAPK kinases serve as a focal point for the transduction of biological signals from the cytoplasm to their nuclear gene targets. Thus, the pathway consists of a cascade of three protein kinase families: MAP kinase kinase kinases (MKKK), MAP kinase kinases (MKK), and MAP kinases, that is, the p38 MAPK proteins (Fig. 1). These proteins are activated in series such that the MKKKs phosphorylate the MKK activation loop serines, which then activate the p38 MAPKs by phosphorylation of Thr180 and Tyr182.

It is through the p38 MAPK that transcription factors are activated on translocation to the nucleus. The p38 MAPKs exhibit differential responses to specific drugs and

inflammatory agents [7,11]. Thus, the four p38 isoforms can target genes in response to specific drugs and inflammatory agents. Its diverse functions are seen, for example, in its role in the Fas-mediated apoptosis of endothelial cells of the murine liver sinusoids [12] versus its role as the MAP kinase required for pathogen defense against *Pseudomonas* infection in *Caenorhabditis elegans* [13]. A most recent study has demonstrated that *B. anthracis* lethal factor selectively induces apoptosis by cleaving the amino-terminal domain of MKKs that serves as a docking domain for the activation of p38 MAPK [13a]. This dismantling of the p38 MAPK–MKK interaction is the mechanism by which *B. anthracis* paralyzes the host inmate immunity. Similarly, in HeLa cells, p38α induces apoptosis while p38β promotes cell survival, suggesting both overlapping and distinct physiological roles for the p38 isoforms, but clearly demonstrating their roles in establishing a biochemical phenotype [14]. One subgroup (p38α and p38β) is inhibited by pyridinyl imidazole derivatives, drugs that inhibit the production of proinflammatory cytokines, while the others (p38γ and p38δ) are insensitive to these drugs [15].

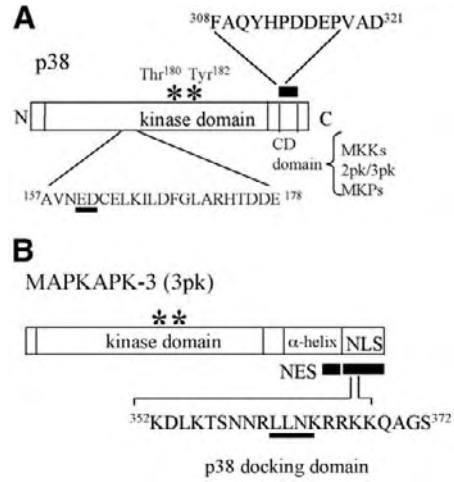


Figure 2 Maps showing the p38 MAPK and 3pk docking sites. (A) The p38α docking sites for upstream activators (MKKs) and downstream substrates (2pk/3pk). (B) A map of the MAPKAPK3 domains. The nuclear localization signal (NLS), nuclear export signal (NES), and p38 docking domains are at the C-terminal end of 3pk.

Transduction of Stress Signals via p38 MAPK Pathway

The activation of p38 MAPK signaling occurs by interaction with one of two specific upstream serine/threonine MAPK kinases, MKK3 and MKK6 (Fig. 1). Targeted disruption of the *mkk3* and *mkk6* genes shows that they have nonredundant functions [15,16]. For example, coexpression of MKK3 with p38β enhances hypertrophy, whereas coexpression with p38α enhances apoptosis [18,19]. This signaling specificity is an example of the generation of specific biological responses by this kinase pathway and emphasizes the importance of how specific complexing of pathway components target and affect specific biological processes.

Although MKK6 is a common activator of p38α, β, γ, and δ, MKK3 activates only p38α, γ, and δ [20]. This exclusive activation of p38β by MKK6 contributes to this pathway's specificity of signal transduction.

The following factors contribute to the specificity of p38 MAPK activation [4,18]: (1) the selective formation of functional complexes between MKKs and the p38 MAPK isoforms, which requires the presence of a MAPK docking site at the N terminus of the MKKs (Fig. 2); and (2) selective recognition of the activation loop (T-loop) or catalytic domain of p38 MAPK isoforms; the T-loop contains the Thr180-Tyr182 residues involved in kinase activation. Together, these provide a mechanism for the selective activation of p38 MAPKs in response to activated MKKs (Fig. 2).

Structural Basis for Protein–Protein Interactions of p38 MAPK and MKK Proteins

Enslen *et al.* (18) showed that interaction with a p38 docking domain in MKK6 contributes to the activation of all four p38 isoforms (Fig. 2). This docking site identified in the N-terminal region of MKK1 consists of highly conserved sequences and has also been identified within the N-terminal region of MKK6. Synthetic peptides based on the primary sequence of the MKK6 docking sites inhibit activation of p38β, indicating that MKK binding is necessary for the activation. MKK3, on the other hand, which lacks this docking site, can activate p38α, p38γ and p38δ, but not p38β; this accounts for the selective activation of p38α but not p38β by MKK3. Differences in the primary sequences of the T-loop of the p38 isoforms also contribute to signaling specificity (discussed later). Thus, selective activation of p38 by MKKs requires multiple molecular determinants present in both kinases, that is, p38 and MKKs.

A conserved docking motif common to interacting substrates, activators, and regulators has been localized to the C-terminal portion of p38 MAPKs and is commonly used for binding to MKKs, nuclear localized MAP-kinase-activated protein kinases (MAPKAPKs; 2pk, 3pk), and MAPK phosphatases (MPK). This common docking (CD) domain is characterized by negatively charged amino acids (MPKs; Fig. 2).

In p38α the CD domain is outside of the active center (Fig. 2). Conceptually, therefore, recognition between p38 MAPKs and interacting proteins involves both the docking interaction domains and the transient enzyme–substrate interaction at the T-loop (Thr180-Tyr182), both of which regulate the efficiency and specificity of the enzymatic (kinase) reactions [4,19–24].

Beside the CD domain, another domain, the ED site, determines docking specificity toward 2pk and 3pk, which are the nuclear enzymes that associate with p38 MAPK and transcription factors [23]. In p38 this site is located at Glu160 and Asp161; exchange of these amino acids with the corresponding residues in ERK2 and vice versa also changes the docking specificity. A 3D molecular model of MAPKs shows the proximity of the CD and the ED domains, which

would explain their ability to serve as a common docking groove [25]. It is proposed that every MAPK-interacting molecule may bind to this docking groove and that each residue therein is differentially involved in each docking interaction [26]. This study suggests that on translocation to the nucleus, p38 isoforms interact with 3pk (or 2pk) to form a complex that targets transcription factors (Fig. 3). In addition, the 2pk and 3pk proteins contain trafficking domains that mediate the import as well as the export of a p38 MAPK–2pk complex from the nucleus to the cytoplasm (Figs. 3 and 4) [26].

Activation of the p38 Catalytic Site

The MAPKs are activated by dual phosphorylation of Thr180 and Tyr182 in the T-loop. Whereas p38α is phosphorylated preferentially on Tyr182 by MKKs that lack a MAPK docking site (e.g., MKK3), those with a MAPK docking site (MKK6) phosphorylate p38α MAPK on both Thr180 and Tyr182 residues. Thus, this differential phosphorylation by MKK3 versus MKK6 participates in the specificity of signaling of these upstream activators. It has been suggested that docking may increase the processivity of MAPK phosphorylation, leading to dual phosphorylation and increased activation [26]. Similarly, differential phosphorylation occurs with MKK4 which preferentially phosphorylates the tyrosine residue of the JNK1 catalytic site while MKK7 phosphorylates the threonine residue of this catalytic site.

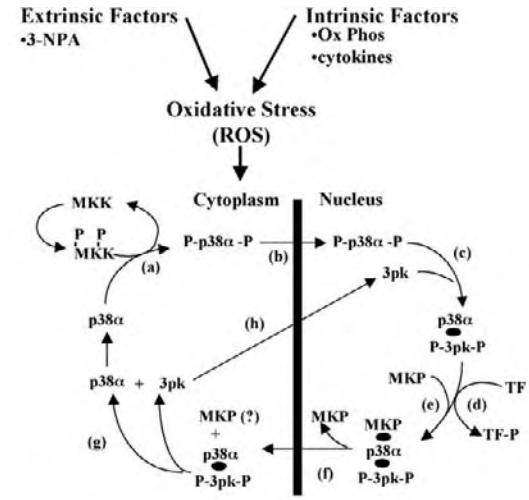


Figure 3 A proposed pathway for the nucleocytoplasmic trafficking of p38α signaling proteins, its upstream activators (MKKs), and downstream substrates (3pk and transcription factors). (a) Activation of p38α in the cytoplasm; (b) translocation to the nucleus; (c) activation of 2pk/3pk; (d) activation of ATF-2 transcription factor (TF); (e) interaction of MAPK-phosphatase (MKP) with the complex and inactivation of signaling; (f) translocation of the complex to the cytoplasm; (g) dissociation of the p38α-3pk-MKP complex; (h) dephosphorylated 3pk is translocated to the nucleus where it reenters the transcription factor activation cycle; p38α is reactivated in the cytoplasm in response to a new stress.

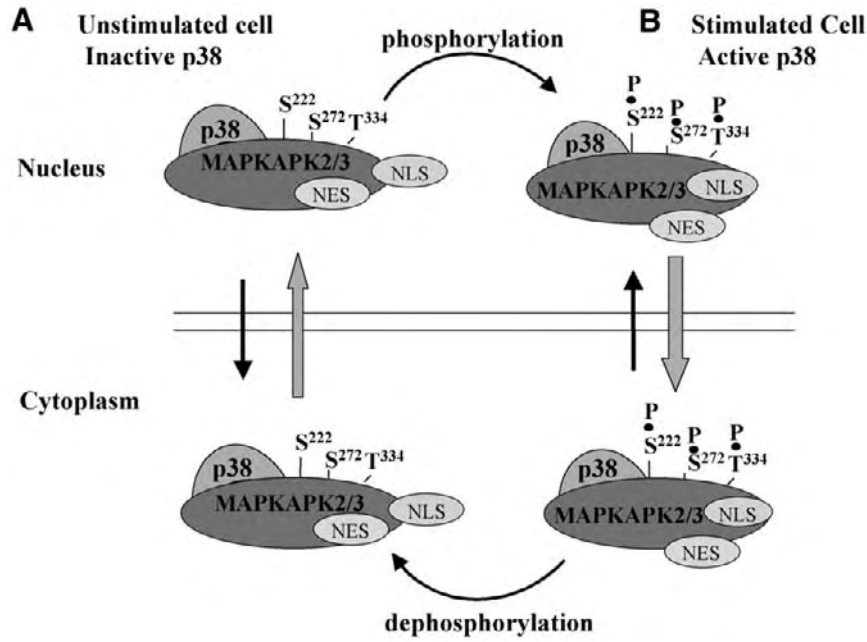


Figure 4 A model of the regulation of nuclear and cytoplasmic localization of a MAPKAPK-2/3–p38 MAPK complex as a mechanism for the activation of p38 MAPK in the nucleus. (A) In the unstimulated cell a MAPKAPK-2/3–p38 MAPK complex is formed in the cytoplasm. The nuclear localization signal (NLS) of MAPKAPK is exposed and mediates the translocation of the complex to the nucleus. (B) In the stimulated cell, the active complex mediates its transcription factor activation. The nuclear export signal (NES) is exposed and the complex is translocated to the cytoplasm. In the cytoplasm of the recovered cell, MAPKAPK-2/3 is dephosphorylated and the NLS is exposed. This results in the translocation of the complex to the nucleus. In the model the p38 MAPK may be phosphorylated in either the cytoplasm or nucleus.

Substrate Specificity of p38 MAPK Isoforms

Binding of the p38 MAPK complex to transcription factors is a critical determinant of kinase specificity. The mammalian MAP kinases (p38 MAPK, JNK, and ERK) require docking sites on transcription factors for correct function. For example, p38α and p38β phosphorylate and activate the muscle-specific transcription factors, MEF2A and MEF2C [27]. These p38 isoforms target transcription factors by a docking domain distinct from the phosphoacceptor motifs that confer responsiveness for p38α and p38β, but not p38γ or p38δ [4,28,29]. Similar binding motifs have been identified in several MAPK substrates, including c-Jun [23], Jun B [30,31], Elk-1 [27,32], NFAT4 [33], and ATF-2 [34,35].

The ability of p38 MAPK to bind to related sequences in both their downstream substrates (transcription factors) and their upstream MKKs enzymes suggests an upstream–downstream competition for binding to the MAPK. This hypothesis predicts that phosphorylation of transcription factors by MAPK can only occur after release of activated MAPK from MKK. However, the complexity of the signaling interactions increases significantly because of the interactions of p38 with the nuclear kinases, 2pk/3pk, which may interact with the transcription factors as well as mediate the nuclear export of p38 (Figs. 3 and 4) [26].

Subcellular Localization and Nucleocytoplasmic Transport of the p38 MAPK Signal

Subcellular localization is an integral part of the functional p38 MAPK signaling pathway (Figs. 3 and 4). Nucleocytoplasmic trafficking facilitates the regulation of transcription factor activity. It is thus important to understand the cellular localization of the p38 MAPK pathway components before and after stimulation. Nuclear substrates of p38 include 2pk, 3pk, MAP-kinase-interacting kinase (Mnk), and a variety of transcription factors, including CHOP, Elk-1, CREB, and Sap-1a. The 2pk and 3pk have both nuclear localization sequences (NLSs) and nuclear export sequences (NESs), and are localized in the nucleus in unstimulated cells, where, on stimulation, they dock with and are phosphorylated by p38 MAPK [16,23,34]. Although this complex phosphorylates (activates) specific transcription factors, it is also exported to the cytoplasm [16,23,34]. For example, phosphorylation of CREB may be mediated by 2pk. Thus, phosphorylation of 2pk and 3pk by p38 MAPK not only activates the kinase so that it can phosphorylate its transcription factor substrates, but it has also been postulated to expose the NES that results in nuclear export of both proteins (Fig. 4) [18,26,36].

The NES and NLS of 2pk and 3pk are located at their C-terminal ends (Fig. 2) [23,36]. In addition, 3pk is localized in the nucleus before osmotic stress and in the cytoplasm upon recovery. The docking of p38 with 3pk and 2pk is essential for their phosphorylation and nucleocytoplasmic export of the complex. Thus, the docking interaction between p38 and 3pk is achieved via direct interaction of the CD domain and the ED site of p38 with the C-terminal portion of 3pk. (Figs. 2 and 4).

Although some data indicate that p38 is activated in the cytoplasm, followed by rapid transport to the nucleus where it phosphorylates its substrates and then is exported to the cytoplasm, there is equally strong evidence that it may also be activated within the nucleus by 2pk or 3pk, followed by translocation of the activated complex to the cytoplasm [26]. Thus, the model in Fig. 4 suggests that a p38–2pk/3pk complex may be formed in the cytoplasm of unstimulated cells and translocated to the nucleus where it may be activated. The fact that there are cytoplasmic substrates (PRAK and Hsp27) that are phosphorylated by p38 in the cytoplasm points to the diversity of p38 MAPK activity for downstream targets. Interestingly, the activation of p38 MAPK by DNA-damaging agents supports the idea that the p38 cascade might be initiated in the nucleus [37]. Activation of p38 in the nucleus, on the other hand, would indicate that MKK3 and MKK6 must be localized there. Ben-Levy *et al.* [26] found that both MKK3 and MKK6 are localized in both the cytoplasm and nucleus. Thus, 2pk and 3pk may serve a dual function, both as effectors of p38 by phosphorylating substrates such as Hsp27 (cytoplasmic) and CREB (nuclear), and as determinants of p38 localization (Figs. 3 and 4).

Feedback Control of MAPK-Regulated Transcription

The dual-specificity MAP kinase phosphatases (MKPs) inactivate MAPKs by targeting their two regulatory phosphorylation sites [38]. There are about nine mammalian MKPs, divided into two groups according to their patterns of transcriptional regulation and subcellular localization [39]. The nuclear MKPs are rapidly and highly inducible by many of the stimuli that activate MAPKs. It is postulated, therefore, that these MKPs play an important role in the feedback control of MAPK signaling in the nucleus [39–41]. Hutter *et al.* [42] showed that MKP-1 interacts with the C-terminal end of nuclear p38 to activate MKP-1 catalytically. This raises the question of whether a 2pk/3pk–p38–MKP complex forms in the nucleus and is exported into the cytoplasm (Fig. 3) and whether this complex is targeted in the process of inactivation of p38 MAPK?

Several cytosolic MKPs can be triggered by direct interaction with MAPK [43,44]. MKP-3 interacts specifically with ERK [19,41]; binding of ERK2 to MKP-3 dramatically enhances the latter's catalytic activity [43]. On the other hand, MKP-4 interacts with all members of the three major MAPK subfamilies to become catalytically activated [43]. These novel mechanisms ensure the tight feedback control of MAPK signaling in the cytosol.

Response of the Aging Liver to Stress Challenges

The free radical theory of aging proposes that endogenous oxygen radicals generated by cells are a basic cause of progressive age-associated declines in cell and tissue function,

and that oxidative stress generated by extrinsic or environmental factors accelerates this decline (Fig. 3) [45–50]. Identification of mitochondria and peroxisomes as major sources of endogenous reactive oxygen species (ROS) and of superoxide dismutases as enzymes that remove superoxide anions supports this theory. More precisely, the theory postulates that accumulated oxidative damage to DNA, RNA, proteins, and lipids leads to the age-associated decline in tissue function and development of the age-associated biochemical phenotype [49–54]. In fact, the accumulation of ROS-damaged macromolecules, for example, nuclear and mtDNA, and carbonylated proteins [55,56] suggests that oxidative damage of macromolecules may be a major contributing factor to the decline in specific biological functions. This idea is strongly supported by studies with nematodes [52], *Drosophila* [49,51], and rodents [53,54,57], showing longevity to be associated with resistance to oxidative stress. Thus, damaged mitochondria release more ROS, initiating a vicious cycle of increasing DNA damage, leading to increased ROS production, more DNA damage, and mitochondrial dysfunction [53,55–59]. Based on this hypothesis, it might be expected that (1) the increased level of persistent (long-term) oxidative stress may, through oxidative damage of macromolecules, affect the function of stress signaling pathways; (2) as aging progresses, the constitutive activity of stress-activated signal pathways (e.g., p38 and SAPK/JNK) would increase; and (3) this new level of activity is stabilized—becoming a basic factor in the development of chronic stress in aged tissues.

Mitochondrial Dysfunction as a Source of Oxidative Stress

Phosphorylation of proteins in the p38 MAPK pathway increases dramatically in the aged liver without an increase in pool levels, suggesting that these modifications may mediate functional changes in these stress-signaling proteins [37,60]. Furthermore, p38 MAPK is activated in the young mouse livers by methyl methane sulfonate, a DNA damaging agent, and 3-nitropropionic acid (3-NPA), a potent inhibitor of complex II (succinic dehydrogenase). Both of these agents are generators of oxidative stress (ROS). In the livers of aged mice, however, this response to oxidative stress does not occur. Thus, the increased constitutive levels of phosphorylation, but failure to respond to the oxidative stress, suggests that the structure of these proteins may be altered in the aged tissue [60]. It has been proposed that changes in the p38 MAPK and SAPK/JNK signaling pathway activities may be a primary causative factor in the age-associated alteration of structure and function of transcription factors targeted by these pathways. A summary of these interactions affecting signaling in the liver is shown in Fig. 5 and listed here: (1) "Locked-in" generation of ROS is a major cause of age-associated mitochondrial dysfunction and a basic factor in the age-associated changes in activity of p38 MAPK and SAPK/JNK signaling pathways; (2) similarly, the increased level of ROS may cause nuclear dysfunction; (3) these structural changes alter the activities of transcription factors targeted by these signaling proteins, resulting in the development of symptoms of chronic stress (oxidative stress); (4) p38 MAPK pathway proteins' failure to respond to MMS or 3-NPA-generated oxidative stress in the aged rodent liver is due to structural changes in catalytic and/or regulatory domains of these kinases, decreasing their ability to interact/dock to form complexes essential for normal signal transduction; and (5) similar age-associated characteristics seen in the aging liver are also observed in other tissues such as brain and muscle.

Mitochondrial Dysfunction and the p38 MAPK Stress Signaling Pathway

3-NPA, a suicide inhibitor of complex II (succinic dehydrogenase), increases ROS by interrupting the electron cascade and preventing phosphorylation [61–65]. 3-NPA increases

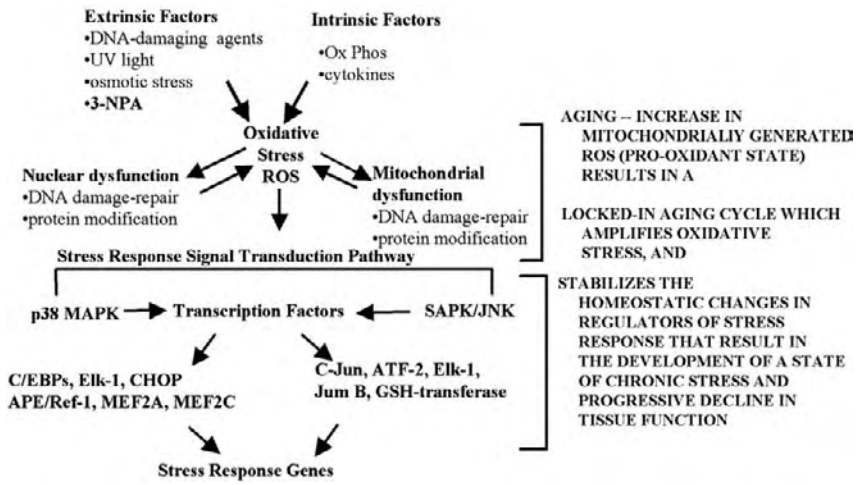


Figure 5 The oxidative chronic stress cycle of aging that depicts the relationship of oxidative stress to mitochondrial dysfunction, activation of stress response signaling pathways, stress response transcription factors, and their targeted genes.

production of free radicals (about twofold) in both mitochondria and microsomes of the liver and brain [68–70].

The mechanism of inactivation of complex II (succinic dehydrogenase) involves the alkylation of an—SH group in the enzyme's catalytic site, located in the 70-kDa subunit [66]. The inactivation mechanism involves a nucleophilic stack by this essential—SH group on the double bond of 3-nitroacrylate (3-NA, the product of SDH oxidation of 3-NPA. This mechanism proposes that an $O^{2\cdot-}$ radical may be the ROS generated by the oxidation–reduction of FAD resulting from the oxidation of 3-NPA [66]. Interestingly, it has been shown that oxidative stress generated by rotenone, an inhibitor of complex I, also activates p38 MAPK [67]. Although these experiments were done with cells in culture, these data demonstrate that ROS generated by dysfunction of components of the mitochondrial electron transport system participate in activation of the p38 MAPK signaling pathway. It has also been proposed that the nitro group of 3-NPA may be reduced by the nitro-reductase system to produce nitro superoxide anions. This initiates lipid peroxidation, leading to cell membrane damage [70]. Thus, 3-NPA can stimulate free-radical production both *in vivo* and *in vitro*. The 3-NPA-mediated increase in ROS (acute oxidative stress) can activate both the p38 MAPK and SAPK/JNK stress response signaling pathways and so induce an inflammatory response [37,60]. These studies suggest a possible linking of mitochondrial dysfunction, that is, generation of ROS to the activation of specific stress response signaling pathway(s) and the targeting of stress response genes. Furthermore, these studies have shown that aging affects the ability of liver cells to respond to this oxidative stress as is indicated by either failure to respond or an increased response (JNKs) to these challenges.

Aging, Oxidative Stress, and Cellular Signaling in the Liver

Both extrinsic and intrinsic signals are transduced to nuclear gene targets by a complex series of protein–protein interactions. These characteristics are determined by 3D protein structures that determine the proximity of specific amino acid clusters and their functional capacity [71,72]. Modifications of these clusters by phosphorylation/dephosphorylation modulate such functions as enzyme activity (activation), docking ability, and intracellular localization. The activities of the stress-activated p38 MAPK and JNK signaling pathways in aged liver suggest that these molecular interactions are altered by and affect the level and efficiency of signal transduction.

SOCS Family of Negative Regulators of Inflammatory Response

Cytokines activate signal transduction processes in response to inflammatory challenges as well as to growth factors and hormones. They initiate their signaling cascades by binding to cell-surface receptors whose cytoplasmic domains activate intracellular signal cascades of the JAK-STAT pathway [73]. In the liver, cytokine receptors (e.g., IL6) are associated with the Janus kinase (JAK) family of tyrosine kinases, for example, TYK2, JAK1, JAK2, and JAK3. Thus, cytokine binding induces receptor aggregation, which then activates JAKs via cross-phosphorylation of tyrosine residues in the cytoplasmic domain of cytokine receptors. This creates recruitment sites for signaling proteins containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains. By this mechanism, cytokine stimulation initiates multiple signal transduction cascades, one of which is the *s*ignal *t*ransducers and *a*ctivators of *t*ranscription (Stats). These signaling pathways then target genes whose activities lead to such biological processes as inflammatory response, cell differentiation, proliferation, and apoptosis.

Induction of SOCS Transcription and the Negative Regulation of Cytokine Gene Expression

Cytokine signaling is negatively regulated by the *s*uppressor *o*f *c*ytokine *s*ignaling (SOCS) family of proteins (SOCS1–SOCS7, CIS) [73]. SOCS gene transcription is up-regulated in response to cytokine stimulation, and the corresponding SOCS proteins inhibit the cytokine-induced signaling pathway. Modulation of this signaling occurs by several mechanisms, including (1) inactivation of the JAKs, (2) blocking access of the Stats to receptor binding sites, and (3) ubiquitination of signaling proteins and targeting to degradation via the proteosome.

The molecular mechanism of negative regulation of cytokine signaling by SOCS proteins is shown in Fig. 6. The models show that the cytokine activation signal is initiated by binding the dimerized cell-surface receptor. This stimulates the JAK kinase phosphorylation of the Stat transcription factors. The dimerized Stat-P translocates to the nucleus where it activates SOCS genes. Transcription and translation of the SOCS mRNA produces the SOCS proteins. Inhibition of the cytokine signal is then mediated by several interactions between a specific SOCS protein and the JAK or Stat proteins.

SOCS mRNAs are induced by cytokines and the corresponding SOCS proteins through a classical negative feedback mechanism that attenuates the signaling pathways that stimulated their production (Fig. 6). Furthermore, because the induction of SOCS proteins by one cytokine can inhibit signaling by another, it appears that these proteins might also mediate an overall down-regulation via their ability to cross-talk between cytokine receptors.

Overexpression of CIS, SOCS1, or SOCS3 in cell lines has shown their inhibitory effect on a wide variety of cytokines, hormones, and growth factors. Transgenic mouse models have revealed the ability of SOCS proteins to inhibit cytokine inducible signaling *in vivo*. For example, transgenic mice that express *cis* under the control of the β-actin promoter exhibit a significant down-regulation of GH-induced

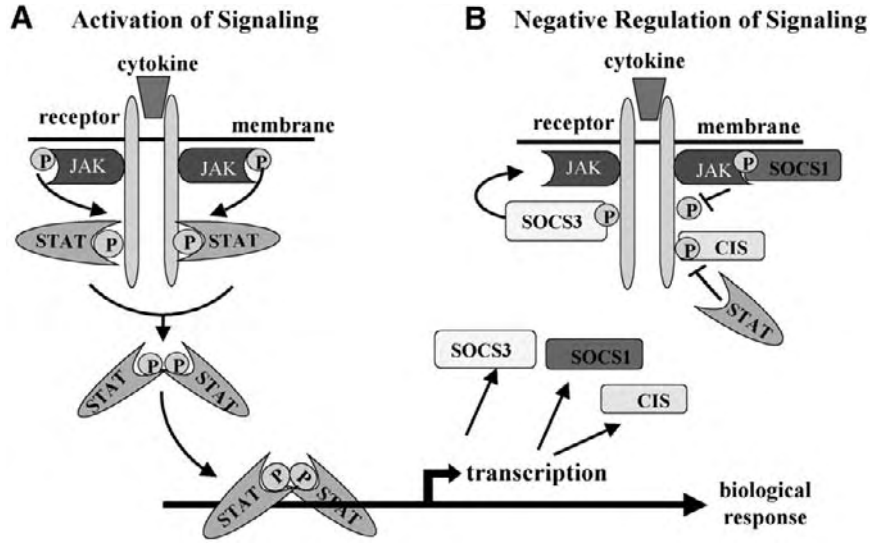


Figure 6 A model showing the cytokine-mediated activation of the JAK/Stat signaling pathway and the SOCS-mediated negative regulation of the JAK/Stat signal. (A) Activation of cytokine signaling is initiated by the dimerization of a cytokine membrane receptor complex. Phosphorylation of JAKs occurs upon dimerization of the receptor. Activation of the JAK/Stat signaling induces SOCS gene expression. (B) The SOCS proteins (CIS, SOCS1, SOCS3) inhibit the signaling pathway by negative feedback. The inhibition by SOCS1 involves binding to the JAKs and inhibition of JAKS catalytic activity. Inhibition by SOCS3 involves binding to the JAKs binding site on the receptor. Inhibition by CIS involves blocking of STAT binding to the receptor.

tyrosine phosphorylation of Stat 5 in the liver [74]. The fact that the phenotype of CIS transgenic mice resembles that of mice lacking Stat5a and/or Stat5b suggests that CIS might contribute to the negative regulation of Stat5 [74].

Physiological Function of SOCS Protein

Understanding of the physiological functions of SOCS genes advanced rapidly upon the successful generation and analysis of several knock-out mice. Although the SOCS1 (*socs1*$^{-/-}$) mutants are normal at birth they exhibit stunted growth and die at 3 weeks of age with a syndrome characterized by severe lymphopenia, activation of T cells, fatty degeneration and necroses of the liver, and macrophage infiltration of major organs [75]. It appears that the neonatal defects of the *socs1*$^{-/-}$ mutants may be due to the overexpression of IFN-γ signaling. More specifically the studies with the mutants suggest that SOCS1 plays a key role in the negative regulation of signaling by IFN-γ. This is supported by the fact that administration of IFN-γ to neonatal wild-type mice induces a pathology similar to that observed for *socs1*$^{-/-}$ mutants. It has been suggested that SOCS1 may regulate I-cell differentiation to prevent the emergence of activated I cells that produce excess IFN-γ, a situation that may have secondary consequences such as the up-regulation of proinflammatory cytokines such as TNF-α.

Mice lacking the *socs2* gene appear normal until 3 weeks after birth after which they grow 30–40% larger than their wild-type littermates. The weight increase is due to a significant increase in visceral organ weight, (not excess fat), carcass weight, long bone length, and body length [75]. Because *socs2*$^{-/-}$ mice are excessively large, it has been proposed that SOCS2 may negatively regulate growth promoting cytokines such as GH and IGF-I. For example, *socs2*$^{-/-}$ mice exhibit abnormalities associated with GH and IGF-I signaling. One outcome of GH signaling is the production of IGF-1; *socs2*$^{-/-}$ mutants exhibit characteristics of deregulation of GH and IGF-I signaling. These include elevated levels of IGF-I in heart, lungs, and spleen and decreased levels of the major urinary proteins (MUPs) [75]. MUPS have been shown to be down-regulated in GH-deficient mice [76,77]. In addition, the regulation of MUPs, which is GH-pulse dependent, is down-regulated in mice that overexpress GH. This is attributed to the disruption of the GH-pulsatile pattern. The decrease in MUPs in *socs2*$^{-/-}$ urine suggests, therefore, that GH signaling might be disregulated in these mice [75]. SOCS2, therefore, is a major component of the SOCS family synthesized in the liver and appears to play an essential physiological role in the regulation of growth, possibly due to its ability to modulate GH and/or IGF-I signaling. Thus, the ability of SOCS2 proteins to regulate GH/IGF-I signaling indicates that it plays a key role in the regulation of multiple pathways of biological processes.

Analysis of *socs3*$^{-/-}$ mice has shown that these mutants, as well as those overexpressing SOCS3, are embryonic lethals. The transgenic mice that overexpress *socs3* lack fetal liver erythropoiesis, whereas the *socs3*$^{-/-}$ embryos exhibit a marked erythropoiesis throughout the embryo [78]. There appears to be some controversy with respect to the characteristics of this mutation since a more recent study

describes severe placental dysfunction as the major consequences of this defect [79]. SOCS3 is especially expressed in fetal liver erythroid progenitors during the stage of erythropoiesis in which the erythropoietin (Epo)-dependent expansion of erythroid lineage cells occurs. It has been proposed, therefore, that the *socs3*$^{-/-}$ gene plays an important role in the regulation of fetal liver erythropoiesis and that the mechanism involves modulation of Epo signaling. Thus, SOCS3 deficiency in the *socs3*$^{-/-}$ mutant results in inability to attenuate Epo signaling. SOCS3 is also expressed in low levels in adult tissues; it is inducible by LPS in adult liver and plays a key role in the regulation of leptin in brain.

Molecular Mechanisms of Negative Feedback Regulation of Cytokine Signaling

The major SOCS family members of the liver, CIS, SOCS1, SOCS2, and SOCS3, inhibit cytokine signaling by several different mechanisms (Fig. 6): SOCS1 reacts directly with JAK1, JAK2, and JAK3, and TYK2, thereby inhibiting their catalytic (kinase) activity [80–83]. SOCS3 also inhibits JAK2 activity, but it binds to JAK2 with a lower affinity, suggesting it is a less efficient inhibitor than SOCS1 [84–87]. CIS and SOCS2 do not bind to or inhibit the JAKs [83,84].

The mechanisms by which SOCS1 inhibits JAK activity involve its binding to phosphorylated Tyr1007 of JAK2, which lies within the JAK2 activation loop, thereby regulating JAK activity [83]. The SOCS1 SH2 domain and 24-amino-acid residues immediately N terminal to the SH2 domain are required for high-affinity binding and inhibition of JAK2 activity [82,83]. There is also a kinase inhibitory region (KIR) within the 24-residue region (both SOCS1 and SOCS3) that is required for inhibition of JAK2 activity [83]. It is proposed that the KIR might inhibit JAK activity by acting as a pseudosubstrate that prevents access of legitimate substrates to the JAK catalytic pocket [83].

The negative regulatory activity of CIS occurs via another mechanism that involves the competition of Stats versus SOCS proteins for the phosphotyrosine binding (PTB) sites within the cytoplasmic domain of the cytokine receptor (Fig. 6). This is supported by the observation that both CIS and Stat5b bind to and compete for an overlapping set of phosphotyrosine residues on the GH receptor [88–90]. Thus, CIS inhibits GH signaling and Epo signaling by binding to their receptors [90].

The ability of SOCS3 to inhibit GH-induced JAK2 activation increases when the expression level of GHR increases [89]. Similarly the ability of SOCS3 to inhibit IL-2-induced JAK1 activation is enhanced in the presence of IL-2 [91]. These studies indicate that the inhibition of JAK activity by SOCS3 is augmented when SOCS3 is bound to cytokine receptors.

Recently, Nicholson *et al.* [92] demonstrated that SOCS3 binds with high affinity to a phosphopeptide corresponding to a region surrounding Tyr757 within gp130 of the IL-6 receptor. Mutation of Tyr757 causes decreased ability of

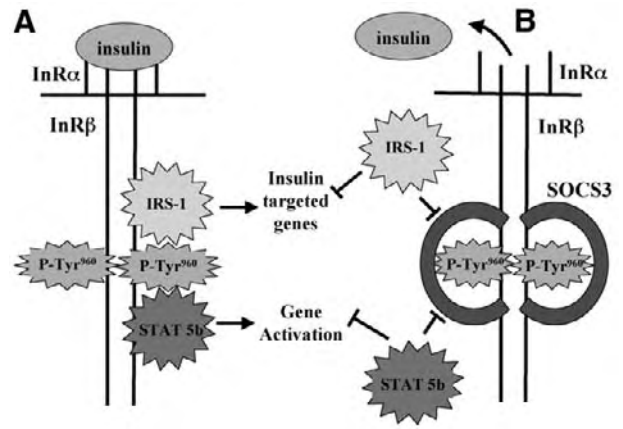


Figure 7 A model of the inhibition of Stat5b and IRS-1 binding to the insulin receptor by SOCS3. (A) The IRS-1, through its PTB domain interacts with the insulin receptor P-Tyr960 and the amino acid residues at the C terminus of the motif containing the P-Tyr. Stat5b interacts with the P-Tyr960 and the amino acid residues at the N terminus of the motif containing the P-Tyr. SOCS3 binds to the cytoplasmic domain of the insulin receptor (InRβ) via its SH2 domain and interaction with P-Tyr960. The binding of SOCS3 to the P-Tyr960 containing motif prevents IRS-1 and Stat5B from binding to the insulin receptor.

SOCS3 to inhibit gp130 signaling. These data suggest that optimal inhibition by SOCS3 occurs when SOCS3 is bound to gp130. A similar mechanism is suggested by the inhibition of the SOCS3 binding site on leptin or Epo receptors, which also interferes with ability of SOCS3 to inhibit signaling from these receptors. Thus, it is proposed that the mechanism of SOCS3 inhibition binding to activated cytokine receptors in the region of the JAKs (Fig. 6). Once localized at the receptor, SOCS3 inhibits JAK activity through its KIR.

Role of SOCS Proteins in Down-Regulation of Insulin Signaling

Insulin induces a transient expression of SOCS3 in liver, skeletal muscle, and white adipose tissue *in vivo*, and in 3T3-L1 cells *in vitro* [92]. Furthermore, SOCS3 antagonizes insulin-induced IRS-1 tyrosine phosphorylation and decreases IRS-1 association with p85 (PI3K). This involves the binding of SOCS3 to Tyr960 of the insulin receptor, thereby preventing Stat5b activation by insulin (Fig. 7).

Thus, the binding of SOCS3 to Tyr960 of InR has multiple regulatory functions, for example, attenuation of STATb targeted genes, attenuation of the downstream activation of IRS-1 tyrosine phosphorylation, and decreases in docking with p85 (PI3K), thereby demonstrating a sequential attenuation of downstream signal transduction.

Analysis of RNA from livers of mice starved overnight and injected with insulin showed that SOCS3 was induced 5.5-fold 1 hr after injection. Expression was sustained for 1 hr and returned to basal levels within 2 hr after injection. Similarly starved mice were injected with TNF-α; SOCS3 was induced in liver within 2 hr after injection and returned to normal at 4 hr. The level of induction of SOCS3 by TNF-α in liver was less than that by insulin.

These studies have shown that SOCS3 interferes with the tyrosine phosphorylation of IRS-1. IRS-1, through its interaction with PI3K, Grb2, and SHP2, controls several major cellular processes regulated by insulin such as glucose uptake, protein and glycogen synthesis, and gene expression. IRS-1 binds to InR using its PTB domain, whereas Stat5b and SOCS3 bind to the InR via their SH2 domains. However, the SH2 domain interacts with P-Tyr960 and amino acids located in the N terminus of the motif containing P-Tyr960, whereas the PTB domain binds P-Tyr960 and is dependent on residues located at the C terminus of the motif containing P-Tyr960. Another difference is that IRS-1 possesses a PH domain that also plays a critical role in insulin-induced IRS-1 phosphorylation in intact cells (Fig. 7) [93].

SOCS3 expression attenuates insulin-induced tyrosine phosphorylation of IRS-1 and its subsequent association with $p85^{PI3K}$. Thus, binding of SOCS3 to Tyr960 prevents the docking of IRS-1 with InR both through its PTB and PH domains. The interference of IRS-1 phosphorylation and p85 docking should attenuate specific biological functions. Through this protein–protein complex, SOCS-3 serves as a potent inhibitor of insulin signaling. It is proposed that SOCS3 expression is increased in several situations associated with insulin resistance.

All reports point toward an important role in IRS-1 phosphorylation on serine residues thought to be responsible for the decrease in insulin signaling [94]. There is a pronounced decrease in SOCS3 expression in (obese) mice lacking TNF-α signaling. Thus, elevated SOCS3 levels (found in obesity) in inflammatory response may be linked to increased TNF-α expression. To explain TNF-α-induced insulin resistance it has been proposed that phosphorylation of serine residues in IRS-1 may be responsible for the decrease in insulin signaling. This is based on the proposal that phosphorylation of serine residues of IRS-1 prevents its docking to form an active signaling complex [95].

The InR mediates its biological effects through tyrosine phosphorylation of several substrates such as IRS, Shc, and Stat5b. This phosphorylation is dependent on a functional coupling between the InR and its substrates. Thus, any molecular event impairing the coupling between the insulin receptor and its substrates should lead to a decrease in the tyrosine phosphorylation of the substrate molecules. These studies have shown that SOCS3 expression reduces insulin-induced DNA binding activity of Stat5b, probably via a competition between Stat5b and SOCS3 for binding to the insulin receptor P-Tyr960. Thus, SOCS3 negatively regulates insulin signaling; increased SOCS3 participates in development of insulin resistance; SOCS3 also regulates Stat5b and its targeted genes.

Role of SOCS Proteins in Down-Regulation of Growth Hormone Signaling

GH resistance is known to develop in certain conditions of severe illness, for example, sepsis and burns. Sublethal doses of LPS have been shown to result in a decreased ability of GH to stimulate Stat5 tyrosine phosphorylation in the liver. This occurs for at least 6 hr after single endotoxin injection [96]. There is no change in Stat5 pool levels in the liver, and the decrease in Stat5 phosphorylation results in decreased GH-mediated gene activation. This is proposed to be the mechanism of the resistance to GH induced by endotoxin or other catabolic states.

It has also been shown that the GH-GHR activation of JAK2 tyrosine kinase by autophosphorylation of tyrosine residues is also decreased in response to LPS treatment [94,97]. In this case, however, there is also an increase in the nonphosphorylated JAK2 pool level, which results from an increase in JAK2 mRNA. Although the mechanism of up-regulation of Jak2 gene transcription, for example, the transcription factors associated with this regulation, is not known the overall mechanism appears to involve both post-translational and transcriptional processes.

The abundance of GHR in the liver is sensitive to such stresses as nutrient regulation (starvation), surgery, sepsis, and so on. GHR levels were not affected by endotoxin 4 hr after treatment at a time when the Stat5 phosphorylation is lowest and JAK2 mRNA level is at its peak. Thus, these changes in Stat5 and JAK2 are independent of GHR abundance in the liver and suggest that resistance to GH occurs in the absence of receptor abundance.

The SOCS proteins are candidates for the endotoxin-induced postreceptor GH resistance. Three possible candidates of the SOCS family that are abundant in the liver are CIS, SOCS2, and SOCS3. These genes respond to cytokines and endotoxins; Northern analyses showed a rapid 4-fold increase in CIS and 10-fold increase in SOCS3 mRNAs but only a small transient increase in SOCS2 after endotoxin treatment. The SOCS3 mRNA remained elevated for at least 6 hr.

The increased SOCS gene mRNAs correlate with endotoxin-induced resistance to GH-stimulated Stat5 and JAK2 tyrosine phosphorylation, suggesting that SOCS proteins mediate these effects on GH signaling. In addition further evidence is provided by SOCS3-mediated inhibition of Spi 2.1 promoter activity induced by GH transactivation. Thus, SOCS3 negatively regulate the GH signaling that activates Spi 2.1. This negative regulation was not seen with CIS.

GH itself induces SOCS3 mRNA in the mouse liver; the resultant SOCS3 protein then functions as a negative feedback loop to attenuate GH signaling. This mechanism is similar to that proposed for the attenuation of cytokine signaling by SOCS proteins. After endotoxin administration, multiple cytokines are elaborated and each may interact with the liver to induce SOCS-family gene expression. Thus, the activation of SOCS genes in the liver may serve as attenuators of GH as well as cytokine signaling via a negative feedback mechanism.

In summary, endotoxin causes the decrease of GH stimulated tyrosine phosphorylation of Stat5 in the liver. This occurs in the absence of any change in Stat5 pool levels and an increase in JAK2 mRNA and protein pool levels. There is also a 50% decrease in the amount of GH stimulated phosphotyrosine per JAK2, which is preceded by the marked

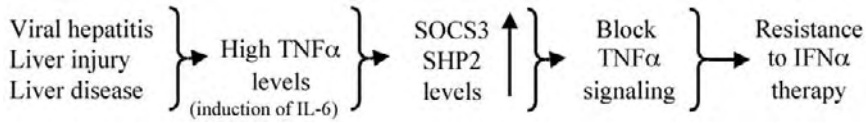


Figure 8 The role of TNF-α in development of resistance to IFN-α therapy. The sequence of biochemical events that leads to resistance to IFN-α therapy in patients with liver disease.

increase in JAK2 mRNA. The GH signaling changes due to LPS-mediated resistance are not due to changes in the abundance of GHR. But SOCS mRNAs (CIS, SOCS2, SOCS3) are rapidly up-regulated by LPS treatment. Thus, the SOCS proteins are involved in LPS-mediated alteration of GH signaling.

Role of SOCS Proteins in the Regulation of Liver Regeneration

Adult animal hepatocytes regenerate following partial hepatectomy in rats. Quiescent hepatocytes rapidly and synchronously reenter the cell cycle and divide until the original mass of liver tissue is restored (~10 days). One of the Stat transcription factors, Stat3, which is associated with cytokine signaling, is activated during the first few hours after PH [97]. For example, Stat3 activation in rat hepatocytes peaks at 3 hr after partial hepatectomy and recovers by 12 hr. The Stat3 activated hepatocytes were initially detected in the periportal zones and then extended to the pericentral zones. This coincides with the transcriptional induction of immediate early growth response genes [96]. This follows the pattern of DNA synthesis [98]. DNA synthesis and Stat3 binding activity, after partial hepatectomy in TNFR-I or IL-6(–/–), are severely impaired. This can be corrected by injection of IL-6 before partial hepatectomy [99–101].

For example, Stat3 is also activated in Kupffer cells as well as sinusoidal endothelial cells prior to activation in hepatocytes. Stat3 is activated rapidly in regenerating or sham-operated livers [101]. Stat1, -4, or -6 is not activated. Thus, SOCS1 and SOCS3 negatively regulate Stat3 signaling in the early phase of liver regeneration.

Role of SOCS and SHP2 in Regulation of Interferon Signaling

Patients having high levels of TNF-α have been found to respond poorly to IFN-α therapy, suggesting that TNF-α may be involved in resistance to IFN-α therapy. The studies by Hong *et al.* [102,103] have shown that TNF-α induces Stat1 protein expression but inhibits IFN-1α activated tyrosine phosphorylation of both Stat1α (91 kDa), and Stat1β, (84 kDa) at Tyr701. These data indicate Stat1 gene expression is activated.

TNF-α inhibition of IFN-α activated Stat1 in the liver occurs 2 hr after injection, which is consistent with new protein synthesis. The role of two protein families [SOCS and PIAS (protein inhibitor of activated Stat)] in the inhibition of IFN-α signaling was examined. The data show that TNF-α specifically induces SOCS3 mRNA expression in the liver; that SOCS3 attenuated IFN-α-induced reporter gene expression. These data suggest that SOCS3 plays a significant role in the TNF-α-mediated inhibition of IFN-α signaling in the liver (Fig. 8).

Protein tyrosine phosphatase (PTP) is another major pathway involved in suppression of IFN-α signaling. Injection of TNF-α into mouse showed that SHP2 is markedly induced, whereas several others were not affected. TNF-α specifically induces SHP2 protein expression in the liver. Coimmunoprecipitation showed that JAK1 and TYK2 coimmunoprecipitate with SHP2 in normal untreated (TNF-α) liver thus demonstrating an *in vivo* complex formation. TNF-α markedly enhances association of SHP2 with JAK1 or TYK2 and increased level of expression of SHP2 protein. These data suggest that induction of SHP2 protein expression and an increase in association of SHP2 with JAKs may contribute to TNF-α mediated inhibition of IFN-α signaling in liver, *in vivo* [103].

Although TNF-α and SOCS3 are components of the TNF-α inhibition of IFN-α signaling in liver *in vivo*, TNF-α does not directly induce SOCS3 expression or inhibit IFN-α signaling. TNF-α does stimulate IL-6 gene expression and it is the IL-6 that markedly stimulates SOCS3 expression in primary rat hepatocytes. These studies have shown that IL-6 is involved in the IFN-α induction of Stat1 protein expression but not in the induction of either SOCS3 or SHP2 in liver. The failure of TNF-α to induce SOCS3 or SHP2 was shown using IL-6(–/–) mutant. TNF-α can induce SOCS3 and SHP2 in IL-6(–/–) mutant livers.

CCl_4 was used to induce liver injury and inflammation to examine whether IFN-α is inhibited by the TNF-α mechanism. CCl_4 markedly induces TNF-α and attenuates IFN-α activated Stat1 in the liver. The Stat1 protein level goes up. Using TNFR1 and TNFR2 knock-out mice, it was shown that CCl_4 attenuated IFN-α-activated Stat1 in wild-type mice but not in knock-out mice. These studies indicate that activation of TNFR1 by TNF-α is involved in liver injury-mediated suppression of IFN-α-activated Stat1 in the liver.

In summary, (1) TNF-α, which is elevated in serum of patients with viral hepatitis (Fig. 8), inhibits IFN-α signaling in the liver *in vivo*; (2) TNF-α is involved in resistance to IFN-α therapy; (3) both SOCS3 and SHP2 are involved in TNF-α-mediated inhibition of IFN-α signaling in liver; (4) three families of proteins are implicated in the down-regulation of the JAK/STAT signaling: SOCS, PIAS, and PTP; (5) TNF-α induces SOCS 3 and SHP2, but not other SOCS and PTPs; and (6) induction of SOCS3 and SHP2 participate in TNF-α-mediated inhibition of IFN-α signaling.

Role of SOCS in Hepatocellular Carcinoma

Abnormalities of the JAK/STAT pathway have been reported in association with cancer; activation of JAK/STAT expression plays a significant role in oncogenesis [104]. Aberrant methylation of promoter regions, which silence transcription, has been shown to be associated with cancer. Using restriction landmark genomic scanning analysis of hepatocellular carcinoma (HCC), Yoshikawa *et al.* [104] identified the SOCS1 gene sequences in the DNA fragment and determined that the *NotI* site was methylated in the HCC. These studies showed that methylation silenced SOCS1 and the growth-suppression activity of SOCS1 protein. Examination of a variety of HCC cell lines showed that the expression status of SOCS1 correlates with the dense methylation of CpG islands in the promoter. Analyses also showed that the matrix association of SOCS1 is altered in association with methylation status and that the matrix attachment was reduced in the SOCS1 methylated HCC cells.

Because SOCS1 inhibits JAK2 tyrosine phosphorylation, there should be functional consequences of the silencing of SOCS1 by methylation. The cells showing silenced SOCS1 also exhibited phosphorylated JAK2. Furthermore, because phosphorylation of Stat3 is in the JAK/STAT pathway, the methylated SOCS1 should result in constitutive Stat3 phosphorylation. The data show that SOCS1 inactivated-JAK2 phosphorylated cells exhibit constitutive phosphorylation of Stat3. Thus, SOCS1 inactivation leads to a basal level of activation of JAK2/Stat3 pathway.

Restoration of SOCS1 in silenced HCC cells selectively suppressed the growth of these cells by inducing apoptosis. Thus, SOCS1 suppresses anchorage-independent growth as well as growth in a monolayer. The following model is proposed: (1) In normal cells, SOCS1 activation blocks JAK activation and terminates cytokine signaling due to stress and growth factor; (2) in cancer cells, SOCS1 is silenced by methylation and is unable to terminate these signaling pathways, making JAK activation constitutive; (3) thus, SOCS-1 silenced cells have unopposed growth stimulation by various cytokines, growth factors, and hormones; and (4) SOCS1 normally functions to suppress hepatocyte growth.

Conclusions

Biological signaling is the highly specific process that enables cells and tissues to regulate their responses to their environment, both intrinsic and extrinsic. This chapter focused on two major signaling pathways: the p38 MAP kinase pathway, which responds to mitogenic and stress activating signaling molecules, and the SOCs family, which "turns off" or mediates the recovery from these signaling stimulations. Although the p38 MAPK stress response pathway was first discovered in response to an inflammatory challenge by bacterial endotoxin (LPS), its diverse functions have been rapidly identified, thus making it a major and critical pathway that regulates numerous biological processes. This is seen in its regulation of differentiation, apoptosis, and immune functions.

The recent demonstration that the mechanism of cellular destruction by anthrax lethal factor involves blocking the p38 pathway adds to the importance of this pathway. Most certainly, there are other equally important signaling pathways that play a key role in the response to stress factors, mitogens, hormones, and so on, such as the ERK and SAPK/JNK pathways and the hormone activated pathways such as insulin/IGF-1 and GH pathways. All of these pathways share several mechanisms of signal transduction: protein–protein interactions (docking), protein modifications (phosphorylation), and intracellular trafficking (nucleocytoplasmic, mitochondrial). Thus, in our discussion of the mechanisms of p38 MAPK and SOCs pathways, our purpose was to familiarize the reader with these mechanisms that occur in most signaling processes. Furthermore, our focus on how aging affects the function of the p38 MAP kinase pathway is meant to demonstrate that in addition to environmental and intrinsic factors, signaling pathways also play a key regulating role in biological processes during the entire life cycle, that is, spanning the embryonic young adult and aging phases of life.

Although signaling is a critical factor in the activation of biological processes, an important part of the signaling process is "turning the signals off." Thus, by describing the negative feedback regulation of signaling by members of the SOCs gene family we demonstrate the basic mechanisms required for this regulation. Again, we see that protein–protein interactions, protein modifications, and intracellular trafficking are important processes in the mechanism. Thus, the regulation of biological signaling, both "in" and "off" plays a key role in establishing tissue homeostasis. It is important therefore, to understand these mechanisms in order to understand the biological processes of growth, development, and aging as well as their role, in many cases, in dysregulation in pathological circumstances.

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CHAPTER 342

Cell–Cell Signaling in the Testis and Ovary

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Introduction

The evolution of multicellular organisms was facilitated by the ability of different cells to communicate and interact. This cell–cell signaling generates a higher order functional state than that possible with individual cell types. Cell–cell interactions have become an essential requirement for the physiology of any organ or tissue and are critical in the regulation of any cell's biology. For this reason, elaborate networks of cell–cell interactions have evolved to control the development and maintenance of tissue functions. The focus of the current chapter is on the regulatory signals that mediate cell–cell interactions in the testis and ovary.

Several previous reviews have discussed the cell–cell interactions in the testis [1,2] and ovary [3,4]. These include a focus on secretory products of the various cell types and actions of individual regulatory molecules. The current chapters briefly discusses the advances in cell–cell signaling in these organs.

Many different types of cell–cell interactions are required for the control of tissue physiology and cellular functions. These have been previously categorized into regulatory, nutritional, and environmental classifications [1]. Regulatory interactions are generally mediated by extracellular factors that through receptor-mediated events cause a signaling event to modulate cell functions. Nutritional interactions generally involve the transport of nutritional substances, energy metabolites, or metabolic substrates between cells. Environmental interactions involve extracellular environmental factors that affect cell contacts and cytoarchitecture. The focus of this chapter is primarily on regulatory-type interactions that involve a receptor-mediated signaling event. It is this type of cellular signaling that regulates a cell's function on a molecular level. The factors involved are generally paracrine and autocrine agents such as growth factors and cytokines.

Both the testis and ovary are endocrine organs. Endocrine hormones from the pituitary [i.e., gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH)] act on various cell types to influence cellular functions and cell–cell interactions. The influence these endocrine hormones have on cell–cell signaling events is briefly reviewed. The testis and ovary are also sites for the production of hormones. These gonadal hormones have an endocrine role in regulating a wide variety of tissues in the body, but can also act in a paracrine manner within the gonads to influence cell–cell signaling and cellular functions. Again the role these gonadal steroids and peptide hormones play in the regulation of cell–cell signaling within the gonad are discussed in this chapter.

Cell–Cell Signaling in the Testis

Testis Cell Biology

The adult testis is a complex organ that is composed of seminiferous tubules that are enclosed by a surrounding interstitium. The seminiferous tubules are the site of spermatogenesis where germ cells develop into spermatozoa in close interaction with Sertoli cells (Fig. 1). The Sertoli cell

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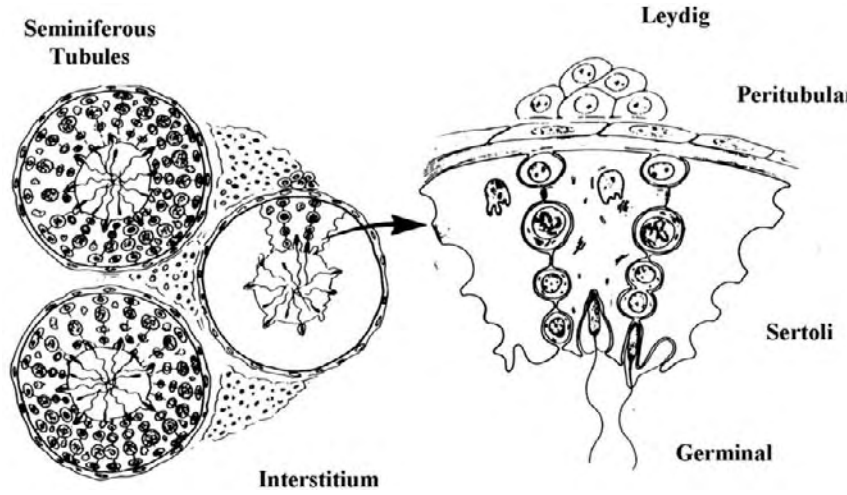


Figure 1 Testis cell biology.

is an important testicular somatic cell that controls the germ cell environment by the secretion and transport of nutrients and regulatory factors. The Sertoli cells [5] form the basal and apical surface of the seminiferous tubule and provide the cytoarchitectural arrangements for the developing germ cells [6]. Tight junctional complexes between the Sertoli cells contribute to the maintenance of a blood–testis barrier [7] and create a unique environment within the tubule [8]. The structure of the Sertoli cell has been reviewed by several investigators (for a review, see [6]), and a three-dimensional reconstruction has increased appreciation for the complexity of the structural relationships between cells within the seminiferous tubule [9]. The biochemical analysis of the Sertoli cell has primarily focused on an examination of the components synthesized and secreted by the cell. The list of products includes steroids such as estradiol [10], metabolites such as lactate [11], and various proteins such as plasminogen activator [12], testicular transferrin [13], testicular ceruloplasmin [14], inhibin, and others (for a review, see [2]). The majority of the secretory products are hormonally regulated and provide useful markers of Sertoli cell differentiation.

Surrounding the basal surface of the Sertoli cells is a layer of peritubular myoid cells (Fig. 1) that function in contraction of the tubule. The peritubular cells surround and form the exterior wall of the seminiferous tubule. Peritubular cells are mesenchymally derived cells that secrete fibronectin [15] and several extracellular matrix components [16]. Both the peritubular and the Sertoli cells form the basement membrane surrounding the seminiferous tubule and their interactions are important in germ cell development.

The interstitial space around the seminiferous tubules contains another somatic cell type, the Leydig cell (Fig. 1), which is responsible for testosterone production. Leydig cells have a major influence on spermatogenesis through the actions of testosterone on both the seminiferous tubule and the pituitary. Although the Leydig cell has numerous secretory products [1], testosterone is the most significant secretory product of the cells. Thus, interaction of all three somatic cells—Sertoli, peritubular, and Leydig—are important for regulation of normal spermatogenic function in the testis (for a review, see [1]).

Testis Development

The process of fetal testis formation occurs late in embryonic development (embryonic day 13 where plug date = E0 (E13) in the rat) and is initiated by migration of primordial germ cells, first from the yolk sac to the hindgut and then from the hindgut to the genital ridge. The first phase of migration is proposed to occur through a mechanism in which transient interactions between fibronectin molecules on the extracellular matrix and corresponding receptors on the primordial germ cells cause movement of the germ cells. The second migration is thought to occur by the release of chemoattractant factors from the genital ridge. Kit ligand and its receptor c-kit appear to be involved first in the migration to the genital ridge and later in the proliferation of germ cells after colonization of the genital ridge. Expression of kit ligand has been localized to cells along the migratory pathway, and c-kit is expressed by primordial germ cells at this time in development (for a review, see [17]). After migration, germ cell differentiation in the gonad is dependent on locally produced factors such as prostaglandins [18] and the induction of specific transcription factors [19]. It is a complex network of cellular interactions that control testis and germ cell development.

The gonad is bipotential after germ cell migration and can be distinguished morphologically from the adjoining mesonephros (E12 in rat), but cannot be identified as an ovary or a testis. A variety of genes such as SRY, SOX-9, SF1, and DMRT1 are involved in the transcriptional induction of sex determination and testis development [20–27]. Two morphological events occur early on E13 to alter the bipotential gonad. First, Sertoli cells, which are proposed to be the first cell in the testis to differentiate, aggregate around primordial germ cells [28,29]. Secondly, migration of mesenchymal cells occurs from the adjoining mesonephros into the developing gonad to surround the Sertoli cell–germinal cell aggregates. The migrating population of cells has been

speculated to be preperitubular cells [30–32]. The mechanism for this migration is unknown, but a signal from the testis is proposed to occur and cause cell migration. This is postulated due to the observation that ovarian mesonephros can also be stimulated to initiate cell migration after close interaction with a developing testis [33]. In addition, using an organ culture system in which mesonephros and embryonic testis were separated by an embryonic ovary, mesonephros cells migrated through the ovary to the testis [30]. Therefore, during early testis development Sertoli–peritubular cell interactions may allow for cord formation to occur. The cords develop neonatally into seminiferous cords and at the onset of puberty develop into the seminiferous tubules. Sertoli cells have been postulated to originate from stem cells in the coelomic epithelium at an early stage in gonadal development. Other cells that may potentially originate from the coelomic epithelium are interstitial or Leydig cells [34].

Seminiferous cords, precursors of adult seminiferous tubules, form as the Sertoli cell–primordial germ cell aggregates become more organized and are fully surrounded by mesenchymal cells. The formation of the seminiferous cords (E14 in rat) is a critical event in the morphogenesis of the testis since this is the first indication of male sex differentiation [26]. During the process of cord formation, Sertoli cells undergo a number of morphological changes including a change in expression of mesenchymal to epithelial cell markers (vimentin to cytokeratin; [35]), a change in expression of cytokeratin 19 to cytokeratin 18 (cytokeratin 21 expressed in ovary; [36]), and expression of Müllerian inhibiting substance (MIS), which inhibits the development of the Mullerian duct, the precursor of the female uterus, cervix, fallopian tubes, and upper vagina [37–39].

Outside of the seminiferous cords, the peritubular layer of cells becomes identifiable from the interstitium or Leydig cells at E15 [38] and 3βHSD production is detected after E15 [39]. Leydig cells have been hypothesized to differentiate after cord formation and Sertoli cell differentiation is completed [40,41]. This is important because the production of testosterone and androgens by the Leydig cells has been demonstrated to stabilize the Wolffian duct derivatives, hence allowing normal male duct development [42,43]. Therefore, appropriate differentiation of somatic cell types in the testis around the time of cord formation is crucial not only to the normal development of the testis, but also for the continued presence of the Wolffian duct and normal male reproductive tract development.

Testis Cell–Cell Interactions

Table I outlines a number of the factors produced locally in the testis that mediate cell–cell signaling events in the control of spermatogenesis and testis function. Several reviews address the topic of cell–cell interactions in the testis and the control of spermatogenesis [1,2,44,45]. Recent observations are cited next.

Transforming growth factor α (TGF-α) is an epidermal growth factor (EGF) superfamily member and is produced by Sertoli, peritubular, and Leydig cells. TGF-α can act as a growth stimulator on all major cell types in the testis [46–48]. As with TGF-α, transforming growth factor β (TGF-β) is also produced by Sertoli, peritubular, and Leydig cells and can act on all the major cells in the testis [49–53]. In contrast, TGF-β primarily acts as a growth inhibitor and can stimulate a variety of differentiated functions. Another example of a factor that is produced by all of the somatic cells and acts on all major cell types in the testis is insulin-like growth factor 1 (IGF-1) [54–56]. IGF-1 plays a general role in regulation of the growth cycle and homeostasis of the testis. A related family member, IGF-2, mediates paracrine interactions between Sertoli cells and germ cells [57]. These are examples of regulatory factors that mediate cell–cell signaling events between the majority of the cell types in the testis.

Several interleukins (IL-1α, IL-1β, IL-6) are produced in the testis by Sertoli cells and Leydig cells. These interleukins can regulate Sertoli, Leydig, and germ cell growth and differentiated functions [58–65]. Although further analysis is needed, interleukins appear to mediate primarily Sertoli–germ cell and Leydig–Sertoli cell interactions, as well as autocrine roles for these factors.

Several hormonal factors produced in the testis also act locally within the testis as paracrine factors. An example is inhibin and its related peptide activin [66–68]. Inhibin is primarily produced by Sertoli cells and can act on germ cells and Leydig cells. Further investigation of the actions of inhibin and related compounds within the testis is needed. Another major endocrine factor produced in the testis is testosterone by Leydig cells that can in turn act on Sertoli, peritubular, and Leydig cells [69]. Androgens have a major role in the maintenance of testis function by inducing cellular differentiated functions.

Fibroblast growth factor (FGF) family members have been shown to be expressed in the testis and regulate the growth and differentiation of a variety of cells [70–75]. FGF receptors are predominant in germ cells and Leydig cells, but are also present in the other cell types [71]. FGF-14 has recently been shown to be expressed in spermatocytes and may influence adjacent Sertoli or peritubular cells. FGF-9 null mutants also suggest a role in early testis development, but remain to be investigated in the adult [74]. Basic FGF is produced by Sertoli cells and can also act on the other cells [75]. The variety of FGF ligands and receptors role in testis function remains to be elucidated.

Platelet-derived growth factor (PDGF) has been shown to be produced by Sertoli cells and influence peritubular cells and Leydig cells [76–79]. Although PDGF in the adult may also be produced by the Leydig cell [76], it appears to be a factor produced within the seminiferous tubules that acts on adjacent peritubular cells and Leydig cells. Another factor that is only produced by Sertoli cells is stem cell factor (SCF)/kit ligand (KL), which has a direct role in regulating spermatogonial cell proliferation [80–83]. Mutations in SCF/KL block the process of spermatogenesis. This is one of the better examples of a somatic–germ cell interaction.

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine that influences stem cell growth and survival. LIF is

Table I Cell–Cell Signaling Factors in the Testis

Signaling factor	Site production	Site action	Functions	Ref
Transforming growth factor α (TGFα)	Sertoli Peritubular Leydig	Sertoli Peritubular Leydig Germ	Growth stimulation	46–48
Transforming growth factor β (TGFβ)	Sertoli Peritubular Leydig	Sertoli Peritubular Leydig Germ	Growth inhibition Differentiation stimulation	49–53
Insulin-like growth factor (IGF1)	Sertoli Peritubular Leydig	Sertoli Peritubular Leydig Germ	Homeostasis and DNA synthesis	54–56
Interleukin-s	Sertoli Leydig	Sertoli Leydig Germ	Growth regulation Cellular differentiation	58–65
Inhibin	Sertoli	Germ Leydig	Cellular differentiation	66–68
Androgen	Leydig	Sertoli Peributular Leydig	Cellular differentiation	69
Fibroblast growth factors	Sertoli Germ Leydig	Germ Peritubular Sertoli Leydig	Growth stimulation	70–75
Platelet derived growth factor (PDGF)	Sertoli	Peritubular Leydig	Growth stimulation Cellular differentiation	76–79
Stem cell factor/Kit ligand (SCF/KL)	Sertoli	Germ	Growth stimulation	80–83
Leukemia inhibitory factor (LIF)	Peritubular Sertoli Leydig	Germ	Growth stimulation Cell survival	84–85
Tumor necrosis factors	Germ Leydig	Sertoli Germ	Cellular apoptosis Cellular differentiation	86–88
Hepatocyte growth factor (HGF)	Peritubular	Leydig Peritubular Sertoli	Growth stimulation Tubule formation	89–91
Neurotropins	Germ Sertoli	Sertoli Peritubular	Growth stimulation Cell migration Cellular differentiation	1,92

predominantly produced by peritubular cells, but also by Sertoli cells and Leydig cells [84]. Although LIF has been shown to influence germ cell growth and survival [85], other functions remain to be elucidated.

Tumor necrosis factors (TNF-α) and related ligands (TRAIL) are produced in the testis by germ cells and Leydig cells. Both TNF and TRAIL have a role in regulating germ cells and Sertoli cells [86–88]. The function of these regulatory factors for the germ cells may be more for apoptosis regulation, unlike for Sertoli cells, which may be more for cellular differentiated functions.

Hepatocyte growth factor (HGF) is generally a mesenchymal-derived factor that acts on adjacent epithelial cells. HGF was found to be expressed by the mesenchymal-derived peritubular cells, and its receptor (c-met) was found on both Sertoli cells and Leydig cells [89–91]. Interestingly, c-met was also found in the peritubular cells. HGF may also have a role in seminiferous tubule formation [91].

Several neurotropins have been shown to be expressed in the testis. Nerve growth factor (NGF) is produced by the germ cells in the adult and appears to act on the Sertoli cells [1]. In embryonic development neurotropin-3 is expressed by Sertoli cells and acts on the migrating mesonephros cells to promote seminiferous cord formation [92]. Further investigations are needed to elucidate the roles of these and other neurotropins in the testis.

Additional factors are anticipated to be identified and have critical roles in testis development. Recently identified factors such as erythropoietin expression in Sertoli cells and peritubular cells [93], or interferon-gamma actions on

Sertoli cells [94], or relaxin-like factor (RLF) expression by Leydig cells [95] are all likely to have roles in cell–cell signaling in the testis. These and other factors such as PModS [96] need to be further investigated to determine their roles in testis cell biology. Clearly, a complex network of cell–cell signaling events and factors regulates testis function and spermatogenesis.

Cell–Cell Signaling in the Ovary

Ovarian Cell Biology

The ability of somatic cells in the gonad to control and maintain the process of gametogenesis is an essential requirement for reproduction. The basic functional unit in the ovary is the ovarian follicle, which is composed of somatic cells and the developing oocyte (Fig. 2). The two primary somatic cell types in the ovarian follicle are the theca cells and granulosa cells. These two somatic cell types are the site of action and synthesis of a number of hormones that promote complex regulation of follicular development. The proliferation of these two cell types is in part responsible for the growth of the ovarian follicle. The elucidation of factors that control ovarian somatic cell growth and development is critical to an understanding of ovarian physiology.

Granulosa cells are the primary cell type in the ovary that provides the physical support and microenvironment required for the developing oocyte (Fig. 2). Granulosa cells are an actively differentiating cell with several distinct populations. Alteration in cellular differentiation is required during folliculogenesis from a primordial stage of development through ovulation to a luteal stage of development. Regulation of granulosa cell cytodifferentiation requires the actions of a number of hormones and growth factors. Specific receptors have been demonstrated on granulosa cells for the gonadotropins FSH [97] and LH [98]. In addition, receptors have been found for factors such as EGF [99,100], insulin-like growth factor [101], and anti-Mullerian hormone [102]. The actions of these hormones and growth factors on granulosa cells vary with the functional marker being examined and the stage of differentiation. The biosynthesis of two important ovarian steroids, estradiol and progesterone, is a primary function of the granulosa cells in species such as the bovine, human, and rodent. Estrogen biosynthesis is controlled by the enzyme aromatase, which requires androgen as a substrate. Progesterone is synthesized from cholesterol by a series of steroidogenic enzymes. As the follicle develops, granulosa cells differentiate and estrogen biosynthesis increases. FSH promotes this follicular development via the actions of cAMP. As the follicle reaches stages before ovulation, the granulosa cells develop an increased capacity to synthesize and secrete progestins under the control of LH. In contrast, the early follicle stage (e.g., primordial) granulosa appear hormone independent and are nonsteroidogenic.

Another important cell type in the ovary is the ovarian theca cell (Fig. 2). These are differentiated stromal cells that surround the follicle and have also been termed theca interstitial cells [103]. The inner layer of cells, the theca interna, has a basement membrane separating it from the outermost layer of mural granulosa cells. One of the major functions of theca cells in species such as the bovine, human, and rodent is the secretion of androgens [104]. Theca cells respond to LH by increasing the production of androgens from cholesterol [105] (Fig. 2). Theca cells also produce progestins under gonadotropin control [106–109]. Other secretory products of theca cells have not been thoroughly investigated. At the primordial stage no theca cells are present; however, during transition to the primary stage, theca cells (i.e., precursor cells) are recruited to the follicle [3].

Follicle Growth and Differentiation (Folliculogenesis)

The control of ovarian follicle development is complex and involves multiple waves of growth [110]. In both the human and bovine ovary, two or three waves of follicles are

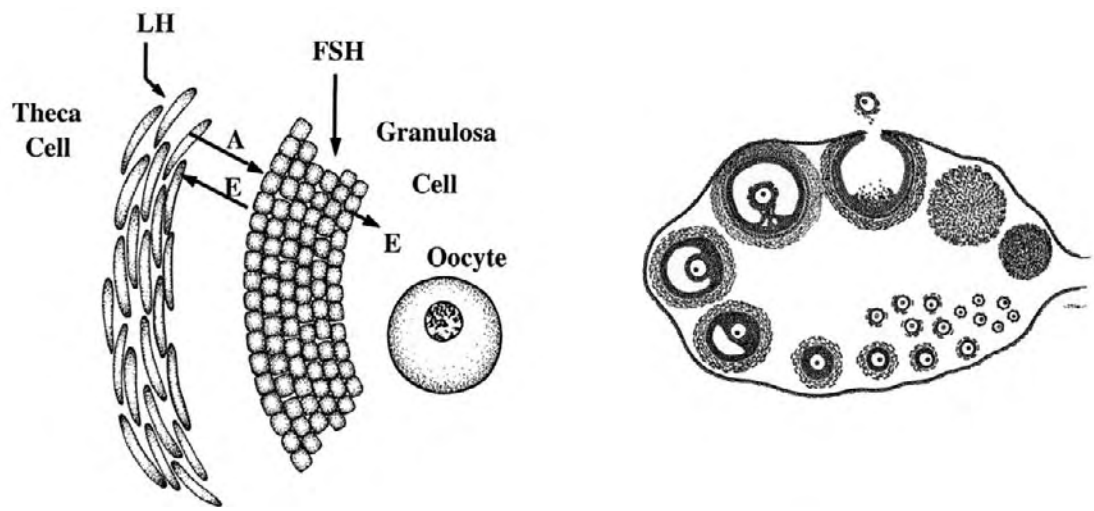


Figure 2 Ovary cell biology.

initiated to develop in a single ovarian cycle [111,112]. For both of these species, follicles expand from several millimeters to up to 2 cm during this process. A combination of granulosa cell growth, theca cell growth, and antrum formation results in the expansion of the ovarian follicle. Although rapid stimulation of cell growth is required for the ovulatory follicle to develop, the vast majority of follicles undergo atresia in which cell growth is arrested at various stages of follicle development. Hormones such as estrogen and FSH have been shown to promote follicle cell growth *in vivo*, however, these hormones alone do not stimulate growth of ovarian cells *in vitro* [113]. The possibility that these hormones may act indirectly through the local production of growth factors is proposed for later stages of development. Therefore, the regulation of ovarian cell growth is a complex process that requires an array of externally and locally derived regulatory agents. Interactions between theca cells, granulosa cells, and oocytes are required for follicular maturation [114]. The individual processes such as dominant follicle selection [115] and follicle cell apoptosis/atresia [116,117] also require integrated cell–cell interactions. A variety of specific growth factors produced in the follicle appear to mediate many of these cellular interactions in later stages of follicle development.

Ovarian Cell–Cell Interactions

Table II outlines a number of the factors produced locally in the ovary that mediate cell–cell signaling events in the control of follicle development and ovarian function. Several reviews address the topic of cell–cell interactions in the ovary and the control of follicle development [3,4,118,119]. Recent observations are cited next.

Table II Cell–Cell Signaling Factors in the Ovary

Signaling factor	Site production	Site action	Functions	Ref
Transforming growth factor α (TGFα)	Theca	Granulosa Theca	Growth stimulation	120–127
Transforming growth factor β (TGFβ)	Theca Granulosa	Granulosa Theca	Growth inhibition Cellular differentiation	128–132
Hepatocyte growth factor (HGF)	Theca	Granulosa	Growth stimulation	133–136
Keratocyte growth factor (KGF)	Theca	Granulosa	Growth stimulation	137–139
Colony stimulating factor (CSF)	Theca	Granulosa Theca	Growth regulation	140–141
Tumor necrosis factor (TNF)	Granulosa Theca Oocyte	Oocyte Granulosa Theca	Apoptosis Growth regulation	142–145
Fas ligand	Granulosa Theca Oocyte	Oocyte Granulosa Theca	Apoptosis	146–149
Nerve growth factor (NGF)	Theca	Granulosa Theca	Growth stimulation Ovulation	150
Fibroblast growth factor (bFGF)	Granulosa Theca Oocyte	Granulosa Theca	Growth stimulation	152–154
Growth differentiation factor -9 (GDF-9)	Oocyte	Granulosa Theca	Cellular differentiation	155–159
Bone morphogenic poteins (BMP)	Oocyte Theca	Granulosa Theca	Cellular differentiation	160–166
Kit Ligand/Stem cell factor (KL)	Granulosa	Oocyte Theca	Growth stimulation	167–171
Leukemia inhbitory factor (LIF)	Granulosa	Oocyte Theca	Growth stimulation Cellular differentiation	172–173
Vascular endothelial factor (VEGF)	Theca Granulosa	Edothelium Granulosa	Angiogenesis	174–178
Interleukins	Granulosa Theca	Granulosa Theca	Cellular differentiation	179–182
Insulin-Like growth factor (IGF-1)	Granulosa Theca	Oocyte Granulosa Theca	Growth stimulation Cellular differentiation	183–185
Inhibin	Granulosa	Oocyte Theca Granulosa	Cellular differentiation	186–187

TGF-α has been shown to be produced by theca cells [120–123] and to influence the growth of both theca and granulosa cells [120,124]. Several *in vivo* experiments have shown that TGF-α can influence follicle development [125,126]. TGF-α appears to be important for follicle development and involves theca cell–granulosa cell interactions. TGF-α has also been localized to isolated granulosa cells but appears predominantly in theca cells [127]. The primary function of TGF-α is growth stimulation. TGF-β is also predominately produced by theca cells [128], but is produced by isolated granulosa cells in selected follicle stages [129]. TGF-α and TGF-β differentially regulate granulosa and theca cell differentiated functions and growth [130–132]. Although TGF-β inhibits TGF-α growth stimulation, TGF-β also can influence cell functions.

HGF is produced by theca cells and acts on granulosa cells to promote cell proliferation and function [133, 134]. This is an excellent example of the role HGF plays in mediating mesenchymal–epithelial interactions in tissues. Interestingly, SCF/KL produced by the granulosa cells can feed back on the theca to regulate HGF production [135,136]. In a similar manner, keratinocyte growth factor (KGF) is produced by theca cells and acts on granulosa cells to regulate cell growth [137,138]. KGF is also expressed in the corpus luteum [139]. SCF/KL was found to also stimulate KGF expression by theca [135]. These factors reflect the importance of the theca cell in the regulation of follicle growth.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) was found to primarily be expressed by theca cells and most cells in the ovary [140,141]. The GM-CSF can influence granulosa cell growth and function. Null mice had abnormal follicle development that suggested effects on the local cell–cell interactions [141].

Apoptosis is an essential aspect of follicle development and ovarian function. The vast majority of follicles undergo atresia and apoptosis. TNF has been shown to be produced by most cell types in the ovary associated with apoptosis [142–145]. TNF can act on all cell types and induce apoptosis and growth regulation. Another death ligand that binds death receptors to induce apoptosis is Fas ligand. Fas is also produced by all the cells associated with apoptosis and acts to promote apoptosis in the atretic follicles [146–149]. These signaling molecules are essential for ovarian function in the promotion of follicle atresia during folliculogenesis.

Nerve growth factor (NGF) was found to be expressed by theca cells and act on theca and granulosa cells [150]. The localization and actions of NGF suggest a potentially important role at the time of ovulation [150]. Other neurotropins (e.g., NT-4) are also expressed at various stages of ovary development [151] and require further investigation.

Basic fibroblast growth factor (bFGF) has been shown to be expressed by granulosa cells and to a lesser extent by theca cells [152]. Basic FGF can regulate both granulosa cell and theca cell growth and differentiated functions [153,154]. During follicle development the expression of bFGF changes, being initially in the oocyte at the primordial stage and then in the granulosa in the primary stage [3]. The role of other FGF family members has not been rigorously addressed.

Growth differentiation factor 9 (GDF-9) is a member of the TGF-β superfamily and is specifically localized to the oocyte. GDF-9 can act on both granulosa cells and theca cells to regulate steroidogenesis and differentiated functions [155–159]. The actions of GDF-9 are follicle stage specific and it appears to be expressed in a variety of species. GDF-9 may regulate the expression of other paracrine factors such as SCF/KL in the developing follicle [159]. This is one of the few oocyte-specific products identified to be involved in cell–cell signaling in the ovary.

Another factor specifically expressed in the oocyte that appears to regulate granulosa cell function is bone morphogenic protein 15 (BMP-15) [160,161]. BMP-15 and GDF-9 may act synergistically during follicle development. Other BMP family members include BMP-4 and -7, which are primarily localized in the theca cells and appear to act on the granulosa cells [162]; BMP-2, which acts on granulosa cells [163]; and BMP-6, which is also expressed in the oocyte and acts on the granulosa cells [164]. The BMP family of growth factors are TGF-β superfamily members and appear to be critical to follicle development [165,166].

SCF/KL is produced by the granulosa cell and acts on the oocyte and theca cells [167–171]. The null mutant suggests a critical role in oocyte viability and recruitment of primordial follicles. In addition to the role in granulosa–oocyte interactions, granulosa KL also influences theca cell function and development [170]. Oocytes appear to have a regulatory role in influencing the expression of KL by granulosa cells [171]. As found in the testis, this is a critical somatic–germ cell interaction. Another factor found to be expressed by granulosa cells that regulates oocytes is LIF [172,173]. LIF is also produced by stromal cells in the ovary. This action of LIF in mediating granulosa–oocyte interactions is supported by levels of LIF that increase in follicular fluid as the follicle develops [172,173].

Vascular endothelial growth factor (VEGF) has a critical role in angiogenesis. This process is important for developing follicles past the primary stage of development. VEGF is primarily expressed in theca cells and to a reduced level by granulosa cells [174–178]. VEGF has a major role in acting on endothelial cells to promote angiogenesis, but also can influence granulosa cell functions [175]. This cell–cell signaling event controlled by VEGF is critical for follicle development.

Cytokines as seen with the testis also influence ovary function. Interleukins 1, 6, and 8 have all been shown to regulate follicle development. IL-1 is expressed by the granulosa and affects granulosa function [179,180]. IL-8 is primarily expressed by the theca and to a lesser extent by granulosa and influences cellular function [181]. IL-6 is also expressed by granulosa cells an acts on various cells, including granulosa [182]. Further investigation of the specific roles of these and other member of the interleukin family is needed.

IGF-1 also has a role in the ovarian follicle [183]. IGF-1 is expressed by granulosa and theca cells and acts on the oocyte,

granulosa, and theca cells [183–185]. Mice with null mutations in IGF-1 have impaired follicle development [184]. Other members of the family IGF-2 and the IGF-binding proteins also have a critical role in follicle development [185].

Inhibin also has a paracrine role in the developing follicle. Inhibin is primarily produced by the granulosa cells and acts on the oocyte, theca, and granulosa cells [186,187]. Related family members such as the activins are also anticipated to have similar roles. This is distinct from the roles these factors have in the endocrine system.

Additional signaling factors are anticipated to be essential for ovarian function and follicle development. One example is anti-Müllerian hormone (AMH), which is expressed by the granulosa cells, but specific biological function remains to be determined [188]. AMH may have a role as a negative regulator of oocyte viability or of primordial follicle development. Local steroid production is also expected to influence the network of local cell–cell signaling events. This includes both androgen and estrogen production [189].

Conclusions

The preceding descriptive discussion of cell–cell signaling in the testis and ovary demonstrate a growing complexity in the networks of cellular interactions and factors. It is anticipated that some of these factors will have compensatory roles to ensure growth and differentiation of the tissues. The list of factors provided is likely to be only partially complete and more will be added as investigation of cell–cell interactions in the gonads expands. Currently, we are primarily in the identification of the site of production and action research phase. The functions of some individual factors are also being analyzed. However, the next research phase of cell–cell signaling will involve a more systems biology type of approach that should tie together all potential interactions and provide more insight into the regulation of testis and ovary function.

The specific cell–cell signaling events identified are shown in most cases to change during development. The requirements and physiology of the embryonic testis and ovary are very different from the adult. Another research area to expand is the elucidation of cell–cell signaling at these different stages of development.

A comparison of the cell–cell signaling events between the testis and ovary is very useful. Some signaling events are the same, for example, the role SCF has in mediating direct somatic–germ cell interaction or the role HGF and KGF play in mesenchymal–epithelial cell interactions. A direct correlation among the cell–cell interactions of the testis and ovary will be invaluable in elucidating the system biology approach to understand gonadal function.

Elucidation of cell–cell signaling events is required for the future development of therapeutic agents to control fertility and treat reproductive diseases. By understanding the signaling events involved in testis and ovarian function, basic information is provided to design more effective therapeutics. Significant advances are anticipated to be in the area of contraceptive and fertility agent development and treatment of diseases such as polycystic ovarian disease or premature ovarian failure. Although an understanding of the intracellular signaling events is essential for understanding how a factor acts, the elucidation of the network of extracellular signaling molecules that regulates a cells function is essential to our understanding of how a tissue or organism functions.

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CHAPTER 343

T Lymphocytes

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Introduction

The immune system provides a highly sophisticated surveillance mechanism to detect diverse antigens and protect the host organism from invading pathogens and altered cells (e.g., virus-infected and tumor cells). Adaptive immune responses depend on the recognition of antigen by specific antigen receptors that are expressed on the surface of T and B lymphocytes. To initiate effector mechanisms, binding of the antigen must induce intracellular signaling cascades that activate the lymphocytes and promote their differentiation to an effector cell type appropriate for the particular antigenic challenge. Importantly, regulatory mechanisms must also be present to safeguard against inadvertent self-reactivity, which could lead to autoimmunity, and to terminate immune responses, thereby avoiding overexposure of the organism to toxic effectors (e.g., cytotoxic T cells, cytokines).

T lymphocytes are derived from the lymphoid lineage of hematopoietic stem cells. T-cell progenitors enter the thymus where they develop into mature T lymphocytes. During thymic development, the immature thymocytes undergo rearrangement of first the β and then the α T-cell receptor (TCR) genes [1]. This process increases the diversity of available TCRs and ensures that each T cell expresses only a single type of TCR. Only those thymocytes that have successfully completed TCR gene rearrangement will be allowed to survive; unsuccessful rearrangement leads to programmed cell death. Following rearrangement, the functionality of the maturing thymocytes is tested by interactions with thymic antigen-presenting cells (APCs).

To be functional, the thymocytes' TCR must be able to engage epitopes formed by short peptides bound to molecules encoded by the major histocompatibility complex (MHC). Because of the large isotypic and allelic variability of MHC molecules within each species, not all recombination events of TCR genes lead to matches with a peptide–MHC complex potentially present in the individual organism. Therefore, a positive selection event is required. Two classes of MHC molecules select T cells with different functions. MHC class I molecules bind peptides derived from proteins synthesized by the presenting cell and proteolytically processed by the cell's proteosome. These peptides can combine with nascent MHC class I molecules in the endoplasmic reticulum. MHC class II molecules bind peptides derived from extracellular, endocytosed proteins that are processed in lysosomes. Nascent and recycling MHC class II molecules bind these peptides while trafficking through lysosomes [2].

Positive selection of thymocytes requires that the interaction between the TCR and peptide–MHC complex induces a signal of sufficient strength and duration. Thymocytes that do not receive a TCR-mediated signal during positive selection die. Positive selection occurs on thymic stromal epithelial cells, which do not present a full complement of all of the possible antigens that the T cell might encounter in its lifetime. Thus, selected T cells have partial self-reactivity and the potential to recognize antigens that will only be encountered later in life. Maturing thymocytes express both CD4 and CD8 coreceptors. Following positive selection, one coreceptor gene is silenced. Recent experimental results suggest that the duration of the selection signal determines lineage commitment with a short duration leading to differentiation to $CD8^+$, and a long duration favoring differentiation to $CD4^+$ thymocytes [3,4].

A separate selection step is a negative test to eliminate thymocytes that overtly respond to self-antigens. Estimates suggest that of all thymocytes that mature up to the

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double-positive ($CD4^+CD8^+$) stage, only about 3% mature and emigrate from the thymus to peripheral lymphoid tissues (e.g., blood, lymph nodes, and spleen). Negative selection is mediated by thymic stromal cells or APCs (mainly dendritic cells) that migrate from peripheral organs into the thymus. These cells provide a large battery, but not a complete complement, of different peptides derived from self and foreign proteins. Thymocytes that vigorously respond to the stimuli presented during negative selection will undergo apoptosis [5,6].

This chapter provides a broad overview of T-cell signaling, with emphasis on the signaling molecules and pathways important for antigen-induced signal transduction and elicitation of effector functions. General principles are discussed, and open questions and areas of current research are also highlighted.

Signaling Receptors in T Cells Form Dynamic Macromolecular Signaling Complexes

Antigen-Specific T-Cell Receptors

The antigen-specific T-cell receptor is composed of multiple polypeptides. Following thymic selection, peripheral T lymphocytes express a TCR that is composed of two transmembrane protein chains linked by a disulfide bridge. Most mature T cells express a recombined α TCR gene combined with a recombined β TCR chain, but some T cells, especially those in mucosal tissues, express a γδ TCR. The αβ TCR is the antigen-recognition unit of the T cell, but it does not have intrinsic signaling capability. The ability to transduce intracellular signals is conveyed to the TCR via its mandatory and constitutive association with a multiprotein structure, termed the CD3-ζ2 complex. Neither of the individual components of the TCR/CD3-ζ2 signaling machine can be transported to the cell surface without the full assembly of the complex [7].

The CD3 complex is composed of four transmembrane polypeptide chains, a γε and a δε heterodimer. These proteins have very short extracellular domains and each intracellular domain contains a conserved protein tyrosine kinase (PTK) recognition motif, termed an *immunoreceptor tyrosine-based activation motif* (ITAM). The associated, disulfide-linked ζ-dimer, sometimes substituted with a ζ-η heterodimer, contains three ITAMs per protein chain. ITAMs are substrates for *src* family PTKs, and their phosphorylation is a determining initiation event for T-cell signaling [8].

TCR Engagement and the Formation of Signalosomes

The binding of ligands to the TCR triggers the activation of receptor-associated *src* family PTKs, such as $p56^{lck}$ and $p59^{fyn}$, leading to the rapid tyrosine phosphorylation of numerous proteins. The phosphorylation of ITAMs located in the cytoplasmic tails of CD3 and ζ_2 generates binding sites for proteins bearing Src homology 2 (SH2) domains, such as the cytosolic *syk* family PTK ζ-*associated protein of 70 kDa* (ZAP-70) [8]. Recruitment of ZAP-70 allows enhanced activation of that kinase. ZAP-70 in turn phosphorylates components of distinct downstream signaling pathways [9–11]. Thus, T-cell activation depends on activation of both *src* family kinases and ZAP-70.

Before we follow the signal transduction cascade further, it is worthwhile to briefly discuss the complex temporal and spatial arrangement of signaling complexes and networks activated by TCR engagement. Slightly different compositions of these signaling machines, also termed *signalosomes* [12], can induce different second messenger signals and lead to drastically diverse cellular responses. In addition, the dynamic assembly and disassembly of signalosomes is likely a major factor in regulating signal transduction networks. TCR signalosomes consist of transmembrane receptors, protein kinases, phosphatases, and their substrates, all of which are organized into signaling machines by anchoring, adapter, and scaffolding proteins. Signalosomes connect events on the plasma membrane to distal signaling cascades, which ultimately modulate T-cell biology. Several protein adapters, in particular, linker of activated T cells (LAT), act as central switches that translate the quality, quantity, and duration of signals into the correct activation of specific downstream pathways [8].

Formation of signalosomes is aided by compartmentalization of the plasma membrane into detergent-insoluble, sphingolipid/cholesterol-enriched microdomains, which promote the recruitment of signal transduction molecules to the TCR-signaling machine upon TCR engagement [13,14] (Fig. 1). These areas of the T-cell surface are also known as lipid "rafts." Palmitoylation constitutively embeds several components, such as Lck, Fyn, and LAT, into these lipid microdomains, whereas others, such as ZAP-70, relocalize into rafts upon TCR engagement [12,13].

Returning to the events following TCR engagement, it is now clear that the relocalization of signalosomes to receptor-associated scaffolds is crucial for effective signal transduction [12,15,16]. Adapter proteins with SH2 domains bind to the phosphorylated ζ chain. Among these proteins is the Src homology 2 protein of beta cells (Shb), which recruits LAT via its central, phosphotyrosine-binding (PTB) domain-like motif [17,18]. LAT contains nine tyrosine phosphorylation sites and is a substrate for ZAP-70 [18]. Tyrosine phosphorylation of LAT leads to the recruitment of additional signaling molecules with SH2 motifs, including the adapters growth factor receptor-bound protein 2 (Grb2) and Gads, as well as the phospholipase Cγ1 (PLCγ1), and the p85 subunit of phosphatidylinositol 3-kinase (PI3K) [18–21] (Fig. 2).

Gads is associated with the SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), and together they form a macromolecular scaffold, which stabilizes the interaction of PLCγ1 with the TCR signalosome [21,22]. SLP-76 is essential in the activation of PLCγ1 and downstream signaling [23]. PLCγ1 is tyrosine phosphorylated in antigen-activated T cells, an event required for its activation. Active PLCγ1 hydrolyzes phosphotidylinositol biphosphate (PIP_2),

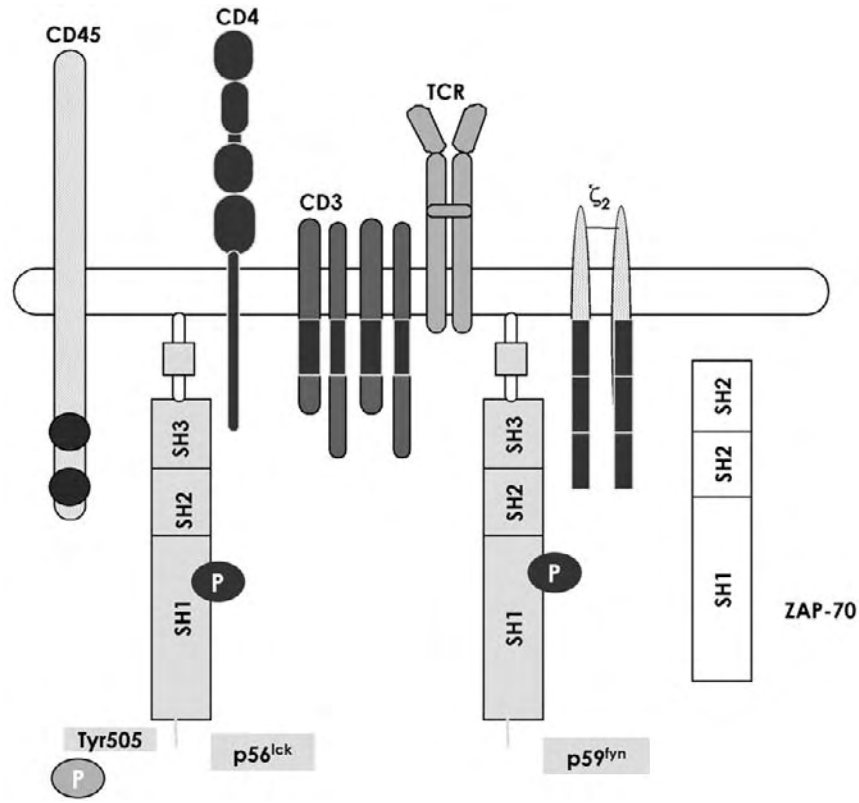


Figure 1 The TCR and its signaling machinery. The initial signalosome consists of the TCR, the associated CD3/ζ_2 complex, the coreceptor (CD4 or CD8), the phosphatase CD45, and the *srk* kinases p56^{lck} and p59^{fyn}. The *syk* kinase ZAP-70 is recruited following phosphorylation of tyrosines located in ITAMs of CD3 and ζ_2 by srk kinases.

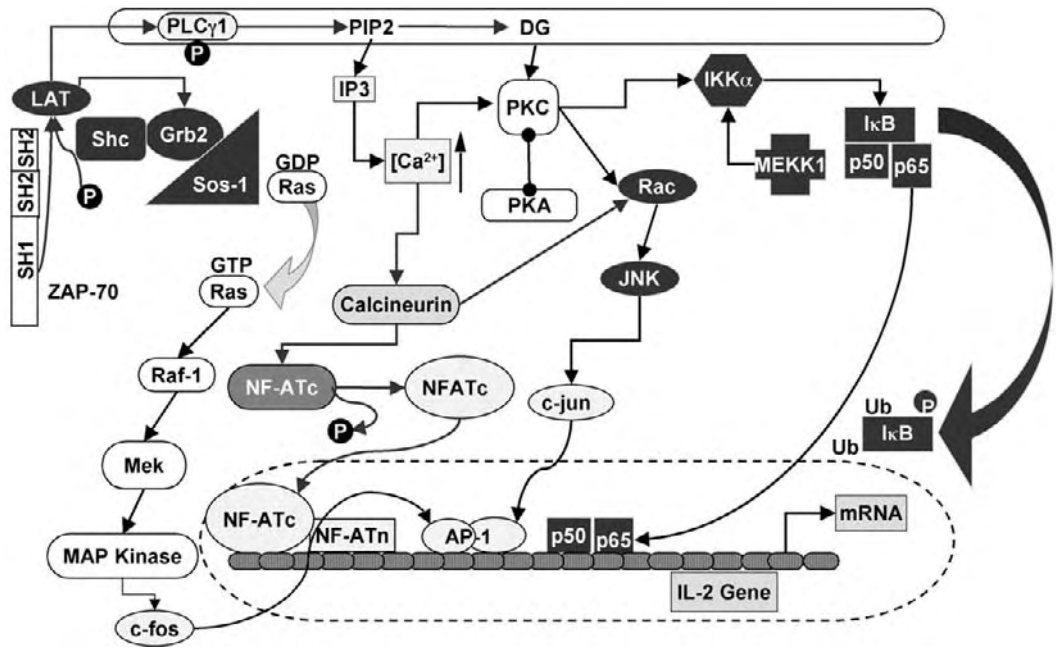


Figure 2 The signaling pathways induced by stimulation of the TCR/CD3/ζ_2 complex mediate translocation of NFAT, AP-1, and NFκB transcription factors to the nucleus, leading to IL-2 gene expression. See text for details.

producing diacylglycerol (DAG) and 1,4,5-inositol triphosphate (IP_3). DAG in turn activates the serine/threonine kinase family of protein kinase C (PKC), while IP_3 induces calcium (Ca^{2+}) mobilization in the cytosol. Thus, ZAP-70 amplifies the TCR signal by specifically phosphorylating downstream components such as LAT [18,20] and PLCγ1 [23].

In this way the signalosome expands in molecular complexity and amplifies the TCR-initiated signal. Importantly, LAT can also bind proteins that negatively regulate TCR signaling. The SH2 domain-containing hematopoietic phosphotyrosine phosphatase, SHP-1, associates with LAT upon TCR stimulation [24,25] and prevents further phosphorylation of the adapter by ZAP-70, suggesting a potential conversion from an "activating" to an "inhibiting" signalosome. Similarly, the C-terminal *src* kinase (Csk) relocalizes to rafts by docking to the transmembrane adapter Csk-binding protein (Cbp), also known as phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG) [26]. In rafts, Csk inhibits *src* family PTKs by phosphorylating their regulatory tyrosines and, thus, blocking TCR-mediated signal transduction [27].

Coreceptor and Costimulatory Proteins Modulate T-Cell Signaling Pathways

Before discussing the second messenger signals induced by TCR engagement, we need to introduce two sets of T-cell surface proteins that crucially modulate TCR/CD3-ζ_2-mediated signals: the coreceptors and the costimulators.

Coreceptors, TCRs, and Formation of Signalosomes

Coreceptors are associated with the TCR/CD3-ζ2 complex upon T-cell activation. Their presence in the TCR multicomponent signaling machine amplifies or modulates the activation signal. Often, their presence is absolutely required, but not sufficient, for productive signaling, that is, signaling that results in cell cycle progression and effector functions.

CD4 AND CD8

CD4 and CD8 are membrane glycoproteins associated with MHC class II and class I restriction, respectively. In mature T cells, their expression is mutually exclusive. In general, $CD4^+$ T helper (Th) cells respond to antigen presented by MHC class II molecules, and $CD8^+$ cytotoxic T (Tc) cells respond to MHC class I-presented antigens. CD4 and CD8 have been thought of as molecules that enhance the stability of the tripartite complex between TCR, antigen, and MHC. However, in recent years, it has become clear that their function is more complex and dynamic. For example, TCR antigen–MHC interactions initiate the formation of a specialized junction between T cells and APCs, the immunological synapse. Stimulation- and cytoskeleton-dependent processes cluster TCR/CD3 complexes in the center of the synapse, also termed the *central zone of the supramolecular activation cluster* (cSMAC), whereas adhesion molecules such as LFA-1 form a ring surrounding the central area called the *peripheral SMAC* (pSMAC) [28,29]. After stimulation of the T cell, CD4 coreceptors are rapidly recruited into the cSMAC, but migrate toward the periphery within a few minutes, whereas TCR/CD3 complexes stabilize within the central area [30]. Both CD4 and CD8 associate with the PTK $p56^{lck}$, and the efficient transport of $p56^{lck}$ into the cSMAC is a major function of these coreceptors [31]. In addition, coreceptor interactions with MHC molecules regulate peripheral T-cell homeostasis and the survival of naïve T cells in the absence of antigenic stimulation [32,33]. The recent discovery that CD4 can induce signals independent of the TCR suggests complex regulatory effects of coreceptors on T-cell function [70].

CD5

The CD5 lymphocyte glycoprotein is expressed on thymocytes and all mature T cells. CD5 can act as a costimulatory molecule for resting T cells by augmenting CD3-mediated signaling [34]. In mature, peripheral T cells, CD5 is present in lipid rafts of the T-cell surface, where it promotes CD3 redistribution into rafts, thus markedly up-regulating ZAP-70 and LAT activation and also Ca^{2+} influx [35]. However, CD5 is also constitutively associated with SHP-1, an interaction that increases upon TCR stimulation and negatively regulates TCR-mediated activation [36]. The differential modulatory properties of CD5 depend on the context of lymphocyte subset and their differentiation stage. A recent review discusses contradictory and complementary reports of CD5-mediated molecular intracellular signaling events [37].

CD45

CD45 is a membrane-bound tyrosine phosphatase present on hematopoietic cells. Multiple exons and differential glycosylation allow the expression of different isoforms in cell- and development-specific fashion. These different CD45 isoforms can be distinguished by monoclonal antibodies specific for splicing- and glycosylation-dependent epitopes [38]. In peripheral human $CD4^+$ T cells, the naïve subset, that is, the cells that have not been stimulated by antigen after thymic selection, expresses the high-molecular-weight form, CD45RA, whereas activated and memory $CD4^+$ T cells express the low-molecular-weight form, CD45RO [38]. Importantly, TCR-dependent intracellular signaling events differ in relation to CD45 isoform expression [39]. Another distinguishing feature of CD45 is its distribution in activated T cells: Whereas the CD4/CD8 coreceptors migrate into the cSMAC before relocating to the pSMAC, CD45 is completely excluded from the immunological synapse [40].

MULTIPLE FUNCTIONS OF CORECEPTORS

Views on coreceptor function have evolved with available technologies. The traditional definition identifies coreceptors as proteins that associate with the TCR upon T-cell stimulation, and stabilize the TCR's interactions with its ligands. However, the ability to identify PTKs and to measure

their activities has demonstrated associations between $p56^{lck}$ and CD4 or CD8, inspiring the realization that these coreceptors enhance and regulate TCR functions by transporting Lck into the TCR/CD3-ζ_2 complex. Similarly, the recently identified coreceptor CD160/BY55, which is expressed by most intestinal intraepithelial lymphocytes and by a minor subset of circulating lymphocytes including NK, γδ TCR, and cytotoxic effector $CD8^+$ T lymphocytes, associates with Lck and tyrosine-phosphorylated ζ_2 upon TCR/CD3 cell activation [41]. In addition, CD5 is associated with SHP-1 upon T-cell activation [36], and CD45 contains intrinsic phosphatase activity. Thus, physiological responses to TCR engagement at the T-cell–APC contact site are the result of localized and finely tuned alterations in the balance between cellular kinases and phosphatases [40].

The ability of coreceptors to transduce signals independent of TCR stimulation suggests that they are not merely scaffolding or transport proteins, but rather exert complex regulatory effects on T-cell activation [70]. Functionally, the distinction between coreceptors and costimulators blurs and the distinguishing feature of coreceptors may be their temporal association with the TCR/CD3-ζ_2 complex.

Costimulatory Receptors and TCR-Mediated Activation

The concept of T-cell costimulation was born of the necessity to explain the phenomenon of self-tolerance within the concept of Burnet's clonal selection theory. Because not all self-proteins traffic to the thymus or are expressed at all stages of development, central tolerance induced by negative selection of autoreactive thymocytes in the thymus cannot fully explain the unresponsiveness of mature T cells to peripheral self-antigens. Therefore, several investigators introduced the two-signal model of activation [42–44]. The model postulates that any naïve lymphocyte stimulated via engagement of only the antigen receptor will enter a state of anergy, which is characterized by unresponsiveness to future antigen-mediated stimulation. Only if a second, costimulatory signal is given during antigen stimulation is the lymphocyte fully activated to display effector functions and to proliferate. Costimulatory signals for B cells and $CD8^+$ cytotoxic T cells are mainly provided by activated $CD4^+$ Th cells, whereas a variety of surface molecules expressed on hematopoietic APCs (e.g., dendritic cells, activated macrophages, activated B cells) can induce costimulation in Th cells.

Th-cell costimulatory receptors do not have to associate with the TCR/CD3 complex to exert their function. They can transduce signals independent of the TCR/CD3. Originally, they were thought to only induce cellular effector functions in combination with TCR-transduced signals. This dogma was recently challenged as a result of the realization that CD28, one of the most important costimulatory receptors for T-cell activation, can up-regulate cytokine gene transcription independently of TCR stimulation [45]. Some of the coreceptors discussed in the previous chapter also have costimulatory functions.

CD28

Interactions of CD28 with its ligand B7 on APCs activate the CD28-responsive element (CD28RE), contained within the interleukin 2 (IL-2) gene promoter, and thus promote induction of IL-2 gene expression. Interaction of CD28 with three intracellular proteins—PI3K, the T-cell-specific Tec-family kinase Itk, and the complex between Grb2 and the guanine nucleotide exchange protein "son of sevenless" (SOS)–activates the MAPK/ERK kinase kinase (MEKK1), which contributes to full activation of the CD28RE and regulates activation of the transcription factors NFκB and AP-1 [46]. The MEKKs are serine/threonine kinases that are upstream regulators of MAPKs (Fig. 2).

The CD28 signal also amplifies activation of PLCγ1 and mobilization of Ca^{2+}. Further, CD28 engagement activates $p56^{lck}$ molecules that are phosphorylated at the regulatory tyrosine residue 505, generally considered to be an inactive form of $p56^{lck}$ [31]. Thus CD28 amplifies signals from the TCR that would otherwise be too weak for T-cell activation. Interestingly, the PTKs $p56^{lck}$ and $p59^{fyn}$ phosphorylate CD28, and Itk binding to CD28 is dependent on the presence of $p56^{lck}$. Thus, $p56^{lck}$ is likely to be a central switch in T-cell activation, with the dual function of regulating CD28-mediated costimulation as well as TCR/CD3/CD4 signaling.

Because CD28 provides cosignals in T-cell responses, a key question is whether the CD28 operates exclusively via TCR/CD3-ζ_2 or also operates as an independent signaling unit. Indeed, CD28 can cooperate with Vav and the SLP-76 adapter to up-regulate IL-2 and -4 transcription independently of TCR engagement [45,47]. In addition, CD28 stimulation alone activates the p38 alpha MAPK [48].

Importantly, engagement of the TCR alone may result in an anergic state or T-cell deletion, both of which can induce tolerance to antigen stimulation. Insight into the regulation of CD28 dependency comes from genetic experiments. T cells that are deficient in the adapter molecule Cbl-b do not require CD28 engagement for IL-2 production. Also, whereas B cells responding to T-cell-dependent antigens cannot undergo isotype switching from IgM to IgG in CD28-deficient mice, T-cell help is fully restored in CD28/Cbl-b double-deficient mice [49]. The function of Cbl-b is to selectively suppress TCR-mediated Vav activation, thus rendering T cells dependent on CD28 costimulation [49].

CD40L

The interaction of the tumor necrosis factor (TNF) family member, CD40L, on activated T cells with its receptor, the TNF receptor family member, CD40, which is expressed on macrophages, dendritic cells, and activated B cells, provides a strong signal for IL-12 production [50]. An important aspect of CD40-CD40L signaling is its synergistic relationship with the CD28-B7 signal. CD40L cell surface expression is up-regulated by CD28 signaling. The subsequent

interaction between CD40L and CD40 induces B7 up-regulation on APCs, enhancing the costimulatory activity of macrophages, dendritic cells, and B cells [51].

Intracellular Signaling Pathways Induced by Antigen Stimulation of T Cells

Calcium Mobilization

The TCR-induced signal transduction leads to activation of PLCγ1, which hydrolyzes PIP_2 to DAG and IP_3. Binding of IP_3 to its receptor in the endoplasmic reticulum membrane induces the release of Ca^{2+} into the cytosol. The subsequent increase in intracellular free Ca^{2+} opens Ca^{2+}-regulated Ca^{2+} channels in the plasma membrane, inducing additional Ca^{2+} influx. Intracellular free Ca^{2+} acts as an essential second messenger for T-cell activation. Its regulatory effects on T-cell activation are mediated via calmodulin, a Ca^{2+}-binding protein expressed in all eukaryotic cells. Effective T-cell activation leading to IL-2 secretion requires that intracellular Ca^{2+} levels be elevated for a period of 1–2 hr. Sustained Ca^{2+} signaling is required for maintaining the transcription factor nuclear factor of activated T cells (NFAT) in the nucleus in an active form [52,53]. NFAT is a key transcriptional regulator of the IL-2 gene and other cytokine genes.

Ca^{2+} signaling is required for various lymphocyte activities, for example, cell mobility, change of cytoskeletal structure, cell death, differentiation, and activation. Thus, a single second messenger can elicit multiple cellular responses. The type of response induced may depend on the amplitude, duration, and temporal fluctuations of Ca^{2+} mobilization. For example, activation of NFκB is induced by high levels of Ca^{2+} because of this transcription factor's low Ca^{2+} sensitivity. In contrast, long-lasting, low levels of Ca^{2+} selectively activate NFAT, because NFAT is highly sensitive to Ca^{2+}, but is rapidly inactivated after Ca^{2+} removal [54].

PKC

Release of DAG stimulates PKC, a family of serine/threonine kinases. In T cells, PKC isoforms α, β, η, δ, ζ, ε, and θ are expressed. One of the major functions of PKC is to induce MAPKs. PKCα directly phosphorylates and activates Raf-1, another serine/threonine kinase. Activation of Raf-1 triggers a protein kinase cascade by directly phosphorylating MAPK kinase. Activation of PKC also mediates the rapid accumulation of the active, GTP-bound form of $p21^{ras}$.

Transcription Factors: Activation and Gene Expression

The multiple signaling pathways originating from T-cell surface molecules initiate the expression of genes responsible for proliferation and immune functions in a cooperative manner. The most extensively studied example is the induction of the IL-2 gene. The IL-2 gene promoter contains at least seven distinct binding sites for transcription factors. Therefore, maximal transcription demands the simultaneous presence of all factors, among which AP-1, NFAT, and NFκB are the best characterized (Fig. 2).

AP-1 is a heterodimer of c-Fos and c-Jun. Maximum activation of AP-1 requires *de novo* synthesis of c-Jun and c-Fos, and phosphorylation by MAPKs of the activation domains of both proteins, leading to translocation into the nucleus.

Activation of NFAT requires dephosphorylation by the serine/threonine protein phosphatase, calcineurin, followed by translocation into the nucleus. In the nucleus, NFAT cooperates with AP-1 in gene transactivation, resulting in a 20-fold increase in the stability of NFAT/AP-1/DNA complexes as compared with NFAT/DNA complexes.

NFκB is a homodimer or heterodimer of a family of structurally related proteins. Each member of this family contains a conserved N-terminal Rel-homology domain (RHD), which mediates dimerization and binding to DNA. The RHD contains a nuclear localization sequence that promotes NFκB translocation to the nucleus following release of NFκB from IκB. In its inactive form, NFκB is sequestered in the cytosol by noncovalent interactions with the inhibitory protein, IκB, which masks the nuclear translocation signal. Phosphorylation of IκB by IκB kinase targets IκB for destruction by the ubiquitin-protease system. NFκB induces IL-2 gene expression, and in a negative feedback loop, promotes transcription of the IκB gene. Importantly, PKCθ associates with IκB kinase to mediate activation of NFκB following TCR/CD28 stimulation [55,56].

Role of Cyclic AMP in Th-Cell Activation

cAMP, Adenylyl Cyclases, and Phosphodiesterases

Cyclic AMP (cAMP) is an intracellular second messenger to a wide variety of hormones and neurotransmitters. In T cells, elevated cAMP levels antagonize activation by inhibiting T-cell proliferation [57,58] and by suppressing the production of IL-2 and IFN-γ [59]. TCR signaling alone (in the absence of CD28 costimulation) elevates cAMP levels and adenylyl cyclase (AC) activity [60,61]. The regulation of cAMP during T-cell activation is also mediated via other T-cell surface molecules, such as CD28 and CD44 [62,63], as well as via the coreceptor CD4 [70].

In T cells, the cAMP level is controlled by two types of enzymes: ACs and phosphodiesterases (PDEs). ACs catalyze cAMP production from ATP, whereas PDEs control the rate of cAMP degradation to AMP. Members of the PDE families 1, 3, 4, and 7 are expressed in T cells. Different mechanisms control the activity of the different families of PDEs: Ca^{2+}/calmodulin stimulates PDE1, cyclic GMP inhibits PDE 3, p70S6 kinase and the MAPK pathway can activate PDE 4, and CD28-mediated signals activate PDE 7 [62]. Importantly, TCR/CD28 stimulation of human T cells transiently up-regulates AC and PDE activities with different kinetics for different PDE isozymes [64]. Thus, an initial

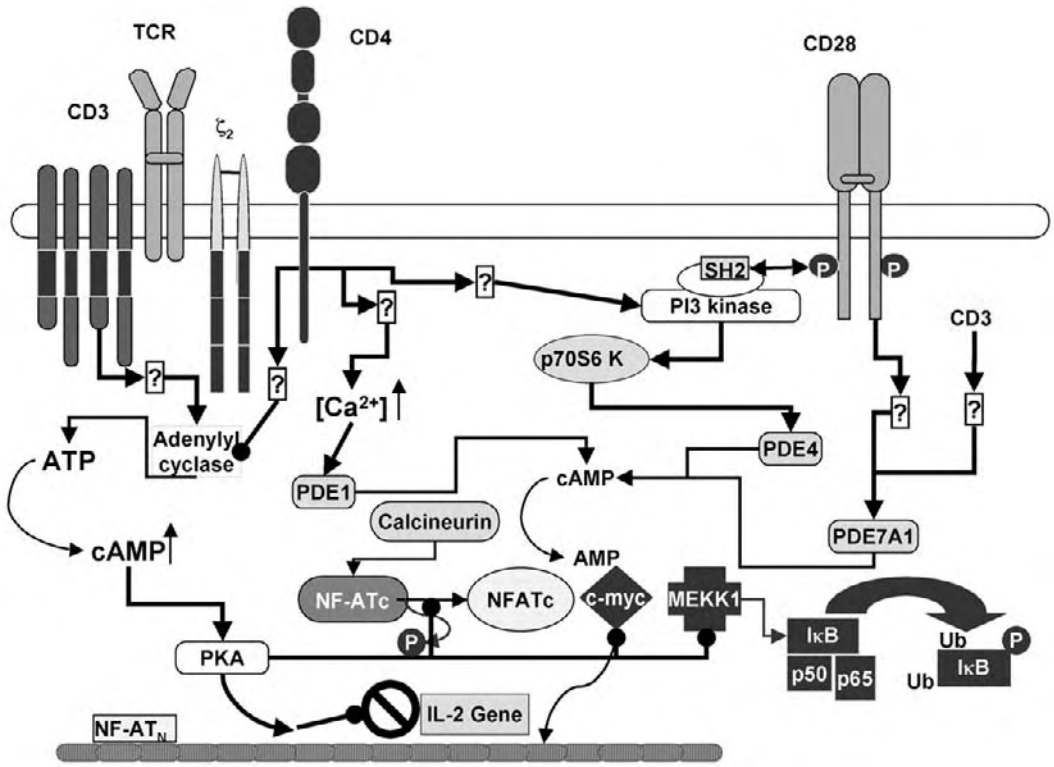


Figure 3 Cyclic AMP exerts multiple regulatory effects on T cell activation and is itself regulated by multiple signaling pathways that activate or block adenylate cyclase and activate phosphodiesterases. Stimulatory effects are indicated by a line ending in an arrow, whereas inhibitory effects are depicted by a line ending in a filled circle. The overall effect of PKA I activation is inhibition of IL-2 gene expression.

increase, followed by a rapid decrease, in intracellular cAMP is required for T-cell activation, suggesting a precise kinetic regulation of cAMP production and degradation (Fig. 3).

cAMP-Dependent Kinase

The cAMP-dependent protein kinase (PKA) is the principal intracellular cAMP receptor. In the absence of cAMP, PKA is an enzymatically inactive, tetrameric holoenzyme, consisting of two catalytic (C) subunits and two regulatory (R) subunits. The cooperative binding of four cAMP molecules to two sites on each R subunit induces dissociation into dimeric R and two monomers of C subunits. Once freed from the R subunits, the C subunits display serine/threonine kinase activity.

PKA I, but not PKA II, mediates the inhibitory role of cAMP on T-cell proliferation induced by TCR signaling [65]. PKA I activates Csk to inhibit Lck activity [27]. It also phosphorylates Ser-43 of Raf-1 to block the MAP kinase pathway [66]. In the nucleus, activation of PKA prevents stable protein–DNA interactions at the NFκB, NFAT, and AP-1 binding sites of the IL-2 enhancer [67] (Fig. 3). In addition, PKA I activity also inhibits cyclin D3 expression and induces the cyclin-dependent kinase inhibitor p27^{kip1} [68]. For T cells to enter the S phase of the cell cycle, D-type cylins, including cyclin D3, are synthesized during the G_1 phase [69]. These cyclins can bind to cyclin-dependent kinase (Cdk) and form an active kinase complex that phosphorylates and inactivates retinoblastoma protein (pRb). Inactivation of pRb then allows cells to pass through the late G_1-phase restriction point and enter the S phase. However, cyclin D/Cdk complexes can associate with the Cdk inhibitor p27^{kip1}, thus be rendered inactive. Therefore, in addition to induction of cyclin D, down-regulation of p27^{kip1} is required for the initiation of T-cell proliferation. Hence, inhibition of cyclin D3 expression and induction of p27^{kip1} by PKA I both block T-cell cycle progression.

Conclusions

Signal transduction research in T lymphocytes has focused on identifying the receptors and intracellular proteins affecting specific signaling pathways. It has become clear that signaling machines in T cells differ in composition depending on the extracellular signal received and the requirements of the stimulated cell. The composition of an established signalosome could also change over time in order to fine-tune or alter effector signaling pathways. In the future, it will be important to determine the kinetics of assembly, the dynamics of composition, and the compartmentalization for all signalosomes induced in T cells, as well as to define the interactions between different signaling pathways. Future research will utilize functional genomics approaches to characterize the *signalome*—the entire complement of a T cell's

signaling molecules and interactions in a temporal and spatial context.

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CHAPTER 344

Signal Transduction via the B-Cell Antigen Receptor: A Crucial Regulator of B-Cell Biology

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Introduction

B lymphocytes express antigen receptors (BCR) on their surface that impart the ability to detect foreign antigens. Binding of antigen to the BCR initiates a signal transduction cascade that in turn leads to cellular activation, proliferation, and ultimately differentiation into antibody-secreting plasma cells. Additionally, the BCR plays an important role in internalization of antigen resulting in its processing and presentation in the context of MHC class II to helper T cells, which promote clonal expansion and differentiation of the antigen-specific B cells [1,2]. Although signal transduction via the BCR is central to the generation of a productive humoral immune response, its role in regulating B-cell biology is more complex and multifaceted. Indeed the BCR is involved in determining the fate of the B cell throughout its development and differentiation [3–5]. During B-cell development in the bone marrow, the pre-BCR is crucial for transducing signals that ensure the formation and expression of a functional, mature BCR on the surface of the B cell. Additionally, the pre-BCR is involved in regulating allelic exclusion to ensure that only BCRs with a single antigenic specificity are expressed on the surface of any given B cell. Once a mature BCR is expressed on the surface of an immature B cell, signals delivered through it can either positively select those cells that will ultimately enter into the periphery to patrol for foreign antigen, or negatively select those cells that recognize self-antigens with a high affinity. Negative selection mediated by signals delivered via the BCR results in editing of the BCR to change its specificity or in deletion of the self-reactive clone [6–8]. Thus the BCR is directly involved in determining the fate of the cell presumably by virtue of its ability to transduce signals that vary both quantitatively as well as qualitatively. Additionally, the response of the B cell to such signals is likely to be regulated in a developmental manner [9,10]. In conclusion, the BCR serves a central role in regulating B-cell biology by virtue of its ability to selectively transduce signals in response to antigen binding. The discussion that follows deals primarily with the basic signal transduction pathways that are activated in response to binding of antigen to BCRs expressed on mature, quiescent B cells.

Initiation of Signal Transduction through the BCR

The BCR complex is comprised of an antigen recognition structure, membrane immunoglobulin (mIg), and an associated signal transducing heterodimer. Membrane Ig consists of two heavy chains and two light chains that are disulfide bonded to one another to form the mature antigen recognition structure. Membrane Ig is noncovalently associated

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with one transmembrane heterodimer consisting of disulfide-linked Igα (CD79a) and Igβ (CD79b) polypeptides [11,12]. The Igα/β heterodimer functions both as a transporter and a signal transducing structure [12]. Initiation of signal transduction through the BCR was originally thought to occur in response to BCR cross-linking mediated by binding of bivalent or multivalent antigen to the bivalent mIg molecule. This was thought to induce BCR dimerization and/or multimerization, which in turn facilitated the ability of associated Src family protein tyrosine kinases (PTK) (e.g., Lyn, Fyn) to phosphorylate tyrosine residues in the cytoplasmic domains of Igα/β [13]. Although the BCR in resting B cells is constitutively associated with Src family PTKs at a low stoichiometry, the net level of Igα/β phosphorylation is presumably minimal due to the fact that individual receptor complexes are distributed throughout the membrane and the phosphorylation of Igα/β is counterbalanced by one or more protein tyrosine phosphatases (PTP). In contrast, BCR aggregation presumably favors a net increase in the total level of protein tyrosine phosphorylation associated with the activation complex due to the physical colocalization of Src family PTKs and their substrates (i.e., Igα/β) [13].

More recently, however, it has been proposed that the BCR exists in the membrane of unstimulated B cells in preformed oligomers and that initiation of signaling occurs when antigen binds to one or more mIg molecules within an oligomer, thereby causing a conformational change or reorganization of the individual subunits that promotes signal transduction [11,12,14,15]. In this regard, the transmembrane region of mIg heavy chains is thought to assume an α-helical conformation in the membrane in which one face of the α-helix is comprised of highly conserved hydrophilic amino acid residues that mediate the interaction with the Igα/β heterodimer [14]. The other face of the α-helix is specific to each of the mIg isotypes and contains several hydrophilic amino acid residues with large polar side groups. Because such hydrogen-binding, hydrophilic moieties exhibit a strong bias against being located within a lipid membrane, it is possible that this face of the helix constitutes an interaction surface that is involved in BCR oligomerization [14]. Although the molecular composition of the resting BCR oligomers has yet to be elucidated, it has been hypothesized that the oligomers are associated with the PTK Syk [16]. This PTK presumably promotes phosphorylation of Igα/β heterodimers within the oligomer, which in turn facilitates binding of Syk to phosphotyrosine residues via its tandem Src homology 2 (SH2) domains. It is further hypothesized that a low basal level of tyrosine phosphorylation is maintained by the action of a PTP called SHP-1, which acts to constitutively down-regulate Syk kinase activity, and perhaps to dephosphorylate Igα/β [13]. Binding of antigen to the complex is thought to cause a conformational change in the resting BCR oligomer such that SHP-1 activity is attenuated, possibly by causing the PTP to become physically dissociated from the BCR complex. Dissociation of SHP-1 would presumably favor a net increase in tyrosine phosphorylation of Igα/β leading to initiation of signal transduction and cellular activation. It has been reported that SHP-1 constitutively interacts with the BCR isolated from resting B cells and is induced to dissociate from the BCR upon activation in agreement with this proposed mechanism [17].

Another recent finding is that binding of antigen causes a rapid translocation of the BCR to glycosphingolipid-enriched microdomains (GEMs) within the plasma membrane. These microdomains are enriched for Src family PTKs including Lyn and it has been hypothesized that translocation of the BCR into GEMs physically localizes the complex with Src PTKs, thereby promoting tyrosine phosphorylation of Igα/β by Lyn [18–20]. Interestingly, translocation of the BCR into GEMs appears to constitute a novel step that precedes signal transduction because translocation does not require the Igα/β heterodimer and is resistant to blockade by PTK inhibitors [21]. Thus, it is possible that binding of antigen to BCR oligomers on resting B cells leads to a reorganization/conformational change in the oligomer that promotes its translocation to GEMs. As stated, localization of the BCR to GEMs promotes Lyn-mediated phosphorylation of Igα/β, as well as phosphorylation of Syk, resulting in potentiation of its catalytic activity. Simultaneously, translocation of the BCR to GEMs may lead to dissociation of SHP-1, thereby causing a net increase in tyrosine phosphorylation of Igα/β and initiation of signal transduction. Alternatively, it is possible that Lyn activity is relatively resistant to the inhibitory action of SHP-1, in which case colocalization of Lyn with the BCR may be sufficient to favor a net increase in Igα/β phosphorylation despite the presence of SHP-1 [14].

Regardless of whether antigen binding promotes BCR aggregation or reorganization of preexisting oligomers, it is clear that the BCR translocates to GEMs where there is a net increase in the tyrosine phosphorylation of Igα and Igβ (Fig. 1). Both of these polypeptides contain immunoreceptor tyrosine-based activation motifs (ITAMs) within their cytoplasmic domains that function as docking sites for SH2 domain-containing proteins including Syk, Shc, and BLNK [13]. The net increase in tyrosine phosphorylation of the Igα/β ITAMs promotes the formation of multimolecular complexes that are targeted to the BCR and are critical for propagation of signal transduction. The formation of these complexes enables the BCR to activate several signaling pathways that are distinct yet interrelated, including the phospholipase C (PLCγ) pathway, the phosphatidylinositol 3-kinase (PI3K) pathway, and signaling processes that are controlled by the Ras, Rac1, and Rap1 GTPases (for reviews, see [22–26]).

Propagation of Signal Transduction via the BCR

Numerous studies have demonstrated that ligand binding to the BCR results in inducible tyrosine phosphorylation and activation of the PTK Syk [27–29]. The critical role that Syk serves during BCR-mediated signaling has been demonstrated by the analysis of Syk-deficient B cells in which

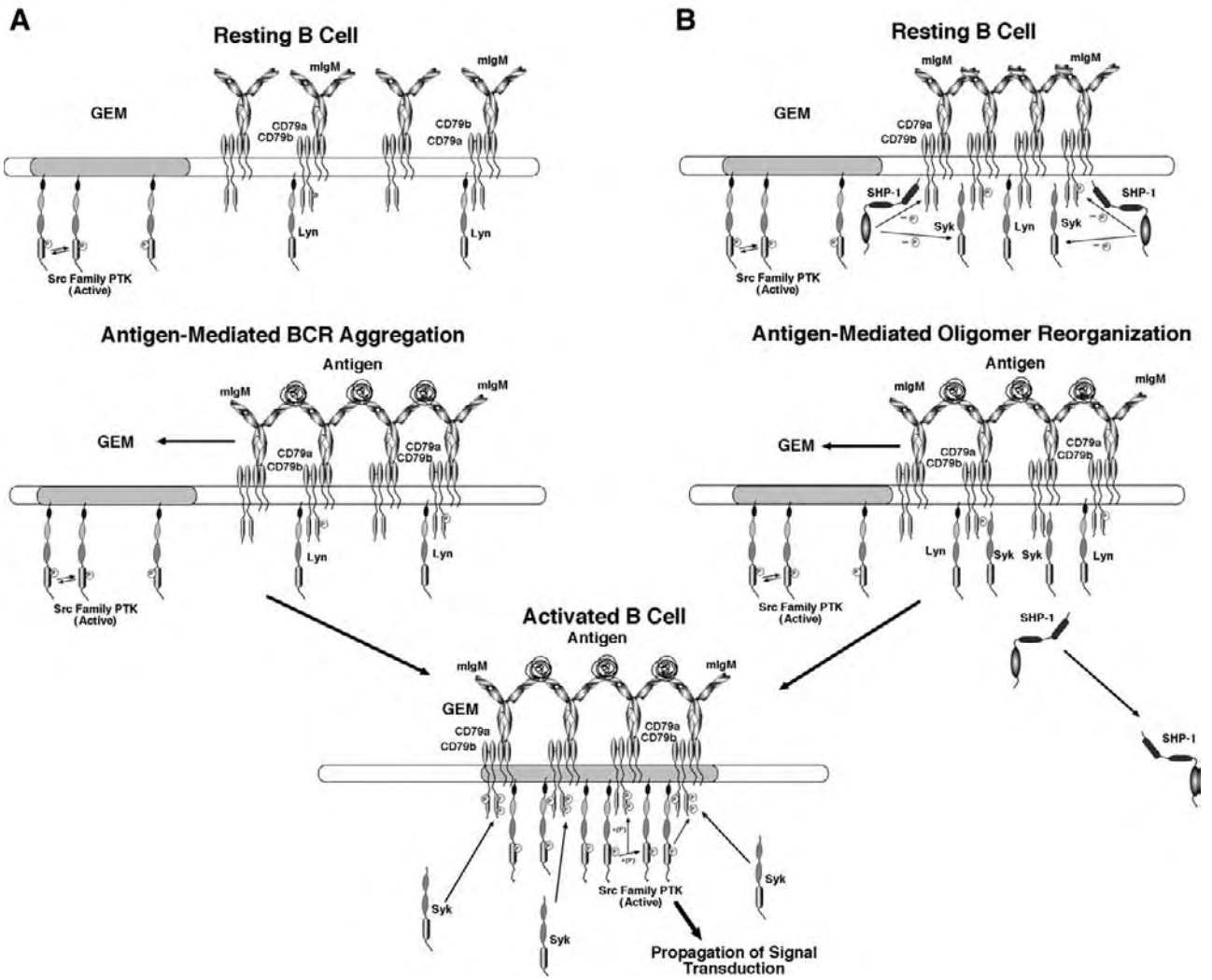


Figure 1 Initiation of signal transduction via the BCR. (A) Initiation of signal transduction in response to antigen-mediated aggregation of BCR complexes. In the resting B cell the BCR exists as monomeric units in the plasma membrane. The net level of tyrosine phosphorylation associated with the BCR is maintained at a low level by the physical dispersal of the BCR complexes throughout the membrane, by the low stoichiometry of interaction with Src family PTKs, and by the action of PTPs (e.g., CD45 or SHP-1), which dephosphorylate the BCR complex. Ligation of the BCR by antigen induces aggregation of the individual BCRs, which then promotes their translocation into GEMs in a signaling-independent manner. Once the aggregated BCRs enter the GEM, they are colocalized with active Lyn, which phosphorylates CD79a/b (Igα/β). This promotes recruitment of Syk and the initiation of signal transduction. (B) Initiation of signaling via antigen-mediated reorganization of preexisting BCR oligomers. In this model, the BCRs on resting B cells exist in preformed oligomers that contain Syk and the PTP SHP-1. Low basal levels of phosphorylation are presumably maintained by SHP-1, which inhibits Syk activation and may dephosphorylate CD79a/b. Antigen binding causes a reorganization of the BCR oligomer that promotes translocation to GEMs, as well as the dissociation of SHP-1 from the complex. Once the BCRs enter the GEM, initiation of signaling can proceed.

abrogation of Syk expression results in the loss of IP_3 generation and Ca^{2+} mobilization [30]. Moreover, studies using bone marrow cells derived from Syk-deficient mice have confirmed that Syk is essential for proper signal transduction via the BCR based on the inability of these cells to develop normally into mature B cells in chimeric mice [31,32]. Recruitment of Syk to the BCR complex is mediated by tyrosine phosphorylation of the ITAMs in Igα and Igβ, creating docking sites that are recognized by the dual SH2 domains of Syk [33–35]. Efficient recruitment of Syk requires that both conserved tyrosines within a given ITAM be present and that they be phosphorylated. Additionally, both SH2 domains of Syk must be present and functional in order to observe optimal association of the kinase with Igα and Igβ and its subsequent activation through the physical interaction with these polypeptides [13]. Binding of Syk to the ITAMs of Igα and Igβ may also play a crucial role in its activation by focusing Syk to the BCR activation complex within GEMs, which effectively brings Syk into proximity with Lyn. Studies support the conclusion that Lyn phosphorylates key tyrosine residues on Syk, resulting in potentiation of its kinase activity [13].

The ability of Syk to phosphorylate downstream signaling effector proteins is dependent on the recruitment of an adaptor protein called BLNK (SLP65, BASH) to the BCR activation complex [25,26,36]. Recent studies have determined

that BLNK is recruited to the BCR complex by virtue of its ability to bind to phosphorylated tyrosine residues in the cytoplasmic tail of Igα that lie outside the ITAM [37,38]. It has been shown that mutation of tyrosines 176 and 204, which flank the ITAM, abrogates BLNK-dependent signaling [38]. Moreover, it was shown that the SH2 domain of BLNK binds directly to tyrosine 204 of Igα [37,38]. Thus it appears that Syk and BLNK are colocalized to the BCR complex through their interaction with Igα. This facilitates the ability of Syk to phosphorylate BLNK, which in turn generates phosphotyrosine-dependent binding sites for recruitment of additional signal transducing proteins [16,39]. The formation of the BCR:Syk:BLNK complex constitutes the basic unit that is required for propagation of signal transduction leading to the activation of several interrelated downstream pathways (Fig. 2).

Activation of PLCγ2-Dependent Signaling

The initial activation of PTKs is responsible for mediating the production of second messengers that subsequently regulate intermediate signaling processes leading to transcription factor activation. Studies have elegantly demonstrated that PLCγ2 is activated in response to BCR ligation and is responsible for the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) [40–42]. These in turn promote PKC activation and mobilization of Ca^{2+}, respectively [22–26]. It has been demonstrated using a nonlymphoid cell reconstitution system that expression of Igα/β, Syk, and PLCγ2 is not sufficient to mediate activation of PLCγ2 and Ca^{2+} mobilization in response to BCR ligation, suggesting that one or more key components were missing [43]. Subsequent work has clearly shown that PLCγ2 is recruited to tyrosine phosphorylated BLNK via its tandem SH2 domains. In cells that lack BLNK, PLCγ2 is not observed to translocate from the cytoplasm to GEMs and exhibits decreased activation [36]. Additionally, mutation of the amino-terminal SH2 domain of PLCγ2 blocks its binding to phosphorylated BLNK and blocks localization to GEMs [44]. Therefore, it is apparent that BLNK provides a scaffold to localize PLCγ2 to the BCR activation complex within GEMs where it can be activated.

Based on numerous findings, it has been concluded that Syk and the PTK Btk act in concert to regulate the phosphorylation and activity of PLCγ2 [45–47]. In addition to the Src family PTKs and Syk, Btk is inducibly activated in response to BCR ligation and plays an important role in signal transduction [46,48]. Like the Src family PTKs, Btk contains contiguous SH3, SH2, and SH1 domains, although it does not possess a carboxyl-terminal negative regulatory tyrosine residue or a myristylation site. In addition to the SH domains, Btk contains an amino-terminal pleckstrin homology (PH) domain and an adjacent proline- and cysteine-rich Tec homology (TH) domain [46]. It has been shown that the SH2 domain of Btk is required for PLCγ2 phosphorylation and activation and that it exhibits restricted binding specificity for tyrosine phosphorylated BLNK. Thus, PLCγ2 and Btk are colocalized to the BCR activation complex by virtue of their interaction with BLNK.

Studies in the mouse and human have clearly demonstrated that Btk expression is essential for BCR-mediated Ca^{2+} mobilization [49,50]. Moreover, expression of Btk has been shown to restore calcium signaling in Btk-deficient XLA B cells. Although expression of Syk is required for normal mobilization of Ca^{2+} in B cells, overexpression of this PTK was not observed to restore calcium signaling in XLA B cells, demonstrating that it cannot compensate for the loss of Btk. This finding indicates that these PTKs must act in concert in a nonredundant manner to regulate PLCγ2 function [36,47]. The ability of Btk to reconstitute calcium signaling is dependent on its catalytic function and the Btk activation loop tyrosine (Tyr551), which is phosphorylated by Syk. Mutation of the Syk transphosphorylation site or the ATP-binding site abrogates Btk-dependent phosphorylation of PLCγ2 [45,47]. Recent studies have demonstrated that specific tyrosine residues in PLCγ2 (tyrosines 753, 759, 1197, and 1217) are direct targets for Btk and that their phosphorylation is essential for optimal activation of its catalytic function [51,52]. In conclusion, it appears that Syk is required for PLCγ2 activation by virtue of its role in phosphorylating BLNK to generate docking sites for Btk and PLCγ2, and through its role in phosphorylating Tyr551 on Btk leading to its catalytic activation. Btk appears to be specifically involved in phosphorylating multiple tyrosine residues on PLCγ2 that are required for optimal catalytic activation, leading to hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce IP_3 and DAG.

The production of IP_3 in response to activation of PLCγ2 results in mobilization of Ca^{2+} from the endoplasmic reticulum (ER) [53]. IP_3-mediated release of Ca^{2+} involves IP_3 receptors (IP_3R) located in the ER, which form heterotetrameric channels. It has been shown that the nature of the calcium mobilization response in B cells may be controlled by the combinatorial function of three IP_3R receptor subtypes [54]. Additional evidence suggests that the function of IP_3Rs may be regulated by phosphorylation, as well as in response to binding of IP_3. A B-cell restricted scaffold protein with ankyrin repeats (BANK) has recently been cloned that is inducibly phosphorylated on tyrosine residues in response to BCR ligation [55]. Overexpression of BANK results in enhanced BCR-induced calcium mobilization. Interestingly, BANK has been shown to interact with Lyn and IP_3Rs, suggesting that this adaptor functions to bring Lyn and IP_3Rs into proximity, thereby promoting tyrosine phosphorylation of IP_3Rs by Lyn [55]. This may play a key role in potentiating calcium mobilization from the ER because it has been shown that tyrosine phosphorylation of IP_3Rs up-regulates their channel activity. The release of Ca^{2+} from intracellular stores has been shown to promote Ca^{2+} influx across the plasma membrane, presumably through Ca^{2+} release activated Ca^{2+} channels (CRAC) [56,57]. CRAC channel activation is mediated by the accumulation of a poorly defined calcium influx factor(s) that is produced in response to depletion of Ca^{2+} from intracellular stores.

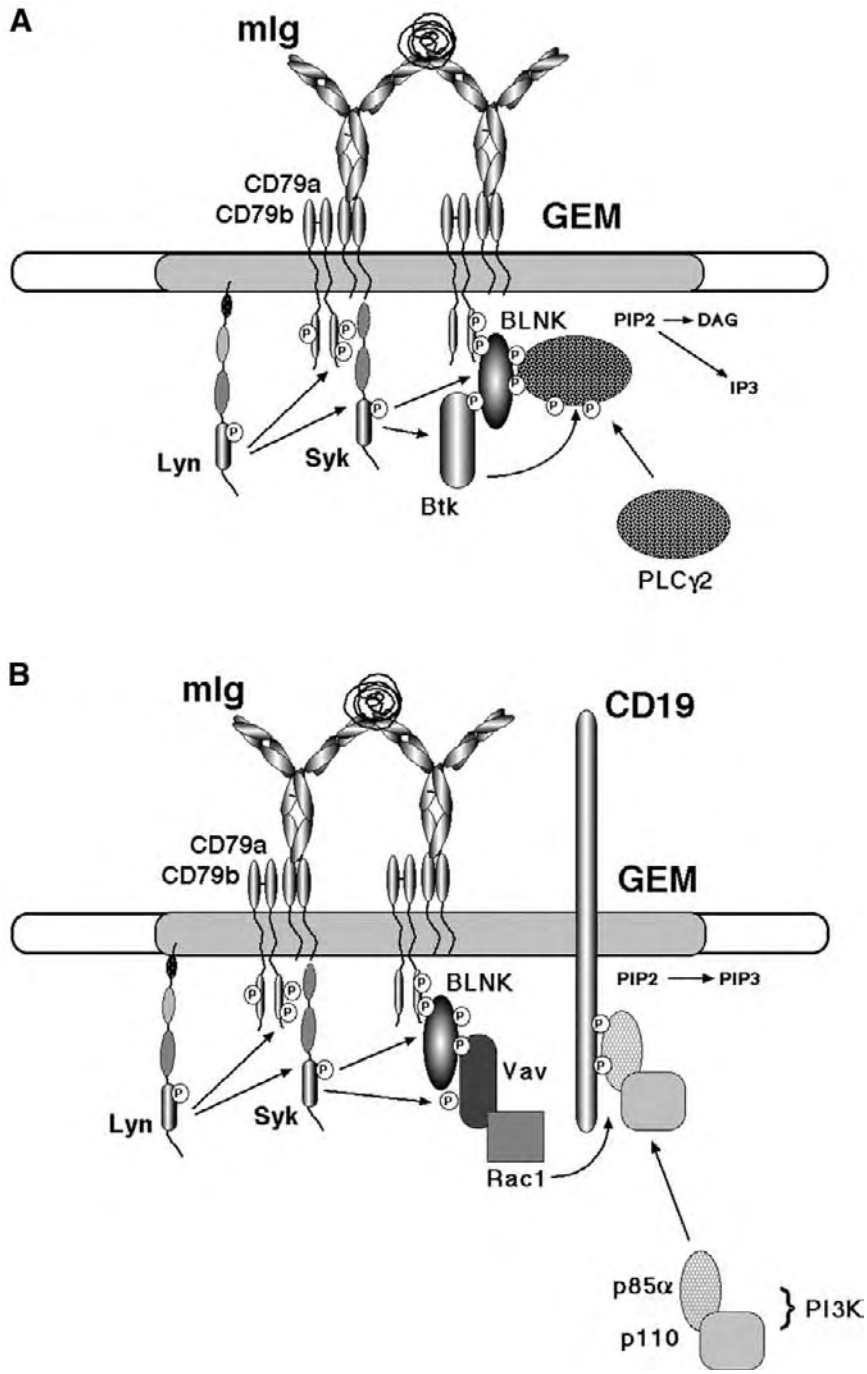


Figure 2 Propagation of signal transduction via the BCR. (A) Activation of PLCγ2-dependent signal transduction. Translocation of the BCR to GEMs facilitates phosphorylation of CD79a/b by Lyn, which in turn promotes recruitment of Syk and BLNK. Syk and BLNK bind to phosphorylated CD79a after which Syk is activated by Lyn-mediated phosphorylation. Syk then phosphorylates BLNK generating phosphotyrosine motifs that recruit Btk and PLCγ2, which bind to BLNK via their SH2 domains. Btk is further activated by Syk and in turn phosphorylates PLCγ2 resulting in its catalytic activation. PLCγ2 then hydrolyzes PIP_2 to produce DAG and IP_3, which mediate PKC activation and Ca^{2+} mobilization, respectively. (B) Activation of PI3K-dependent signal transduction. CD19 is inducibly phosphorylated on tyrosine in response to BCR ligation, enabling it to recruit PI3K to the membrane. Phosphorylated BLNK associated with the BCR complex recruits Vav, which binds to BLNK via its SH2 domain. Vav is phosphorylated and activated by Syk and then activates Rac1. Activated Rac1 binds to PI3K and potentiates its catalytic activity. Active PI3K phosphorylates PIP_2 to form PIP_3. PIP_3 recruits numerous PH domain-containing signaling proteins to the membrane including Btk, PLCγ2, Vav, and the PDK1/Akt kinases.

One of the most important downstream targets of the calcium signaling pathway in B cells is the transcription factor NFAT [58], although increased Ca^{2+} levels in the cell also play a role in the activation of protein kinase C (PKC) family members and in the activation of small molecular weight G-proteins [22–26].

The production of DAG by PLCγ2 leads to the activation of members of the PKC family. Both conventional (PKC-α, β, βII, γ) and novel (PKC-δ, ε, η, θ, μ) members of the PKC family require DAG for their activation, whereas the former require Ca^{2+} as well [59]. Studies have detected PKC-α, β, γ, δ, ε, η, θ, and μ expression in B cells and have also shown that several of these kinases (PKC-α, βII, δ, ε) translocate from the cytoplasm to the membrane in response to BCR ligation [59,60]. It has further been shown that PKC-μ interacts with the BCR and exhibits increased specific activity in response to BCR ligation [61]. The activation of members of the PKC family has been shown to play a role in regulating activation of several transcription factors in B cells [22–26]. PKC activation leads to activation of the mitogen-activated protein (MAP) kinase ERK2 via the classical Ras/Raf-1/Mek/ERK2 pathway [22–26]. ERK2 translocates to the nucleus where it regulates phosphorylation and activation of transcription factors including Elk-1 and members of the Ets family [62,63]. Additionally, it has recently been shown that novel PKC members PKC-θ and δ may play a critical role in B-cell activation by virtue of their ability to regulate the activation of the transcriptional regulator NFκB and the MAP kinase JNK [64].

Activation of Phosphoinositide 3-Kinase-Dependent Signaling

Another major signaling pathway that is activated upon BCR ligation involves the lipid kinase PI3K. Mice lacking the p85 subunit of PI3K exhibit severe defects in B-cell development as well as impaired proliferative responses to stimulation through the BCR [65,66]. Thus it is apparent that PI3K plays a critical role in signaling via the BCR. Recruitment and activation of PI3K to the BCR complex have been shown to occur via the transmembrane protein CD19 [67]. BCR ligation leads to tyrosine phosphorylation of several tyrosine residues in the cytoplasmic domain of CD19. Two of these residues, Tyr482 and Tyr513, have been shown to mediate binding of the p85 subunit of PI3K via its tandem SH2 domains [68]. Because CD19 localizes to GEMs in response to B-cell activation, its interaction with PI3K provides a mechanism to focus this kinase in regions of the cell that are enriched for its substrate phosphoinositide 4,5-bisphosphate (PIP_2) [69]. It is important to note, however, that loss of CD19 expression causes a less severe defect in B-cell development and function than is observed in B cells that lack the p85 subunit of PI3K. This suggests that additional adaptor proteins other than CD19 may mediate PI3K recruitment and activation [25].

Recent studies have identified a protein called B-cell adaptor for phosphoinositide 3-kinase (BCAP) that is inducibly phosphorylated by Syk and Btk in response to BCR ligation [70]. Of the 31 potential tyrosine residues that can be phosphorylated on BCAP, four are contained within consensus motifs for binding to the SH2 domains of the PI3K p85 subunit. Mutation of these tyrosine residues ablates the ability of BCAP to interact with p85 or to restore Akt activation in cells that lack BCAP [70]. Additionally, loss of BCAP expression has been shown to attenuate the recruitment of PI3K to GEMs suggesting that this adaptor plays an important role in targeting PI3K to the BCR activation complex. Nevertheless, loss of BCAP expression does not entirely abrogate the production of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) or the activation of the downstream target Akt [70]. Therefore, it is clear that CD19 as well as other potential adaptors may be able to promote PI3K recruitment to GEMs where it is activated. A candidate for such an adaptor is Gab1, which is inducibly phosphorylated on tyrosine residues in response to BCR ligation mediating its direct interaction with the SH2 domains of PI3K, Shc, and the PTP SHP-2 in a phosphotyrosine-dependent manner [71]. Overexpression of Gab1 in B cells was observed to potentiate BCR-mediated phosphorylation of Akt, which is a PI3K-dependent response. Importantly, it was observed that the pleckstrin homology domain of Gab1 is required for its ability to translocate from the cytoplasm to the plasma membrane and for its ability to potentiate activation of PI3K [72]. PH domains have been shown to bind to PIP_3, which is produced in response to activation of PI3K. Thus it is likely that Gab1 functions as an amplifier of PI3K-dependent signaling due to the fact that its recruitment to the membrane and function require the initial production of PIP_3 by PI3K [72].

Activation of PI3K is mediated by virtue of its ability to bind to tyrosine-phosphorylated proteins such as CD19 and BCAP via the SH2 domains of the p85 subunit. This in turn promotes translocation of PI3K to GEMs, where it is able to access its substrate PIP_2, which is enriched in these microdomains of the plasma membrane. Although PI3K binding to adaptor proteins and translocation is required for activation, it is not sufficient for full activation of PI3K catalytic function. Studies have indicated that Vav family members may play a role in potentiating the activation of PI3K [25]. Generation of B cells lacking Vav3 resulted in significant decreases in PIP_3 production and activation of Akt [73]. These defects could be corrected by expressing Vav3 or Vav2 in Vav3-deficient B cells. However, the guanine nucleotide exchange factor (GEF) mutant of Vav3 was not able to restore PI3K function, indicating that Vav3 may regulate PI3K activity through its target Rac1 [73]. This was indeed found to be the case based on several experimental strategies. In conclusion, Vav appears to be important for potentiating PI3K activity in response to BCR ligation. Nevertheless, it has yet to be formally determined whether all members of the Vav family share the ability to potentiate PI3K activity through activation of Rac1, although it seems likely based on the available experimental evidence.

Activation of PI3K results in the phosphorylation of PIP_2 to form PIP_3, which regulates the activation of numerous

downstream signaling proteins that contain PH domains [74]. It has been shown that activation of PLCγ2, Btk, Rac1, and the kinase network including PDK1, Akt, GSK-3, and mTOR is regulated by the ability of these proteins to bind to PIP_3 via their PH domains [22–26]. Presumably, the production of PIP_3 by PI3K plays an important role in promoting recruitment of PH domain-containing proteins such as PLCγ2 and Btk to the membrane where they are colocalized through their interaction with BLNK. Additionally, it is possible that the production of PIP_3 functions to maintain activated PLCγ2 and/or Btk at the membrane once they have dissociated from BLNK, thereby prolonging the signal transduction response. It is now well documented that PI3K-dependent production of PIP_3 plays a crucial role in activation of the PDK1/Akt/GSK-3 kinase cascade, which in turn promotes cell survival [74]. PIP_3-dependent recruitment of PDK1 and Akt to the membrane facilitates the ability of PDK1 to phosphorylate Akt on serine/threonine residues, which in turn leads to activation of Akt [74]. Numerous studies have documented activation of Akt in response to BCR ligation [75–78]. Kinetic studies have demonstrated that Akt transiently translocates to the plasma membrane in B cells where it is activated and then migrates to the cytoplasm and nucleus where it presumably can interact with potential substrates [79]. Akt is a serine/threonine kinase that phosphorylates numerous downstream substrates including Bad and GSK-3. Phosphorylation of the apoptosis-inducing Bad protein creates a binding site for 14-3-3 proteins preventing Bad from binding to Bcl2 and Bcl-X_L thereby releasing them to mediate cell survival [74]. Phosphorylation of GSK-3, which is a serine/threonine kinase, inactivates its catalytic function. GSK-3 is constitutively active in resting cells and phosphorylates numerous proteins including c-myc and cyclin D, maintaining them in an inactive state [74]. GSK-3 has also been shown to phosphorylate NFAT causing a change in its conformation that reveals a nuclear export signal [80]. Thus, inhibition of GSK-3 activity by Akt promotes retention of NFAT in the nucleus. Therefore, phosphorylation of GSK-3 by Akt promotes the activation of proteins that regulate cell cycling and cell survival.

Activation of Small Molecular Weight G-Proteins and Their Signaling Pathways

BCR ligation promotes the activation of small molecular weight G-proteins that in turn regulate signal transduction pathways that control transcription factor activation and cytoskeletal reorganization, which affects cell morphology and motility. In many instances it appears that BLNK plays a role in colocalizing critical signal effector proteins leading to activation of G-proteins. It has been shown that BLNK interacts with the adaptor protein Grb2, which appears to bind constitutively to a proline-rich region on BLNK via one of its SH3 domains [81,82]. The interaction between Grb2 and BLNK can also be potentiated in a tyrosine phosphorylation-dependent manner in which the SH2 domain of Grb2 binds to phosphotyrosine on BLNK. In either case, Grb2 recruits the guanine nucleotide exchange factor (GEF) Sos to the complex. Another potential mechanism whereby Sos is recruited to the membrane involves the formation of a Shc/Grb2/Sos complex in which Shc is inducibly phosphorylated in response to BCR ligation promoting binding of Grb2 via its SH2 domain. It has been proposed that Shc in turn binds via its SH2 domain to tyrosine residues within the cytoplasmic domain of Igα/β [13,22,23]. An alternative mechanism by which the Shc/Grb2/Sos complex may be recruited to the membrane is through binding of Shc to tyrosine phosphorylated Gab1 [71]. Regardless of the specific mechanism by which Sos is recruited to the plasma membrane, its localization leads to direct activation of the small molecular weight G-protein Ras by virtue of its GEF activity. GTP-bound Ras controls the activation of a kinase cascade that culminates in the activation of the MAP kinases ERK1 and ERK2 [22,23,59]. This is mediated by binding of activated Ras to the Raf-1 kinase, which then phosphorylates and activates MEK1 and 2, and these phosphorylate the ERKs. Activated ERK1 and ERK2 translocate to the nucleus where they phosphorylate and regulate the activity of transcription factors including Elk-1 and Sap1a. The Ras/Raf-1/ERK pathway also functions to regulate cell cycle progression by virtue of its ability to up-regulate the expression of cyclins D and E1 while at the same time inhibiting the expression of the cell cycle inhibitor $p27^{Kip1}$ [83–85]. The function of the Ras/Raf-1/ERK pathway is negatively regulated by the Rap1 G-protein, which is inducibly activated in response to BCR ligation via a DAG-dependent mechanism [86]. Rap1 possesses the same effector binding domain as Ras, suggesting that it can compete with Ras for binding to downstream proteins in the Ras/Raf-1/ERK cascade. Indeed Rap1 has been shown to bind to Raf-1, but does not activate it [87]. Thus Rap1 may sequester components of the Ras/Raf-1/ERK pathway thereby blocking activation of ERK1 and 2. Alternatively it has been proposed that Rap1 activates a distinct pathway that may antagonize the function of the Ras pathway [88].

Activation of the Rac1 GTPase is mediated through the GEF activity of Vav [89]. Vav is effectively recruited to the plasma membrane by virtue of its PH domain, which binds to PIP_3, as well as its ability to interact with adaptor proteins via SH2/phosphotyrosine-dependent interactions. It has been shown that Vav is recruited to tyrosine phosphorylated BLNK where it is colocalized with the PTK Syk [36]. Phosphorylation of Vav by Syk potentiates its GEF activity leading to activation of Rac1. Additionally, it has been shown that Vav interacts with CD19, suggesting that this may constitute another mechanism for targeting it to the membrane. Rac1 is important for regulation of receptor-induced actin polymerization and cytoskeletal reorganization [90]. Such processes are likely to play an important role in organization of signaling proteins into effective complexes that promote B-cell activation. Rac1 activation also plays an important role in linking BCR signaling to activation of the downstream MAP kinases JNK and p38 [22,23,59]. Studies have

shown that activation of these kinases is abrogated in cells that lack BLNK and that activation cannot be restored by reconstitution of PLCγ2 signaling alone [36]. This indicates that binding of Vav to BLNK and its activation by Syk is crucial for subsequent activation of Rac1 and the JNK and p38 MAP kinases. Activated forms of these MAP kinases translocate to the nucleus where they play an important role in regulating the function of transcription factors through phosphorylation. JNK can activate Elk-1 and Sap1a transcription factors as well as c-Jun. It has been shown that p38 can regulate the activation of ATF-2, Sap1a, CHOP, and MEF2C transcription factors [59].

Conclusions

It is clear that the BCR is able to access several distinct, yet interrelated signaling pathways in response to binding of antigen. The initiation of signaling is dependent on antigen-driven changes in the organization of the BCR complexes expressed on the surface of a quiescent B cell. Although the exact nature of the changes that are elicited in response to antigen binding have yet to be elucidated, it is apparent that perturbation of the BCR complexes, whether they exist as individual monomeric structures or in preformed oligomers, leads to translocation to GEMs. This is critical for promoting a net increase in tyrosine phosphorylation of the BCR-associated Igα/β heterodimer and for recruitment of Syk and BLNK, which form the central initiation complex. Formation of this initiation complex leads to propagation of signaling transduction via pathways that are regulated by activation of PLCγ2, PI3K, PKC, Ras, and Rac1. These pathways in turn lead to activation of numerous transcription factors that regulate gene expression.

The ability of the BCR to control the various biological outcomes associated with B-cell development, selection, and activation is ultimately regulated by both intrinsic as well as extrinsic factors that affect the qualitative and quantitative nature of the overall signal transduced via the BCR. Extrinsic factors include the physical nature of the antigen, the duration of exposure to antigen, and the B cell's previous exposure to that antigen. Intrinsic factors include the developmental state of the B cell, which may affect the response of the cell to a given antigenic signal at the genetic level.

Additionally, it is clear that the maturational and differentiation states of the B cell dramatically alter its response to antigenic challenge. This can occur through changes in the expression of proximal BCR signaling proteins such as PTKs and PTPs, as well as through changes in the expression of other transmembrane receptors that function as coreceptors for the BCR. Examples of these include CD19, CD22, FcγRIIb, and PIR-B. These coreceptors have the ability to provide contextual information to the B cell through their ability to detect extracellular ligands that affect their ability to engage the BCR and to recruit signal transducing proteins that modify the nature of the signal transduced via the BCR [91–94]. Thus it is clear that numerous mechanisms exist for modulating the basic signal transduced via the BCR and this in turn alters the complement of transcriptional regulators that are activated as well as the genes that may be accessible to them. This then dramatically alters the expression of key regulators that determine whether the B cell undergoes apoptosis or mounts an active immune response.

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CHAPTER 345

Signaling Pathways in the Normal and Neoplastic Breast

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Introduction

Deregulated cell and tissue growth is a defining feature of all neoplasms, both benign and malignant. Malignant neoplasms have the capacity to invade normal tissues, to induce the development of a local vasculature, to metastasize and to grow at distant body sites. Deregulated growth occurs as a result of perturbed signal transduction. Signal transduction pathways include, in their broadest sense, all biochemical cellular pathways that modulate or alter cellular behavior or function. Consequently, cancers do not necessarily arise as a result of an increased rate of cellular proliferation. Rather, carcinogenesis is a combination of defects in cell cycle progression (cellular division), immortalization, genomic instability, programmed cell death (apoptosis) cell–cell and cell–substrate adhesion, and angiogenesis. During normal embryonic development and in adult life, signaling needs to be precisely coordinated and integrated at all times, because properly regulated differentiation signals are critical for preventing oncogenesis.

Studies of the normal and neoplastic breast during the last several decades of the twentieth century focused on identification of the mechanisms of action of estrogen and progesterone at the local tissue level, in the normal breast, and early promotion and later progression to malignancy. Breast tissue regulation by these hormones is modulated in a rather complex fashion by autocrine and paracrine growth factors and by a variety of transcription factors (such as coactivators and corepressors), controlling epithelial cellular differentiation, epithelial cell–cell and cell–stromal adhesion. A large body of breast cancer research has been focused on understanding the complex interactions among growth factors, deregulated growth regulatory genes (oncogenes or proto-oncogenes), in mediating or modulating endocrine steroid action in breast cancer.

A second important topic has been the involvement of growth factors in facilitating malignant progression of the disease. One area of research has examined defective tumor host interactions, resulting in aberrant stromal collagen synthesis (desmoplasia), epithelial cell invasion, and vascular infiltration (angiogenesis) to promote distant metastasis. Also, studies have found that certain growth factors may suppress the host immune response to the tumor and may influence a tumor's response to therapy.

In the past few years, significant progress has been made in understanding the roles of different signaling molecules in normal and malignant breast. However, for the purpose of this chapter we focus only on several major molecules and pathways, such as the epidermal growth factor family (EGF), TGF-β family/Smads, and some other families of growth factors that have been shown to be important in breast signaling. In addition, three major proliferation/survival molecules/pathways, Mek/Erk, PI3-K/Akt, and Stats, are discussed. We do not address the roles of cell adhesion molecules or steroid hormones in signaling; other reviews covering these topics are available [1–3].

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Signaling Molecules: A Class of Growth Factors

Epidermal Growth Factor Family

Two major classes of structurally and functionally distinct TGF-families were initially characterized by the prototypic transforming growth factor α (TGF-α) and transforming growth factor β (TGF-β). TGF-α is closely homologous to epidermal growth factor (EGF), and both bind to the common EGF receptor (EGFR) [4]. EGFR is tyrosine-kinase that, on activation, stimulates several cellular responses including survival, proliferation, motility, and differentiation [4]. The EGF family members that bind to EGFR are EGF, TGF-α, amphiregulin (AR, a heparin-binding factor), heparing-binding EGF (HbEGF), epiregulin, and β-cellulin [5]. Cripto (CR-1) is another EGF family member with a demonstrated role in embryogenesis and mammary gland development and it has been overexpressed in several human tumors [6,7]. Recently, the nature of its receptor has been identified as an ALK4, a type I serine/threonine kinase receptor for activin. CR-1 binds to cell surface ALK4 that is expressed on mammalian epithelial cells [8]. Also recently, molecules known as *heregulins* (human) and NDFs (*neu* differentiation factor, from rat) were cloned and identified [9,10]. *Neuregulin* is a widely used, common term for both NDFs and heregulins. Unlike most of the other EGF family members that bind directly to EGFR, neuregulins bind to two other EGF family members termed c-Erb-B3 and c-Erb-B4, which are structurally related [11]. Whereas c-Erb-B4 is a tyrosine kinase, c-Erb-B3 is kinase defective. A fourth member, c-Erb-B2 (HER2/neu), binds no secreted ligand and is activated through heterodimeric interaction with other family members. c-Erb-B2 is particularly important as an oncogene in breast cancer [12]. All four members of the EGFR family (Table I), and most of the EGFR-related growth factors, have been shown to play a role in tumor growth and development and in progression of human breast cancer.

Recently, mouse models have provided important corroboration and new insights regarding the role of EGF family members in mammary gland development. The view emerging from recent work is that signaling by EGFR and possibly c-Erb-B2 is critical for ductal outgrowth in pubescent glands, while signaling by c-Erb-B2, c-Erb-B3, and c-Erb-B4 is important for alveolar morphogenesis and lactation. Currently, it is unclear whether EGFR has a significant role in the latter processes. In a recent study only EGFR and c-Erb-B2 proteins appeared to play a role in the developing virgin gland [11], although all four members of the receptor family were expressed and maximally phosphorylated during pregnancy and lactation. Results of several studies indicate that members of the EGF family could act as local mediators of ductal morphogenesis. Analysis of triple null mice lacking EGF, TGF-α, and AR, alone or in various combinations, suggested a physiological role for AR ligand, a very surprising result in light of this ligand's reduced affinity for EGFR [13]. These results from animal models demonstrate a requirement for activation of EGFR by AR in ductal development in the adolescent mammary gland. Elongating ducts of adolescent mice contained high levels of the AR transcript, whereas expression was not detected in surrounding fibroblasts or fat cells, indicating that the epithelium is the principal source of AR in the developing mammary gland [13]. In contrast, EGFR protein and mRNA were detected in all cellular compartments, including epithelial cells, fibroblasts, and adipocytes. Thus, ductal outgrowth could be stimulated by autocrine and/or paracrine activation of EGFR. However, available evidence supports a paracrine model. Furthermore, although the role of EGFR in mammary gland development is obvious, until recently it was less clear whether this receptor also functions in alveolar morphogenesis and lactation. Current evidence supports such a role. Overexpression of TGF-α from WAP-driven transgene induced precocious alveolar development and delayed involution in transgenic mice [14], suggesting that these processes might be normally regulated by EGFR. EGFR levels and EGFR's phosphorylation were also shown to coordinately peak in late pregnancy and lactation [15]. In addition, aberrant epithelial differentiation and impaired lobular expansion were observed in the mammary gland of the AR knock-out mice, a phenotype that was aggravated by additional loss of EGF and TGF-α [13].

Steroid–growth factor interactions have been studied in human mammary tissue only in the context of malignant epithelium, although they are almost certainly crucial as well in the regulation of the normal gland and in the development of cancer. In hormone-responsive human breast cancer cells, estrogen-induced proliferation is accompanied by an increase in growth stimulatory TGF-α, AR, and IGF-II, modulation of IGF-binding proteins, induction of EGF and IGF-I receptors, inhibition of IGF-II and c-Erb-B2 receptors, and inhibition of TGF-β [16].

As mentioned earlier, the role of EGF family members in tumor onset and progression is well established [5]. TGF-α has been shown, both in cell lines *in vitro* and in experimental animal models *in vivo*, to be a positive modulator of cellular transformation. These effects of TGF-α are due to its effects on proliferation, survival, and motility, as well as

Table I Members of the EGFR Family and Their Ligands[a]

Receptor	Ligands
EGFR (c-Erb-B1)	Epidermal growth factor (EGF), transforming growth factor-α, amphiregulin, heparin-binding EGF-like growth factor (Hb EGF), betacellulin (BC), epiregulin (EP)
c-Erb-B2 (HER2)	Not established
c-Erb-B3 (HER3)	Neuregulins, Hb EGF, BC
c-Erb-B4 (HER4)	Neuregulins, Hb EGF, BC

[a]The four ErbB receptors are shown along with their ligands. Note that ErbB2 does not have a direct binding ligand.

modulation of differentiation [17]. Several laboratories, including our own, previously developed and/or utilized mouse models in which the EGFR ligand, TGF-α, was overexpressed in the mammary gland under control of MMTV LTR or WAP promoter [18–20]. The presence of the transgene in the mammary gland caused anomalous development: Alveoli appeared precociously, and postlactational involution was impaired, resulting in persistent epithelial structures, termed *hyperplastic alveolar nodules.* The mice also developed focal mammary tumors with high efficiency and short latency [18], indicating that ErbB signaling leads to neoplastic progression in this tissue. Recently, Humphreys and Hennighausen [19,21], compared TGF-α-induced mammary tumorigenesis in wild-type mice versus those lacking a functional Stat5a gene. They found that the absence of Stat5a delayed hyperplasia and tumor development and, coincidentally, promoted more epithelial regression. These effects were not observed in TGF-α transgenic mice containing Stat5a.

Our own studies and the research of other laboratories have been focused on understanding the cooperation and synergy between TGF-α and c-Myc that is frequently amplified and overexpressed in human breast cancer. c-Myc is a downstream effector of the c-Erb-B2 oncogene. Bitransgenic c-Myc/TGF-α mice developed multiple aggressive mammary tumors with dramatically shorter latency compared to either single transgenic lineage [18]. These results indicate a strong synergy between TGF-α and c-Myc that could reflect the latter's ability to amplify autocrine growth circuits, including those involving EGFR, in cultured cells. In addition, our recent data indicate that there is another possibility involving cooperation between cyclin D1 and TGF-α [22]. It appears that the early up-regulation of cyclin D1 by TGF-α might circumvent the normal ability of c-Myc to repress this cell cycle regulator, perhaps obviating the need for genetic alterations that would otherwise uncouple c-Myc and cyclin D1 during neoplastic progression. Work by Shroeder *et al.* [20] focused on identifying genes that cooperate with TGF-α in mammary tumorigenesis. Based on their results it appears that TGF-α caused up-regulation of the Wnt 3 gene, suggesting that the synergy between EGFR and Frizzled signaling pathways might contribute to neoplastic progression. Both Wnt and ErbB receptors can regulate β-catenin activity leading to cellular disaggregation. Therefore, it is possible to speculate that the observed synergy involves this pathway.

Besides the role of TGF-α in mammary tumorigenesis, other EGFR ligands might have a role in the proliferation process. The closely related factor, EGF, has been shown to act as an oncogene-like molecule when transfected and overexpressed in immortalized rodent fibroblasts [11]. Furthermore, it has been shown that besides TGF-α, both AR and CR-1 may be important in early stages of the onset of breast cancer [15,23–27]. Recently, a new transgenic model of EGFR overexpression in mammary gland has been described [28]. Mammary glands of virgin mice harboring an EGFR transgene, under the control of the MMTV or β-lactoglobulin (BLG) promoters, developed abnormally and displayed epithelial hyperplasias. With multiple pregnancies, dysplasias and tubular adenocarcinomas were also observed. Differentiation of mammary epithelium was perturbed in response to deregulated EGFR, as reflected by fewer alveoli developing in whole mount organ cultures. Similar to EGFR, overexpression of c-Erb-B2 in the transgenic mouse, or in the transgenic mammary gland after retroviral transfer, also leads to pregnancy-induced mammary tumors [11]. Some limited studies have also addressed the function of the c-Erb-B/c-Erb-B4 ligand family and neuregulins in breast cancer [11, 29].

Clearly, signaling by the ErbB network is important to the complex overall regulation of the mammary gland. It appears that all the models described above have firmly established that altered regulation or signaling of ErbB receptors and ligands can facilitate tumor promotion and/or neoplastic progression in the mammary gland. Future issues to be solved will include determination of definitive roles of c-Erb-B3 and c-Erb-B4 in mammary tumorigenesis, exploration of cooperative roles and interaction of EGFR and c-Erb-B2 in carcinogenesis, and the interactions of the c-ErbB family with other oncogenes and hormones in human breast cancers. In addition, it will be of interest to establish the identity of critical intracellular signaling pathways downstream of these receptors in human breast cancer at various steps in malignancy progression. Our own preliminary work and the work of others indicate that both the PI3K and MEK/Erk pathways are two key survival and proliferation signaling pathways, conveying signals downstream of the EGFR and possibly other members of the family. These two pathways are discussed later in this chapter.

Transforming Growth Factor β Family

The TGF-β family consists of several related gene products, each forming 25-kDa homodimeric or heterodimeric species, found in both normal mammary epithelium and in breast cancer. Three membrane-binding proteins interact with this family of growth factors. These were initially termed *receptors* (type I, II, and III), but type III receptors seem to be nonsignaling proteins, whereas the other two types (type I and II) are serine/threonine kinases and have been shown to deliver intracellular signals [30–34]. Four different type II receptors have been cloned, and they each may associate with one of several type II receptors. The function of type II receptors is determined on the basis of which type I receptor is recruited into dimer formation. TGF-β ligands only directly bind to type II receptors.

The epithelial-inhibitory TGF-β family is found in normal and malignant mammary epithelium and human milk [35]. TGF-β clearly has a negative effect on ductal epithelial proliferation and lactation in mouse mammary glands *in vivo* [36]. It is important to note that the production of TGF-β increases as breast cancer progresses; its accumulation appears to be important in the characteristic fibrous desmoplastic stroma of the disease [37], in immune suppression, and in tumor angiogenesis. It seems that, although TGF-β clearly serves a growth-inhibitory role in

the normal gland, in which it may be tumor suppressive, overproduction of TGF-β may contribute to aberrant tumor–host interactions in the later progression of breast cancer [35].

In the review by Wakefield and Roberts [38] on TGF-β signaling in mammary gland development and tumorigenesis, several important issues have been summarized. First, ligands of the TGF-β superfamily are unique, in that they signal through transmembrane receptor serine/threonine kinases, rather than the tyrosine kinases of many other well-known growth factors in the breast. The TGF-β receptor complex couples to a signal transduction pathway involving a novel family of proteins, the Smads. On phosphorylation, Smads translocate to the nucleus where they modulate transcriptional responses. However, TGF-β can also activate the mitogen-activated protein kinase (MAPK) 4 pathway, and the different biological responses to TGF-β depend to varying degrees on activation of either or both of these two pathways. The Smad pathway is a nexus for cross-talk with other signal transduction pathways and for modulation by many different interacting proteins. Despite compelling evidence that TGF-β has tumor suppressor activity in the mammary gland, neither TGF-β receptors nor Smads are genetically inactivated in human breast cancer, although receptor expression is reduced. Work by Xie *et al*. [39] provides new information on the role of Smads in human breast cancer. It shows that alterations of Smad signaling in human breast carcinoma are associated with poor outcome. Among 456 cases of human breast carcinoma assembled in tissue microarrays, the majority (92%) expressed Smad2, Smad2P, and Smad4, indicating their ability to proliferate within a microenvironment that contains bioactive TGF-β.

Although some of the roles of TGF-β in the mammary gland signaling are obvious, some of the mechanisms in which TGF-β might play a role in development of human breast cancer are poorly understood. This needs to be addressed in future research initiatives.

Other Families of Growth Factors

In addition to EGFR and TGF-β growth factor families, at least five other growth factor stimulatory molecules are found to play a role in breast cancer. These are summarized in Table II: insulin-like growth factors (type I and II) [40], members of the Wnt (wingless) growth factor receptor family (Wnt-2, Wnt-3, Wnt-4, Wnt5a, and Wnt-7b) [41], platelet-derived growth factors A and B [42], and the fibroblast growth factor (FGF) family [43]. Each of these growth factor classes binds to one or more specific tyrosine kinase-encoding receptors. Vascular endothelial growth factor (VEGF, a member of a different family of tyrosine kinase receptor-binding factors) [44], pleiotrophin (a developmental, neurotropic factor) [45], and hepatocyte growth factor (HGF; also called scatter factor) and its tyrosine kinase encoding receptor, c-Met [46] are all produced by breast cancer. In addition, breast cancer cells also produce the hormone prolactin [47] and mammary-derived growth factor 1 (MDGF-1) [48].

Table II Diverse Group of Growth Factors and Other Molecules Thought to Play Roles in Breast Cancer[a]

Signaling molecules	Cellular response
Insulin-like growth factors (type I and II)	Stimulatory/tumor cells
Wnt growth factor family	Stimulatory/tumor cells
Platelet-derived growth factors A and B	Stimulatory/stromal cells[b]
Fibroblast growth factors	Stimulatory/tumor cells
Vascular endothelial growth factor	Angiogenesis/vascular cells[b]
Hepatocyte growth factor, receptor c-Met	Stimulatory/tumor cells
Prolactin	Stimulatory/tumor cells
Mammary-derived growth factor 1	Stimulatory/tumor cells
Pleiotrophin (developmental neurotropic factor)	Stimulatory/tumor cells[b]

[a]Proposed autocrine and paracrine factors in breast cancer. Tumor cells release variety of growth factors that might play autocrine roles *in vivo* (this is based mostly on their activity *in vitro*). Several of the same factors also are known to play paracrine and endocrine roles as well.

[b]Additional, angiogenic effects of these factors.

Although the roles of some of the molecules just mentioned are well established in breast signaling, much more research is needed to fully understand their role in both mammary gland development and in human breast cancer.

PI3K/Akt, MEK/Erk, and Stats: Major Proliferation/Survival Molecules Downstream of Growth Factor Receptors in Breast

Information is limited on the signaling pathways linking EGFR and other growth factor receptors to the regulation of cellular survival in mouse and human mammary epithelial and carcinoma cells. However, studies in some nonmammary epithelial cells (hepatic carcinoma cells and keratinocytes) have identified two survival pathways downstream of EGFR: phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal regulated kinase (Erk1/2). In most cases, the PI3K/Akt pathway delivers the most potent survival signals downstream of EGFR [49,50].

Akt is a serine/threonine kinase, downstream of PI3K, that delivers strong survival signals in many cell types [51–53]. Both growth factors and integrins activate Akt through activation of PDK1 and a putative PDK2 kinase, which subsequently phosphorylate Akt at Thr308 and Ser473, respectively [54]. There are several isoforms of Akt (Akt1, Akt2, and Akt3); each has been shown to be expressed at different levels in various tissues [55]. The targets of Akt in epithelial cells, including mouse and human breast cells, include proteins involved in cell growth, metabolism, and apoptosis. The Akt targets involved in apoptosis include Bad, a proapoptotic member of the Bcl-2 family of proteins [56], caspase-9 [57], and the forkhead transcription factor [58]. Recently, Akt also has been reported to up-regulate the expression of antiapoptotic proteins in lymphoid cells

such as Bcl-x_L [59], Bcl-2 [60], and Mcl-1 [61]. Akt activates NFκB in both fibroblasts and epithelial cells [62,63].

Similar to Akt, in most cell types, both growth factors and integrins are capable of activating the MAPK/Erk pathway. Of particular relevance to our studies involving models of human breast cancer is the fact that the MAPK/Erk pathway was previously shown to convey survival signals in response to EGF [64,65].

Recently, it has been shown that the PI3K/Akt and the MAPK/Erk pathways can cooperate in the inhibition of Bad in some cell types [66]. However, the prosurvival targets and the interactions of EGFR-activated PI3K/Akt and MAPK/Erk pathways have not been established in murine mammary epithelial cells (MMECs), human breast epithelial cells, or human carcinomas. Our earlier studies using mouse mammary epithelial cells [67a], established that activation of EGFR can promote survival in association with upregulation of Bcl-x_L a prosurvival family member of the Bcl-2 family. However, the mechanism(s) responsible for up-regulation of Bcl-x_L is unknown.

In our recent studies [67] we have chosen proapoptotic, c-Myc-overexpressing MMECs derived from MMTV-c-Myc transgenic mouse tumors to address these issues. We demonstrated that EGF strongly activates both Akt and Erks over a similar time frame and that this activity is dependent on EGFR kinase activity. The importance of Akt and Erk1/Erk2 in EGF-mediated survival signaling in c-Myc-overexpressing cells was further confirmed through the finding that prolonged inhibition of PI3K and MAPK/MEK activity leads to apoptosis. Apoptosis, induced by inhibition of PI3K activity, was paralleled by down-regulation of protein expression of both Akt and Erk1/Erk2. In addition, overexpression of constitutively activated Akt (Myr-Akt) prevented c-Myc-mediated apoptosis, triggered by the inhibition of the EGFR tyrosine kinase activity. The mechanism through which Akt, and possibly Erk, promote survival in c-Myc-overexpressing cells appears to involve the up-regulation of Bcl-x_L, suggesting that Bcl-x_L might be a novel target of Akt in MMECs (Fig. 1). With the emerging role of activated EGFR family in human breast cancer, it would be important to determine the antiapoptotic targets of EGFR-stimulated Akt and Erk in MMECs and human breast cancer cells. In support of *in vitro* studies, work by Hutchinson *et al*. [68] directly assessed the role of Akt in mammary epithelial development and tumorigenesis, using transgenic mice. The results indicate that activated Akt provides a critical cell survival signal required for tumor progression, without causing metastatic progression.

Another group of very important molecules that plays a role in mammary gland signaling is the Stats, transcription factors that are sequestered in the cytoplasm in an inactive form. Two members of the Stat family of transcription factors play a vital role in mouse mammary gland development. Stat5a was originally described as a regulator of milk protein gene expression and was subsequently shown to be essential for mammary development and lactogenesis. In contrast, Stat3 is an essential mediator of apoptosis and

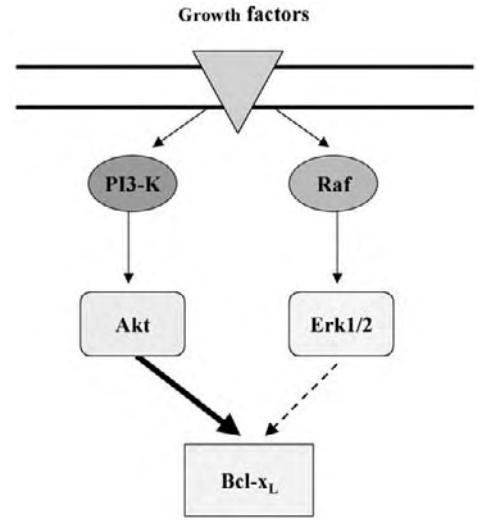


Figure 1 Proposed model of Bcl-x_L regulation by Akt and possibly Erk. Activated Akt up-regulates Bcl-x_L protein by a presently unknown mechanism in mouse mammary epithelial cells overexpressing c-Myc. This might lead to inhibition of apoptosis.

postlactational regression. Other members of the Stat family may have specific, but as yet undemonstrated, functions in mammary development. However, since Stat1 activity is regulated during mammary development in a pattern different from Stats 3 and 5, this factor also may have a functional role. Phosphorylation by activated cytokines and growth factor receptors leads to dimerization and translocation to the nucleus where Stats can activate specific sets of genes [69]. Constitutive activation of Stats has been associated with invasive breast carcinomas and breast cancer cell lines, but not with *in situ* carcinoma or benign lesions. Watson [70] postulates that cross-talk between Stat pathways and the ErbB family and Src may be an important regulator of breast cancer progression.

Stats have been shown to have a binding site in the Bcl-x_L promoter region [71]; therefore, when activated Stats might also contribute to cellular survival through up-regulation of this important prosurvival molecule. It has been reported that STATs 1 and 3 expression in primary breast carcinomas correlate with EGFR, HER2, p53, ER, PR, p21/waf1, Bcl-x_L, and Ki-67 expression [72].

Conclusions and Future Prospects

This chapter highlighted the importance of some of the molecules in breast signaling. Clearly, the importance of signaling by the c-ErbB family, TGF-β/Smads, several other growth factors, PI3K/Akt, MEK/Erk, and Stats in mammary gland development and mammary tumorigenesis has been well documented. Despite the fact that the recent insights revealed a significant amount of evidence for their role, mostly by research using *in vitro* human cellular models and mouse models of human breast cancer, it appears that many more questions remain unanswered. Indeed, studies to date

have only started to answer some of the fundamental questions about how the signaling molecules mentioned here contribute to physiological and pathological activities in the breast tissue. New and more sophisticated insights will undoubtedly provide crucial information that will help our understanding of the role of these molecules in breast cancer and will help in providing valuable information for future therapies.

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CHAPTER 346

Kidney

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Overview of Kidney Function and Cell-to-Cell Interactions

The kidney is a complex organ comprising diverse cell types that work in coordination to perform a broad spectrum of functions, including the maintenance of body fluid and electrolyte balance, pH regulation, secretion of renin and erythropoietin, activation of vitamin D, excretion of numerous drugs and toxins, and regulation of blood pressure. Several of these functions are related and involve the process of urine formation, which takes place in the functional unit of the kidney, the nephron. Each human kidney has about one million nephrons that operate in parallel. The nephron consists of a glomerulus and a tubule arranged in series. The glomerulus is formed by a capillary network (glomerular tuft) extending between the afferent and efferent glomerular arterioles. The capillary loops are separated by intercellular material and surrounded by a layer of specialized epithelial cells (*podocytes*) attached to the basement membrane of the capillaries. The epithelial layer reflects on itself in the vascular pole, forming the Bowman space (or urinary space) that is continued by the lumen of the proximal tubule (Fig. 1). Urine formation is the result of filtration at the glomerulus and reabsorption or secretion at the tubule. The net flux from tubule lumen to blood constitutes reabsorption and the net flux in the opposite direction constitutes secretion. The renal-tubule cells can perform net transport between solutions of very similar or identical composition because they are polarized, that is, different transport proteins are expressed in the apical (lumen-facing) and basolateral (blood-facing) membrane domains, allowing them to carry out directional transport of specific solutes. Water transport is osmotic and occurs in the direction of net solute transport. Further details about renal organization and function can be found in recent texts [1,2].

Glomerular filtration, secretion, and tubule transport are finely regulated processes. The proximity of renal tubules to each other, as well as to capillaries and interstitial cells, facilitates paracrine interactions between different cell types. The glomerular urinary space is in series with the lumen of the renal tubule segments, so that cells in consecutive structures communicate with each other via the luminal fluid. The composition of this fluid can be changed by the rate of filtration (that increases the NaCl load) or by the secretion of signaling molecules into the lumen. Both changes in luminal NaCl concentration and the presence of signaling molecules are sensed by downstream tubule segments. This signaling mode, unique to the kidney, provides functional integration at the level of the single nephron. This theme is one of the central topics in this article.

As in other organs, cell-to-cell communication in the kidney is largely mediated by paracrine and autocrine agents that may act in the extracellular or intracellular compartments, activating signal transduction systems. These complex interactions unify membrane transport processes and urine formation with homeostatic regulation of renal hemodynamics and glomerular filtration rate (GFR) [3–5].

The kidney is divided in two regions: the superficial cortex and the interior medulla (Fig. 1, top panel). The cortex, 70% of the renal parenchyma, contains three classes of cells: *vascular* (large and small arteries, arterioles, capillaries), *epithelial* [proximal convoluted tubule (PCT), loop of Henle (LH) of short-looped cortical nephrons, distal convoluted tubule (DCT), cortical collecting tubule (CCT)], and *interstitial* cells. The medulla comprises 30% of the kidney mass

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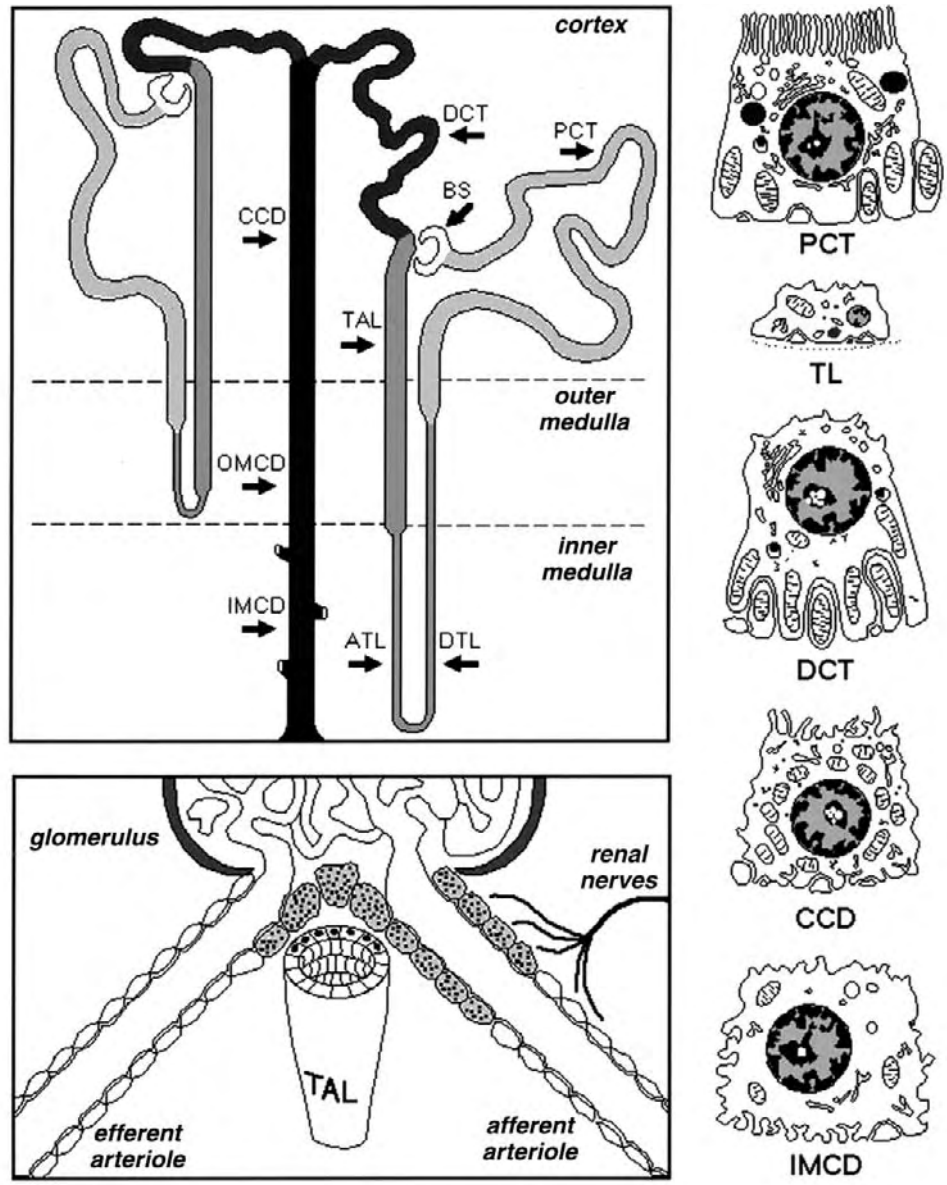


Figure 1 (Top) Scheme of the structure of deep and superficial nephrons. BS, Bowman's space; PCT, proximal convoluted tubule; DTL, descending thin limb of Henle's loop; ATL, ascending thin limb of Henle's loop; TAL, thick ascending limb of Henle's loop; DCT, distal convoluted tubule; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct. Shown on the right are simplified drawings portraying major structural differences between epithelial cells along the nephron. (Bottom) Scheme of the juxtaglomerular apparatus, which consists of vascular smooth muscle cells of afferent and efferent arterioles, juxtaglomerular granular cells at the glomerular end of an afferent arteriole, macula densa cells of the thick ascending limb of Henle's loop (TAL), glomerulus and extraglomerular mesangial cells.

and contains vasa recta capillaries, long loops of Henle of deep (juxtamedullary) nephrons, collecting ducts (CD), and interstitial cells. Thin-descending and thin-ascending LH are present in the inner medulla. The thick ascending limb of Henle's loop (TAL) resides in the outer medulla and cortex.

The vascular resistance of the kidney is lower than those of other organs, explaining the high blood flow of the organ, on average 1200 mL/min (about 25% of cardiac output) or 4 mL/min·g kidney wt. The renal artery and its branches carry 85–90% of the total renal blood flow to the cortex, where the microvasculature consists of two capillary beds arranged in series: the glomerular capillaries (after the afferent arteriole of the glomerulus) and the peritubular capillaries (after the efferent arteriole of the glomerulus). Afferent and efferent arterioles regulate blood flow and glomerular capillary hydrostatic pressure, the latter being a major determinant of the GFR. The transcapillary hydrostatic pressure gradient exceeds the plasma colloid osmotic pressure, thus causing fluid ultrafiltration all along the length of the glomerular capillaries. The glomerular-capillary wall is formed, from

blood to Bowman's space, by the endothelial layer (that is fenestrated), the basement membrane, and the epithelial layer (foot processes separated by slits). The basement membrane, that is, the effective barrier of the glomerular-capillary wall, has a low permeability to plasma proteins and other molecules larger than albumin, whereas its permeability to water, ions, and other small solutes is considerably higher than in systemic capillaries. Fluid flows from Bowman's space into the proximal tubule, where solutes and water are reabsorbed by transport across both the cell membranes and the intercellular pathway into the peritubular space and then the capillaries. The plasma colloid osmotic pressure in the peritubular capillaries (elevated because in the glomerulus water is filtered, but not protein) exceeds the transcapillary hydrostatic pressure gradient and thus the reabsorbate readily flows into peritubular capillaries, both in the cortex and in the medulla. The capillaries in the latter are the hairpin-shaped *vasa recta*.

Medullary blood flow is 10–15% of the total renal blood flow. Its control is essential for the operation of the countercurrent multiplication and exchange mechanisms that accumulate solute in the medullary interstitial space providing the driving forces to concentrate or dilute the urine. In addition to upstream control by efferent arterioles, local blood-flow regulation is provided by smooth muscle-like pericytes around descending vasa recta. The medullary blood vessels are more sensitive to vasoactive agents than the cortical resistance vessels. Abnormal regulation of the renal-medullary circulation can lead to salt retention and hypertension [6–8].

Redundant and complementary control mechanisms form the basis of structural–functional relationships that are central to two of the primary functions of the kidney, namely, the maintenance of salt and water balances and the control of blood pressure. In the steady state, the output of fluid and electrolytes (excretion) matches the input (intake plus production and administration), so that the volume and composition of the extracellular fluid remain constant [3,5,9]. In a normal hydration state, the renal tubules reabsorb more than 99% of the glomerular filtrate. The rate of glomerular filtration in adult humans is about 120 mL/min, or 170 L/day. About 70% of the reabsorption occurs in the PT, 15% in the LH, and the rest in the distal nephrons (DCT, CCD, MCD). Figure 1 outlines the nephron segments and the basic structural features of the epithelial cells of each segment. The luminal fluid flows from PT to the descending and ascending loops of Henle, DCT, and CD. The transport processes and permeability properties of each nephron segment are regulated by paracrine factors generated by vascular and epithelial cells. NaCl is actively reabsorbed in all nephron segments, except the thin loops of Henle. The basolateral membranes of all renal-tubule cells express Na^+-K^+/ATPase, or Na^+ pump, a primary-active transport protein that extrudes Na^+ and takes up K^+, establishing chemical and electrical gradients at the luminal membrane that favor passive Na^+ entry. The luminal membranes of the different tubule segments express diverse Na^+ transport proteins [10–19]. The main mechanisms of Na^+ entry across the luminal membrane are: Na^+-H^+ exchange in the PCT (mediated by the antiporter NHE3, sensitive to high concentrations of the drug amiloride), Na^+-K^+-$2Cl^-$ in the TAL (mediated by the symporter NKCC2, sensitive to the diuretic furosemide), Na^+-Cl^- cotransport in the DCT (mediated by the symporter NCC, sensitive to diuretics of the thiazide family), and channel-mediated entry in the principal cells of the CD (via ENaC, the epithelial Na^+ channel, blocked by low concentrations of amiloride). Reabsorption of Na^+ is accompanied by anion (largely Cl^- and HCO_3^-) transport in the same direction and/or transport of another cation (H^+ or K^+) in the opposite direction to Na^+. The transport of Na^+ is coupled to transport of other solutes at the molecular level (examples given earlier) or by changing the driving force for transport of the other solute (i.e., transcellular Na^+ absorption favors paracellular Cl^- absorption).

Vasopressin, secreted by the pituitary, acts on CD principal cells to elicit exocytotic insertion of aquaporin (AQP2) and urea transporter (UT1) in the luminal membrane, thus increasing the permeability to both urea and water. Aldosterone, secreted by the adrenal cortex, also exerts its main action on the principal cells of the CD, stimulating Na^+ reabsorption by genomic and nongenomic mechanisms that enhance the number of Na^+ channels at the luminal membrane and the number of Na^+ pumps at the basolateral membrane. Because of the differences in the salt and water permeabilities among renal-tubule segments, the fractional reabsorptions of salt and water are somewhat different in specific segments. In normal individuals, fractional Na^+ reabsorption is about 67% in the PT, 20% in the LH, 7% in the DCT, 5% in the CD, and 1% in the IMCD.

In summary, the kidney is a highly sophisticated organ serving multiple functions ranging from synthesis and release of the proteolytic enzyme renin to maintenance of extracellular fluid volume and composition. Several of these functions depend critically on cell-to-cell communication. Homeostasis is regulated by multiple systems featuring coordination of hormonal, neural, paracrine, and autocrine signals as they act to control both the renal microcirculation and epithelial transport along the renal tubule.

This chapter focuses on cell-to-cell communication within the kidney. The extrarenal neural-hormonal regulation and other important functions, such as regulation of acid–base and calcium and potassium balances, are not discussed (for recent reviews, see [5,6,9,14,16,20–22]). Signal transduction among different cells is highlighted, at the expense of in-depth discussion of mechanisms underlying effector cell responses. Due to space limitations, not all intrarenal events are discussed. The reader is referred to recent reviews on contractile mechanisms of vascular smooth muscle cells [23–27] and basic transport mechanisms in renal epithelial cells [10–19].

Vascular Endothelial Cells

Endothelial cells [28–30] are in contact with blood elements and subjected to hemodynamic forces such as

Table I Summary of Major Receptors and Signaling Pathways in the Renal Vasculature

Hormone/paracrine/ autocrine agents	Receptor	G-protein	Intermediates/messengers
Vasoconstrictor Agents (direct actions on VSMC)			
Adenosine	P_1-A_1	$G_{\alpha i}$	↓cAMP/PKA
ATP	P_{2X}	—	↓K channel activity/ Depolarization Non-selective cation channel/Ca^{2+}
	P_{2Y}	$G_{\alpha q/11}$	Ca^{2+}/PKC Ca^{2+}/PLA_2/Cyt P450/HETE
Angiotensin II	AT_1	$G_{\alpha q/11}$	Ca^{2+}/PKC Ca^{2+}/PLA_2/Cyt P450/HETE
Catecholamines	α_1 α_2	$G_{\alpha q/11}$ $G_{\alpha i}$	Ca^{2+}/PKC ↓cAMP/PKA
Endothelin	ET_A	$G_{\alpha q/11}$	Ca^{2+}/PKC Ca^{2+}/PLA_2/Cyt P450/HETE
	ET_B	$G_{\alpha q/11}$	Ca^{2+}/PKC Ca^{2+}/PLA2/Cyt P450/HETE
PGE_2	EP_1 EP_3	$G_{\alpha q/11}$ $G_{\alpha i}$	Ca^{2+}/PKC ↓cAMP/PKA
$PGF_{2\alpha}$	FP	$G_{\alpha q/11}$	Ca^{2+}/PKC
Thromboxane	TP	$G_{\alpha q/11}$	Ca^{2+}/PKC
Vasopressin	V_1	$G_{\alpha q/11}$	Ca^{2+}/PKC
20-HETE	*	—	K channel (Ca^{2+} activated)/ depolarization
Vasodilator Agents (direct actions on VSMC)			
Adenosine	P_1-A_2	$G_{\alpha s}$	cAMP/PKA
Catecholamines	β	$G_{\alpha s}$	cAMP/PKA
Dopamine	D_1	$G_{\alpha s}$	cAMP/PKA
PGE_2	EP_4	$G_{\alpha s}$	cAMP/PKA
PGI_2	IP	$G_{\alpha s}$	cAMP/PKA
EDHF	*	—	K channel (Ca^{2+} activated)/ hyperpolarization
11,12-/14,15-EET	*	—	K channel (Ca^{2+} activated)/ hyperpolarization
Nitric oxide	sGC	—	cGMP/PKG cGMP/↓ PDE/↑ cAMP ↓ Cyt P450/↓ 20-HETE/ hyperpolarization
Carbon monoxide	sGC	—	cGMP/PKG
Vasodilator Agents (with preferential actions on Endothelial Cells)			
Endothelin	ET_B	$G_{\alpha q/11}$	Ca^{2+} NOS/NO Ca^{2+} PLA_2/COX/PGs PLA_2/EET
Bradykinin	B_2	$G_{\alpha q/11}$	Ca^{2+} NOS/NO Ca^{2+} PLA_2/COX/PGs PLA_2/EET
Acetylcholine	M	$G_{\alpha q/11}$	Ca^{2+} NOS/NO Ca^{2+} PLA_2/COX/PGs PLA_2/EET
Vasoconstrictor Agents (that also stimulate production of vasodilator substances by Endothelial Cells)			
Adenosine	P_1-A_1	Gαq/11	Ca^{2+} NOS/NO Ca^{2+} PLA_2/COX/PGs PLA_2/Cyt-P450/EET
Angiotensin II	AT_1	$G_{\alpha q/11}$	Ca^{2+} NOS/NO Ca^{2+} PLA_2/COX/PGs PLA_2/Cyt-P450/EET
ATP	P_{2Y}	$G_{\alpha q/11}$	Ca^{2+} NOS/NO Ca^{2+} PLA_2/COX/PGs PLA_2/Cyt-P450/EET
Vasopressin	V_1	$G_{\alpha q/11}$	Ca^{2+} NOS/NO Ca^{2+} PLA_2/COX/PGs PLA2/Cyt-P450/EET

Abbreviations in Table I:
cAMP, cyclic adenosine monophosphate
ATP, adenosine triphosphate
CO, carbon dioxide
COX, cyclooxygenase
cGMP, cyclic guanine monophosphate
Cyt-P450, cytochrome P450 enzyme
EET, Epoxy-eicosatrienoic acid
HETE, Hydroxy-eicosatrienoic acid
HO, heme oxygenase enzyme
NO, nitric oxide
NOS, nitric oxide synthase
PDE, phosphodiesterase
PGs, prostaglandins
PLA_2, phospholipase A_2
SGC, Soluble guanylyl cyclase
*, undetermined
↓, inhibits

hydrostatic pressure and shear stress. The hydrostatic pressure tends to distend the arterioles, causing a contractile myogenic response, whereas the shear stress, depending on the flow velocity, causes release of vasodilator agents including nitric oxide (NO), cyclooxygenase products and EDHP, and vasoconstrictor factors including endothelin-1, cytochrome P450 (Cyt-P450) products, and COX II derivatives. The interplay of these mechanisms determines the basal tone in resistance arteries and allows for rapid adaptations to fluctuations in flow and pressure.

Endothelial cells constitute a barrier whose permeability varies in different organs, largely depending on the presence of fenestrate and the properties of the tight junctions that separate the endothelial cells. Endothelial cells synthesize paracrine agents (gases, fatty acids, and peptides) that act on both nearby vascular smooth muscle cells (VSMC) and epithelial cells.

The release of both relaxing and constricting vasoactive factors modulates the degree of contraction of VSMC (Table I). Vasodilators include NO, PGI_2, and endothelium-derived hyperpolarizing factor (EDHF). The most potent vasoconstrictor is endothelin (ET). In addition, endothelial cells express angiotensin-converting enzyme (ACE), tethered to the blood-facing membrane; this enzyme catalyzes the local production of the vasoconstrictor angiotensin II (Ang II). Under physiological conditions, the actions of dilator agents predominate, whereas constrictor systems tend to prevail in stressful and disease states. The physiological actions of

vasodilator agents are more important in counteracting the actions of constrictor systems, for example, angiotensin II and norepinephrine, than in producing vasodilation per se.

Nitric Oxide

Nitric oxide [31–34] synthase (NOS) is activated by hypoxia, shear stress, cell deformation, or vasoactive substances, to produce NO from L-arginine and molecular oxygen. NO is an important mediator of communication between endothelial cells and VSMC, as well as between endothelial cells and tubule cells. Constitutively active endothelial NOS (eNOS) is richly expressed in vessels and IMCD. Hemoglobin rapidly inactivates NO. Vasoactive agents such as Ang II and bradykinin stimulate eNOS, and ATP can stimulate NOS isoenzymes. The activation by these agents is mediated by G-protein-coupled receptors and involves activation of phospholipase C (PLC), increase in cytosolic calcium concentration ($[Ca^{2+}]_i$), and calmodulin-dependent enzyme activation. Thus, many vasoconstrictor-ligand-receptor interactions leading to increased $[Ca^{2+}]_i$ elicit primarily VSMC contraction but also stimulate endothelial production of the vasodilator NO, which limits the degree of constriction. The final balance of these two effects depends on the agonist and the density of receptors/enzymes in endothelial cells and VSMC. Ang II produces net vasoconstriction by virtue of the dominant action of AT_1 receptors on VSMC, whereas bradykinin and ATP cause net vasodilation due to their dominant effects on endothelial cells and NO production.

The low renal vascular resistance, compared to other vascular beds, is explained in part by a high dependence on NO. Inhibition of all three NOS isoforms (type I or neuronal, type II or inducible, and type III or endothelial) reduces renal blood flow (RBF) by about 30%, reduces GFR to a lesser extent, and increases arterial pressure by about 30 mm Hg. Constriction occurs along both afferent and efferent glomerular arterioles, and the glomerular filtration coefficient (hydraulic conductivity per glomerulus = capillary surface area × hydraulic conductivity per unit surface area) is reduced.

Endothelin

Endothelins [35–37] are potent paracrine vasoconstrictors produced by endothelial cells. They are 21-amino-acid peptides and three isoforms have been described, ET-1 being the most prevalent one in humans. Human ET is derived by successive proteolytic steps from preproendothelin (212 amino acids), which is hydrolyzed by a specific endopeptidase to a 37- to 39-amino-acid molecule named prepro-ET or big-ET; ET-1 is formed by the action of endothelial-bound ET converting enzyme on pro-ET.

The stimuli for endothelin secretion are bradykinin, ATP, shear stress, and cytokines. ET is predominantly secreted toward adjacent VSMC and the local effects of endogenous ET are more pronounced than those elicited by the usually low concentrations of circulating ET. Both ET_A and ET_B receptors are found in renal VSMC and both activate $G_{\alpha q/11}$ promoting an increase in $[Ca^{2+}]_i$ and triggering a contraction more pronounced and longer than those elicited by norepinephrine or Ang II, due to higher affinity of ET to its receptor. Only ET_B receptors are expressed in endothelial cells, where their activation increases $[Ca^{2+}]_i$ and stimulates NOS to produce the vasodilator NO; generation of vasodilator prostaglandins may also increase. Whether ET exerts a dilator or a constrictor action on arterioles depends on the relative abundance of ET receptors on the endothelium versus the VSMC. ET is thought to be primarily a vasoconstrictor agent in pathological conditions such as congestive heart failure and chronic renal failure, acting on both afferent and efferent arterioles of the glomerulus.

Arachidonic Acid Metabolites

Eicosanoid production is governed by the availability of the membrane fatty acid arachidonic acid. Its release is mediated by calcium-dependent, cytosolic phospholipase A2 (PLA_2) and the activity of enzymes of the cyclooxygenase (COX), lipoxygenase, and Cyt-P450 families. COX I and II synthesize prostaglandin H_2, from which different eicosanoids (PGE_2, PGI_2, and thromboxane TxA_2) are produced by the action of PGE_2-isomerase, prostacyclin synthase, and thromboxane synthase, respectively. The lipoxygenase family generates leukotrienes. Very little is known about lipoxygenase activity in physiological conditions. Finally, the Cyt-P450 monooxygenase family yields epoxy-eicosatrienoic acids (EETs) and hydroxy-eicosatrienoic acids (HETEs).

Arachidonic Acid-COX Metabolites

Prostaglandins (PGs) and thromboxanes (TXs) are synthesized by endothelial cells and act on vascular and tubule cells to function as autocrine or paracrine agents, contributing to the regulation of renal hemodynamics, renin release, and salt and water balance. When renin levels are normal, the net effects of COX metabolites [38–40] are vasodilator and natriuretic (i.e., promoting Na^+ excretion), reflecting the predominant production and actions of PGE_2 and/or PGI_2 over $PGF_{2\alpha}$ and TxA_2. In the cortical vasculature, PGI_2 is the major COX metabolite whereas tubule cells, especially IMCD cells, produce primarily PGE_2.

Prostaglandins activate G-protein-coupled receptors. In the renal vasculature, the main isotypes are EP_4 for dilator PGE_2, IP for dilator PGI_2, FP for constrictor $PGF_{2\alpha}$, and TP for constrictor TxA_2. EP_4 and IP receptors signal through $G_{\alpha s}$-proteins and cAMP/protein kinase A (PKA) cascade to relax VSMC. Under resting conditions, vascular endothelial cells primarily produce PGI_2. Descending vasa recta have dilatory IP and EP_4 receptors. TP receptors favor platelet aggregation and are vasoconstrictors, stimulating $G_{\alpha q/11}$proteins to activate PLC. However, PGE_2 can have

a vasoconstricting effect via EP1 and EP3 receptors (see Table I).

Vasoconstrictors such as Ang II and ET increase $[Ca^{2+}]_i$ in endothelial cells as well as in VSMC. The $[Ca^{2+}]_i$ increase stimulates PLA_2 to release arachidonic acid, the rate-limiting step in eicosanoid production. Vasodilator COX metabolites (PGE_2 and PGI_2) are important in limiting the degree of vasoconstriction and thus maintaining adequate renal blood flow during low-salt diet and pathophysiological conditions such as heart failure and chronic renal failure. Production of vasodilating prostaglandins is increased by chronic salt restriction, an effect presumably mediated in part by high levels of Ang II and continuous elevation of $[Ca^{2+}]_i$ in VSMC. COX inhibition increases the vasoconstrictor effect of Ang II. The enhanced vasodilator effect of endogenous PGs during the course of a low-sodium diet is due to a combination of Ang II stimulation of PG production and apparent up-regulation of EP_4 receptors. A greater density of EP_4 receptors also may contribute to elevated renin release during extracellular volume contraction.

ARACHIDONIC ACID-CYT-P450 METABOLITES

In renal endothelial cells, VSMCs, and tubule cells, arachidonic acid metabolism by Cyt-P450s generates EETs, diHETEs, and 20-HETE, substances that are autocrine agents and second messengers active in the kidney [29,40–46]. These agents are lipophilic, bind to proteins, and partition into phospholipids. Their actions may be independent of conventional receptors. Large cortical arteries produce 20-HETE via the Cyt-P450-4A hydroxylase gene family. Smaller arterioles produce a combination of vasodilator EETs and constrictor 20-HETE. The metabolites 5,6-EET, 11,12-EET, and 14,15-EET are derived from the P450-2C epoxygenase family. Vasodilator 11,12-14,15-EETs are primarily produced in the renal cortex, whereas 20-HETE is preferentially synthesized in the medulla, especially the TAL.

Endothelium-Dependent Hyperpolarizing Factor

In response to stimulation by bradykinin or acetylcholine, endothelial cells release a vasodilating agent distinct from prostaglandins and NO. Endothelial-derived hyperpolarizing factor (EDHF) [29,45,46] is a diffusible factor that relaxes VSMC by hyperpolarization caused by activation of high-conductance, Ca^{2+}-dependent K^+ channels or by propagation of the endothelial-cell hyperpolarization via myoendothelial gap junctions. Recent evidence implicates 11,12-EET and 14,15-EET as EDHFs, with greater relaxing effects in the microcirculation than in conduit arteries.

Heme Oxygenases/Carbon Monoxide System

Microsomal heme-oxygenase (HO) [47,48] catalyzes the metabolism of heme to CO, biliverdin, and free iron. Two isoforms, HO-1 and HO-2, can be expressed in the kidney. Constitutive HO-2 is ubiquitous, present in the renal vasculature and in almost all nephron segments in both the cortex and medulla. Under basal conditions, inducible HO-1 appears to be at low levels or absent from renal structures. CO contributes to the renal vascular reactivity by acting as a vasodilator agent counteracting the vasoconstriction produced by Ang II, catecholamines, or pressure-induced myogenic tone. CO activates calcium-sensitive K^+ channels and increases the membrane potential of VSMC, therefore reducing Ca^{2+} entry. Little is known about the effects of endogenous CO on tubule transport and renal excretion of salt and water. Oxidative stress and injury, as well as chronic activation of AT_1 receptors by Ang II or V_1 receptors by vasopressin (AVP), induce HO-1 mRNA by Ca^{2+}- and perhaps PKC-dependent mechanisms.

Endothelial Cell Connections: Connexins and Gap Junctions

Electrical signals are transmitted along thin sheets of endothelial cells as well as to the surrounding musculature [49]. Gap junctions (GJs) underlie this communication. GJs are cell-to-cell channels permeable to molecules up to 1000 Da, thus allowing for the rapid exchange of water, ions, and other solutes between adjacent cells. GJs are formed by membrane proteins called connexins (Cx), of which about 20 isoforms appear to exist in humans. Cx37, -40, and -43 (number denotes approximate molecular weight in kilodaltons) are expressed in vascular endothelial cells. Cxs can form homomeric or heteromeric channels with different gating and permeability properties. GJ-mediated intercellular communication is stimulated by hypoxia and inhibited by low intracellular pH. PKC-mediated Cx phosphorylation reduces gap-junctional communication. Myoendothelial GJs render endothelial cells and VSMC into a coordinated functional unit. Little is known about gap junctions and their function in the renal vasculature. Cx40 and Cx43 are the main isoforms in VSMC, and Cx40 predominates in the renal preglomerular vasculature. Gap junctional communication between afferent arterioles from different glomeruli can occur via a common interlobular artery.

Summary

Vascular endothelial cells play an important role in regulating renal vascular resistance as well as ion and water transport by the renal tubules. Endothelial cells produce vasodilator and vasoconstrictor agents whose actions are finely balanced to regulate extracellular fluid homeostasis and blood pressure. Under physiological conditions, endothelium-derived vasodilators such as PGI_2, nitric oxide, carbon monoxide, and EDHF counteract the renal vasoconstrictor effects produced by circulating agents such as vasopressin, and norepinephrine, and local, as well as circulating, angiotensin II. In disease states such as congestive heart failure, chronic renal disease, and hypertension, the vascular endothelium can produce large amounts of constrictor

substances such as ET and TxA_2. Our understanding of cell–cell connections between endothelial and VSMC, as well as the paracrine influences of vascular cells on tubular cells, is still quite limited.

Vascular Smooth Muscle Cells

VSMC encircle endothelial cells and contract to regulate the blood-vessel diameter and thereby its resistance to blood flow. Contraction and relaxation of VSMC in interlobular arteries and in afferent and efferent glomerular arterioles are the primary determinants of total renal vascular resistance. In addition to circulating hormones and nerve activity, paracrine/autocrine factors regulate the contractile function of VSMC of arteries, arterioles, and mesangial cells (cells of smooth muscle origin located between the glomerular capillary loops.) Vasomotor tone is regulated by communication between endothelial cells and the contractile VSMC. Just before an afferent arteriole enters a glomerulus, there is a short segment of juxtaglomerular granular cells responsible for the production, storage, and release of renin and Ang II. Renin release depends on signals from the macula densa cells of the TAL, activity of sympathetic nerve terminals, and baroreceptor-like effects of luminal pressure directly on juxtaglomerular granular cells (see Fig. 1, bottom panel).

Vasoconstrictor Mechanisms

The VSMC of renal microvessels respond to local paracrine factors in addition to circulating hormones and sympathetic nerve activity [3,5,23–26]. Contraction of VSMC is regulated by physical and chemical factors, with multiple signal transduction pathways coupling agonist-receptor interactions to $[Ca^{2+}]_i$, followed by activation of the actin-myosin contractile machinery. G-protein-coupled receptors play a prominent role in these processes. Heterotrimeric G-proteins transduce stimulatory or inhibitory signals to the cell interior. Table I shows a list of representative cell-surface receptors found in afferent and efferent arterioles. Ligand binding to the receptor triggers the intracellular signals, inducing changes in vasomotor tone and also causes receptor inactivation by phosphorylation (G-protein-coupled receptor serine/threonine protein kinases), thus limiting the duration of the effect of the agonist. Most of the receptors mediating vasoconstriction couple primarily with $G_{\alpha q/11}$ to activate PLC, form inositol trisphosphate (IP_3) and diacyl glycerol (DAG), and thereby elevate $[Ca^{2+}]_i$ and activate protein kinase C (PKC). Rapid increases in $[Ca^{2+}]_i$ are mediated by mobilization from internal stores and more sustained elevations result from increased Ca^{2+} entry via plasma membrane ion channels. The Ca^{2+} sensitivity of the contractile machinery of VSMC is enhanced by activation of PKC and Rho kinase. A variety of cell-surface receptors for vasoconstrictor agents are phosphorylated and inactivated or desensitized indirectly by PKC-mediated phosphorylations.

Vasodilator Mechanisms: Responses to Endothelium-Derived Agents

EICOSANOIDS

Vasoconstrictor agents may elicit weak vasodilation mediated by $[Ca^{2+}]_i$ stimulation of PLA_2 to form prostanoids and of NOS to produce NO [27,38,39,50–52]. Dilator PGE_2 and PGI_2 activate EP_4 and IP receptors, respectively. Both receptors are coupled to $G_{\alpha s}$-proteins and the cAMP-PKA signaling pathway. Prostanoids and NO exert similar effects on afferent- and efferent-arteriole VSMC. 5,6-EET appears to elicit vasodilation secondary to formation of the COX metabolites PGI_2 and PGE_2. Bradykinin and acetylcholine are thought to produce vasodilation due to their ability to produce endothelial-derived EET and di-HETE (or EDHF) in addition to stimulating COX and NOS.

NITRIC OXIDE AND CARBON MONOXIDE

The gases NO and CO [27,31,33,47,52] reduce vasomotor tone by cGMP-dependent and cGMP-independent mechanisms that ultimately reduce $[Ca^{2+}]_i$ in VSMC. NO and CO rapidly permeate plasma membranes and bind to the heme moiety of soluble (no membrane tether) cytosolic guanylyl cyclase to form a heterodimer of α and β subunits that catalyze production of cGMP.

cGMP/PKG reduce IP3-mediated Ca^{2+} release and stimulate the sarcoplasmic-reticulum Ca^{2+}-ATPase. Both effects lower $[Ca^{2+}]_i$, reducing smooth muscle contraction. PKG also inhibits a cGMP-dependent phosphodiesterase responsible for catabolism of cAMP, thus contributing to the reduction of $[Ca^{2+}]_i$. A third vasodilatory mechanism is NO inhibition of Cyt-P450-mediated production of the vasoconstrictor 20-HETE. In disease states, NO is involved in the generation of vasoconstricting agents such as superoxide radicals and other reactive oxygen species.

Vasoactive Paracrine/Autocrine Agents Produced by Smooth Muscle Cells: Cyt-P450 Metabolites

EETs and HETEs can exert dilator and constrictor actions respectively on the VSMC and, hence, can have opposing effects on tubule transport [40–44]. Vasoconstrictors such as Ang II and ET increase vascular production of 20-HETE that reinforces agonist-induced vasoconstriction by receptor-mediated increases in $[Ca^{2+}]_i$ and activation of PLA_2, with 20-HETE formation via Cyt-P450 ω-hydroxylation of arachidonic acid. 20-HETE produces vasoconstriction due to increased $[Ca^{2+}]_i$ and reduces the open-state probability of a Ca^{2+}-activated high-conductance K^+ channel, causing depolarization and activation of voltage-gated Ca^{2+} channels.

Summary

VSMC contract and relax in response to a host of vasoactive factors to change the diameter of the interlobular arteries and glomerular arterioles, the major renal resistance vessels. The balance of afferent and efferent arteriolar tone determines

glomerular capillary pressure and in turn glomerular filtration rate. In a regulated fashion, tubular reabsorption returns between 90% and 99% of the filtered fluid and solutes to peritubular capillaries and vasa recta and eventually the systemic circulation. Most of the constrictor agents such as Ang II, vasopressin, and norepinephrine activate specific G-protein-coupled cell-surface receptors leading to activation of a phospholipase C that elicits IP3 and DAG signals that lead to increased intracellular [Ca^{2+}] and stimulation of PKC. Vasodilator agents such as PGE_2 and PGI_2 activate specific receptors linked to the cAMP/PKA signaling pathway and reductions in intracellular [Ca^{2+}]. On the other hand, the vasodilators nitric oxide and carbon monoxide exert their relaxing effects on VSMC through a cGMP/PKC pathway, as well as mechanisms independent of cGMP. Dilator EETs and constrictor HETEs act by stimulating or inhibiting K^+ channels, respectively, to change membrane potential and thus the activity of L-type Ca^{2+} channels.

Tubulovascular Interactions: The Juxtaglomerular Apparatus

The juxtaglomerular apparatus (Fig. 1, bottom panel) [3–5,53–55] is a structural and functional unit in which paracrine signals are transmitted between the macula densa cells (differentiated tubule cells at the end of the TAL), the extraglomerular mesangial cells (interposed between the glomerular arterioles and the macula densa), the arteriolar VSMC, and the renin-producing granular cells in the afferent arteriole.

Renin Release: Juxtaglomerular Granular Cell Signaling

Vascular and Neural Control of Renin Secretion

Granular cells at the end of the afferent arteriole synthesize and release renin by a process regulated by local changes in arteriolar hydrostatic pressure and cell stretch (or tension) and β-adrenoceptor stimulation by norepinephrine released from perivascular sympathetic nerve terminals [20,56–60]. Increased afferent arteriolar pressure inhibits renin release, an effect presumably due to stretch of JGA cells causing an increase in Ca^{2+} influx that, in contrast with the effects in most secretory cells, inhibits renin secretion. β-Adrenoceptors activate a $G_{\alpha s}$-protein linked to the cAMP-PKA messenger pathway, which stimulates renin secretion.

Circulating hormones and paracrine factors also influence renin secretion, some of them acting via the cAMP/PKA signaling pathway. Endothelium-derived PGE_2 and PGI_2 and β-adrenergic receptor agonists are potent stimuli of renin secretion. Dopamine stimulates renin release from granular cells via D_1 receptors and cAMP generation. Interactions such as Ang II with AT_1 receptors, AVP with V_1 receptors, or ET with ET_A receptors activate a $G_{\alpha q/11}$-protein, $[Ca^{2+}]_i$ increases, and PKC is activated, causing a decrease in renin secretion. It is not clear whether the key signal is increased $[Ca^{2+}]_i$ by itself, Ca^{2+} activation of Cl^- channels and a fall in $[Cl^-]_i$ per se or via the depolarization associated with Cl^- efflux, or increased PKC activity.

NO has biphasic effects on granular cells. Acute increases are inhibitory, whereas long-term exposure stimulates renin release. In this regard, endothelial NO appears to have a tonic effect, mediated by the ability of cGMP to elevate cAMP by inhibiting phosphodiesterase and thereby cAMP breakdown. Under certain conditions, NO can inhibit renin release via cGMP-dependent protein kinase (PKG) activity. In contrast, NO generated from nNOS in TAL and macula densa cells does not mediate changes in renin secretion in individuals on a low-sodium diet and renin secretion is normally regulated in eNOS-deficient mice.

Macula Densa Control of Renin Release: Arachidonic Acid Metabolites

The macula densa participates in the regulation of renin release from juxtaglomerular granular cells [38–41,61]. Renin secretion depends on NaCl delivery to and reabsorption by the macula densa cells at the end of the TAL. Inhibition occurs when solute delivery to this section of the renal tubule is high, and stimulation is associated with low solute delivery. Renin release leads to increased concentration of Ang I and Ang II in the adjacent interstitial compartment as well as in the systemic circulation. This is a regulatory mechanism mediated by macula densa metabolites of inducible COX II and Cyt-P450 enzymatic pathways. The COX II metabolites PGE_2 and PGI_2 stimulate cAMP and PKA, thereby enhancing renin secretion. COX II expression can be induced by either chronic sodium restriction or by inhibition of TAL NaCl reabsorption by furosemide. In contrast, the Cyt-P450 metabolite 20-HETE inhibits renin secretion, presumably by elevating $[Ca^{2+}]_i$ in effector cells.

Macula Densa Control of Renin Release: Purinergic Agents

Macula densa cells may signal granular cells to inhibit renin release by secreting adenosine and/or ATP in response to increased sodium delivery [56,62]. The precise mechanism of this effect is not clear. Adenosine stimulates granular cell A_1 and A_2 receptors. A_1-receptor activation inhibits renin secretion via stimulation of PCL, by increasing $[Ca^{2+}]_i$ and reducing cAMP/PKA. As stated earlier, this is an exception in secretory cells. Conversely, A_2 receptors stimulate renin secretion via cAMP/PKA signaling.

Summary

The juxtaglomerular apparatus has unique anatomical and functional properties. Macula densa cells at the end of the thick ascending limb of Henle's loop respond to low lumen [NaCl] by signaling to juxtaglomerular granular cells at the end of the afferent arteriole to increase renin secretion. Other stimuli for renin secretion are high sympathetic nerve activity, low systemic and afferent-arteriole pressure, and

increased PGE_2. High rates of NaCl reabsorption by macula densa cells inhibit renin secretion. The signal mediating the functional connection between macula densa cells and afferent-arteriolar granular cells has been elusive. Attractive candidates are adenosine and ATP.

Tubulovascular Interactions: The Juxtaglomerular Apparatus and Tubuloglomerular Feedback

Tubuloglomerular feedback (TGF) [3,53,63], a process mediated by the juxtaglomerular apparatus (Fig. 1, bottom panel), is the direct relationship between the NaCl concentration in the tubule fluid at the macula densa and the capillary pressure and filtration rate of the same nephron. In functional terms, an increase in distal delivery of lumenal NaCl produces contraction of the afferent arteriole and a decrease in glomerular filtration.

The kidney regulates RBF and GFR during changes in arterial pressure by an intrinsic mechanism consisting of adjustments in preglomerular vascular resistance. This is referred to as *autoregulation* and is mediated by two mechanisms. First, a myogenic response intrinsic to the afferent-arteriole VSMC. These cells contract in response to the increase in tension of the wall when stretched by the higher blood pressure. Second, TGF. Primary increases in arterial pressure increase glomerular filtration and fluid delivery to the TAL, where the macula densa cells respond by sending a vasoconstricting signal to the afferent-arteriole VSMC. The nature of the signal mediating TGF is uncertain. Current postulates include ATP, adenosine, and other factors. As NaCl transport by the macula densa cells increases, so does $[Ca^{2+}]_i$, which can stimulate PLA_2 and nNOS and also elicit ATP release from mitochondria. The Cyt-P450 metabolite 20-HETE and NO may also play roles in adjusting preglomerular tone, and extraglomerular mesangial cells may be also involved. These agents may act as either mediators or modulators. For example, NO generated by macula densa nNOS acts as a modulator of TGF responsiveness as it inhibits macula densa NaCl reabsorption at high tubule flow rates, compared to little or no effect when [NaCl] at the macula densa is low. NO is thought to act directly on macula densa cells suppressing release of a constrictor agent, rather than diffusing to the afferent arteriole. Also, NO produced at upstream nephron sites may contribute to inhibition of TGF due to its ability to inhibit NaCl transport by macula densa cells. On the other hand, nNOS deficit in transgenic animals does not affect TGF.

It is clear that Ang II does not mediate TGF. An increase in renin release and Ang II formation occurs when NaCl delivery to the macula densa is low and the preglomerular vessels dilate, not contract. However, Ang II modulates TGF sensitivity and glomerular vascular reactivity by a mechanism that remains elusive. Other vasoconstrictors, such as norepinephrine and ET, have little effect on TGF. An inverse relationship exists between the degree of TGF and chronic salt intake. High renin and Ang II levels are associated with strong TGF during salt restriction. The attenuated TGF during a high-salt diet is attributable to enhanced NO production and low Ang II levels.

The involvement of eicosanoids in TGF is uncertain. COX II is present in macula densa and TAL cells; its expression is enhanced during chronic sodium restriction and COX II metabolites diminish the vasoconstriction accompanying increased NaCl delivery. However, TGF is normal in animals null for COX II or thromboxane receptors.

Purine Nucleotides and Purinoceptors: Role in Tubuloglomerular Feedback

The purinergic agents adenosine and ATP can be released from epithelial cells into the interstitial compartment, providing a metabolic link between epithelial cell NaCl transport and preglomerular resistance [62,64–67]. As load-dependent reabsorption of NaCl by the macula densa increases, so does ATP hydrolysis and hence adenosine production. Adenosine diffuses to the afferent arterioles to activate vascular P_1 (adenosine) receptors and thereby elicit vasoconstriction. ATP can be released by exocytosis from nerve terminals or may exit from endothelial cells, VSMC, and epithelial cells, probably through membrane channels, to activate P_2 (ATP) receptors.

Extracellular adenosine acts on P_1-A_1 purinergic receptors that respond to AMP but not to ADP or ATP. P_1-A_1 receptors couple to $G_{\alpha i}$-proteins and decrease cAMP/PKA activity in VSMC. In epithelial and endothelial cells, P_1-A_1 receptors couple to $G_{\alpha q/11}$, stimulating PLC signaling, increasing both $[Ca^{2+}]_i$ and PKC, and activating Ca^{2+}-dependent eNOS in endothelial cells. Afferent arterioles have more P_1-A_1 receptors than efferent arterioles. Renal P_1-A_2 receptors linked to $G_{\alpha s}$-proteins stimulate adenylyl cyclase to activate the cAMP/PKA pathway. P_1-A_2 receptor stimulation by adenosine causes vasodilation of both afferent and efferent arterioles and a natriuresis without a change in the filtered sodium load. Adenosine has a greater affinity for P_1-A_1 receptors. Therefore, low adenosine concentrations elicit vasoconstriction, whereas high concentrations produce vasodilation. A_1-receptor antagonists attenuate TGF activity, as do mutations of A_1 receptors. In the medulla, adenosine activation of epithelial A_1 receptors appears to be antinatriuretic, compared to A_2-receptor-mediated increase in medullary blood flow and natriuresis. The balance between P_1-A_2 vasodilating receptors and P_1-A_1 vasoconstricting receptors varies with salt intake. P_1 receptor stimulation is more effective in animals maintained on a low-salt diet. In certain pathological conditions, Ang II and adenosine are synergistic, that is, Ang II enhances the vasoconstrictor response to adenosine and vice versa.

ATP can be released from nerve terminals, endothelial cells, VSMC, and epithelial cells and acts locally to produce vasoconstriction. Extracellular ATP and ADP preferentially activate P_2 receptors, which have less affinity for adenosine or AMP. P_2 receptors are present on preglomerular vessels (endothelial cells and VSMC), glomerular mesangial cells, and tubule cells (PCT and CD). The P_{2x} and P_{2y} subtypes are

the most common. P_{2x} receptors are ligand-gated ion channels with two membrane-spanning domains. In preglomerular VSMC, P_{2x} receptors depolarize the plasma membrane by inhibiting K^+ channels and triggering Ca^{2+} entry through voltage-gated L-type Ca^{2+} channels. ATP activation of P_{2x} receptors activates Cyt-P450 production of 20-HETE. P_{2y} receptors are classic $G_{\alpha q/11}$-protein-coupled receptors. Endothelial P_{2y} receptors are coupled to $G_{\alpha q/11}$-proteins that activate PLC to mobilize Ca^{2+} from sarcoplasmic reticular stores and stimulate PKC. Ca^{2+}-dependent eNOS and COX I produce the vasodilators NO and PGI_2. Intra-arterial administration of ATP elicits variable responses. The effects of circulating ATP on the renal vasculature involve vasodilator receptors on endothelial cells and vasoconstrictor P_{2x} receptors on VSMC. When the vasodilating component (P_{2y} receptors) is eliminated by inhibition of NO production, then the net response becomes vasoconstriction (P_{2x} receptors). ATP constricts the afferent arteriole considerably more than the efferent arteriole. Intracellular ATP can modulate vascular resistance by regulating ATP-sensitive K^+ channels; high ATP levels lead to VSMC hyperpolarization and vasodilatation.

Intrarenal Angiotensin II Production, Storage, and Actions

Circulating Ang II is formed by the action of angiotensin converting enzyme (ACE) on plasma Ang I in the lungs and the renal vasculature [56–58,66,68,69]. Ang I can be generated from angiotensinogen by renin and other proteolitic enzymes, for example, chymase and catepsin. Recent evidence suggests important formation, storage, and actions of intrarenal Ang II. All necessary substrates and enzymes for Ang II production are present in juxtaglomerular granular cells and in PCT cells. ACE lines endothelial cells and luminal and basolateral membranes of PCT cells. About 20% of circulating inactive Ang I is converted to biologically active Ang II in the kidney. The PCT can produce Ang II from circulating Ang I, concentrates Ang II by AT_1-receptor-mediated uptake, and stores it in the cells. Ang II is secreted into the lumen, where it can act locally or downstream, binding to luminal-membrane receptors in TAL, DCT, and CD. The effects are to increase Na^+, Cl^-, and HCO_3^- reabsorption.

There are two classes of Ang II receptors. AT_1 receptors, the predominant if not exclusive class under normal conditions, are present along the renal vasculature, including juxtaglomerular granular cells, glomeruli and vasa recta, multiple nephron segments (PCT, TAL, DCT, CD), and medullary interstitial cells. AT_1 receptors couple to $G_{\alpha q/11}$-proteins, PLC and the classical IP_3-Ca^{2+} and DAG-PKC pathways to cause vasoconstriction and decrease salt excretion.

Elevated Ang II levels, as seen with chronic salt restriction, constrict both afferent and efferent arterioles, reducing the renal blood flow (RBF) more than GFR. High Ang II concentrations desensitize the blood vessels by reducing the AT_1 receptor density and thus reducing the chronic effects of increased levels of the vasoconstrictor. In contrast, high endogenous Ang II concentration up-regulates tubule AT_1 receptors, favoring Na^+ retention. AT_1 receptors on granular cells exert short-loop feedback inhibition of renin release in association with increased $[Ca^{2+}]_i$ and PKC activation. Stimulation of AT_1 receptors activates the Ca^{2+}-dependent enzymes PLA_2 and NOS in endothelial cells, promoting production of prostanoids and NO. AT_1 receptors stimulate Na^+-H^+ exchange in PCT and TAL. Renal vascular AT_2 receptors are rare in a healthy, adult kidney. They may be up-regulated during conditions of a low-salt diet, with vascular and tubule functions opposite those of the predominant AT_1 receptors.

Vascular Actions of Bradykinin

Intrarenal bradykinin has vascular actions in the cortex and medulla mediated primarily, if not exclusively, by B_2 receptors [3,33,70]. The main action of bradykinin, vasodilatation, is mediated by a predominance of B_2 receptors on endothelial cells, which trigger PLC and increase $[Ca^{2+}]_i$ to stimulate eNOS activity and perhaps release EDHF/EET. A smaller population of constrictor B_2 receptors coupled to $G_{\alpha q/11}$ reside on VSMC.

Summary

Tubuloglomerular feedback is an adaptive mechanism that links the rate of glomerular filtration to the concentration of salt in the tubule fluid at the macula densa. A high [NaCl] at this level causes contraction of the afferent arteriole and a reduction in GFR. This autoregulatory mechanism is intrinsic to the kidney; that is, it does not require neural or humoral agents. The nature of the signal is still controversial. It could well involve adenosine or ATP. A renin-Ang II system exists within the kidney, with substrates and enzymes localized to proximal tubular cells in addition to juxtaglomerular granular cells. The major effects of local and circulating Ang II are vasoconstriction and Na^+ retention, both of which are primarily mediated by AT1 receptors.

Vasculotubular Communication

Receptors in the basolateral membranes of renal-tubule cells convey chemical information from the blood, vascular cells, and interstitial cells. Interactions between extracellular matrix and cell functions are beginning to be understood. In general, vasodilating agents inhibit Na^+ reabsorption by one or more nephron segments. NO, PGE_2/PGI_2, dopamine, and bradykinin conform to this oversimplified notion. Vasoconstrictors such as Ang II and norepinephrine usually stimulate Na^+ reabsorption. Notable exceptions are ET and 20-HETE, both of which are natriuretic. Paracrine agents produced by vascular cells exert actions on tubule cells by receptor-mediated events such as those elicited by agents produced by epithelial cells. (To minimize duplication, paracrine control of carrier proteins is discussed later under the heading of Tubule–Tubule Communication.)

Pressure Natriuresis

The mechanisms by which acute changes in arterial pressure influence tubule transport and Na^+ excretion are incompletely understood [3,6]. Increased vascular hydrostatic pressure may release NO and PGE_2 from endothelial cells. Blood flow in the inner medulla is more sensitive to changes in arterial pressure than cortical blood flow, and increased medullary blood flow is thought to elevate capillary and interstitial hydrostatic pressure and decrease colloid-osmotic pressure. Together, these changes cause a reduction in net salt and water reabsorption along the loops of Henle and collecting ducts.

Tubule–Tubule Communication: Paracrine Agents Released from Epithelial Cells

Paracrine Control of Solute Carrier Proteins

The major nephron segments produce and release autocrine and paracrine factors that modulate transepithelial solute and water transport [10,13,38,39,71,72]. The main transport mechanisms for solutes and water are listed in Table II. The PCT produces dopamine, NO, and Ang II; the TAL synthesizes ET, 20-HETE, NO, and PGE_2; the DCT generates kallikrein 20-HETE, NO, and COX II metabolites; and the CD produces ET, NO, and PGE_2. All renal-epithelial cells consume ATP and produce adenosine and purine nucleotides.

Angiotensin II

Ang II exerts important effects on water and electrolyte transport by indirect and direct mechanisms [15,44,58,73]. Its main indirect effect is the stimulation of secretion of the sodium-retaining hormone aldosterone by the adrenal cortex. Ang II directly stimulates NaCl reabsorption in PCT, TAL, DCT, and CD. In the PCT this effect is mediated by activation of AT_1 receptors expressed on the basolateral membrane. Receptor activation inhibits the cAMP/PKA pathway via $G_{\alpha i}$-protein and stimulates IP3 and PKC formation via $G_{\alpha q/11}$-protein. Ang II is synthesized in the PT and secreted to the lumen, reaching concentrations 100- to 1000-fold higher than in the systemic concentration ($<10^{-12}$ *M*). The effects of Ang II on the PCT are dose dependent: Physiological concentrations stimulate salt and fluid reabsorption, whereas pharmacologically high concentrations are inhibitory. The stimulatory effect in the PCT is mediated by activation of several target transport proteins: In the apical membrane, the Na^+/H^+ exchanger; in the basolateral membrane, the Na^+-K^+/ATPase, the Na^+/HCO_3^- cotransporter, and the K^+ channels all transport proteins that coordinately account for Na^+ reabsorption. The inhibitory effect of high levels of Ang II is mediated by binding to AT_2 receptors, resulting in PLA_2-mediated AA release. In the TAL, low Ang II concentrations ($<10^{-12}$ *M*) stimulate 20-HETE production and inhibit Na^+ reabsorption by inhibiting the apical-membrane K^+ channel, an effect that decreases K^+ recycling and K^+ availability to the Na^+-K^+-$2Cl^-$ cotransporter. $NaHCO_3$ transport in this segment is also inhibited by Ang II (10^{-8} *M*) via a mechanism that can be inhibited by Cyt-P450 blockade and by inhibitors of AA metabolism and by intracellular events that are not yet understood. Higher Ang II concentrations stimulate AT_1 receptors, elevating PLC and PKC activities and stimulating the Na^+-K^+-$2Cl^-$ cotransporter in the apical membrane. There are additional binding sites for Ang II in the DCT and cortical and medullary CD consistent with an action of Ang II in these segments. A significant effect of Ang in the distal segments is demonstrated by studies of

Table II Major Renal Epithelial Proteins Responsible for Transport of NaCl, Water and Urea and Regulated by Hormones and Paracrine/Autocrine Agents

Tubule segment	Apical membrane	Basolateral membrane
Proximal Tubule	Na^+-H^+ exchanger (NHE3) Na^+-glucose cotransporters (SGLT1, 2)	Na^+-K^+-ATPase Na^+-$3HCO_3^-$ cotransporter (NBC1)
Descending Limb of Henle's Loop	None	None
Thick-Ascending Limb of Henle's Loop	Na^+-K^+-$2Cl^-$-cotransporter (NKCC2) K^+ channel (ROMK, Kir 1.1) Na^+-H^+ exchanger (NHE3)	Na^+-K^+-ATPase Cl^- channel (CLC-K2)
Distal Tubule	Na^+-Cl^--cotransporter (NCC) Na^+-H^+ exchanger (NHE) Na^+ channel (ENaC) K^+ channel (ROMK, Kir 1.1) H^+-K^+-ATPase	Na^+-K^+-ATPase Cl^- channel (CLC-K1)
Collecting Duct (Principal cell)	Na^+ channel (ENaC) Aquaporin (AQP2) Urea transporter (UT-1) K^+ channel (ROMK, Kir 1.1)	Na^+-K^+-ATPase
(Intercalated cell)	H^+-ATPase Cl^--HCO_3^- exchanger Na^+-H^+ exchanger	Na^+-K^+-ATPase

knock-out mice for tissue ACE; the mice exhibit a defect in urine concentration that is concomitant with a decrease in transport proteins (UT-A, CIC-K1, NKCC2/BSC1, and AQP1).

In summary, Ang II modulates water and electrolyte transport indirectly, via the stimulation of aldosterone secretion, and directly by stimulating NaCl reabsorption in PCT, TAL, DCT, and CD. In the PCT this activation is mediated by AT_1 receptors that inhibit the cAMP/PKA pathway via $G_{\alpha i}$-proteins and stimulate IP3 and PKC formation via $G_{\alpha q/11}$-protein. In the PCT, physiological concentrations of Ang II stimulate salt and fluid reabsorption, whereas in pharmacological concentrations, high concentrations inhibit; the latter effect is mediated by AT_2 receptors. In the TAL, low concentrations of Ang II stimulate production of 20-HETE and inhibit Na^+ reabsorption by intracellular mechanisms that seem to differ for the different Na^+ transporters in the segment. At high concentrations, Ang II, elevates PLC and PKC activities and stimulates the Na^+-K^+-$2Cl^-$ cotransporter in the apical membrane. Effects in more distal segments are suspected from the existence of Ang II receptors in the cells of these segments and studies in ACE knock-out mice.

Dopamine

Dopamine [74–77] is an intrarenal hormone or paracrine agent that elicits vasodilation and natriuresis. Renal dopamine is generated mainly in the PCT (from L-dopa by the action of aromatic l-amino acid decarboxylase) and secreted across both apical and basolateral membranes, to the lumen and the blood, respectively. Renal nerves are an additional, minor source of dopamine. During salt loading, dopamine production and excretion are increased in parallel with sodium excretion.

Dopamine receptors are classified as D_1-like and D_2-like receptors. Both classes have been identified in the kidney: D_1-like receptors comprise D_1 and D_5. D_2-like receptors comprise D_2, D_3, and D_4. D_1 receptors predominate in the vasculature (arcuate arteries, interlobular arteries, afferent and efferent arterioles), which also expresses D_2-like receptors (D_3 and D_4). Both D_1-like and D_2-like receptors are present in the renal tubule cells. PTC cells express predominantly D_1, but also D_5 and also D_3. The mTAL expresses D_5. The CCD, expresses predominantly D_5 and less D_1, D_3, and D_4. The overall effect of dopamine is inhibition of Na^+ reabsorption. Activation of D_1-like receptors stimulates both the cAMP/PKA and the PLC pathways, with downstream activation of the IP3-Ca^{2+} and DAG-PKC signaling pathways. Both paths inhibit the Na^+-K^+/ATPase. D_2-like receptors inhibit cAMP/PKA production, which stimulates the luminal Na^+-H^+ exchanger and the basolateral Na^+-K^+/ATPase. Thus, D_1-like and D_2-like receptor actions on salt reabsorption oppose each other, but in some instances D_2 receptors enhance D_1 receptor effects, perhaps by shifting the effect of D_2 from inhibition of adenylcyclase to stimulation of phospholipase A_2, resulting in an increase of AA production. Nevertheless, the dominant effect of dopamine is to reduce Na^+ reabsorption. An additional inhibitory effect on the apical-membrane Na^+-H^+ antiporter in the PCT has been reported, which also contributes to the natriuretic effect [76]. In the TAL, D_1 receptors inhibit the Na^+-H^+ exchanger and the basolateral Na^+-K^+/ ATPase, with weaker stimulation of Na^+-K^+-$2Cl^-$ cotransport via the cAMP/PKA pathway. The net effect is a decrease in Na^+ transport due to the predominance of the inhibition of the Na^+ pump. Finally, D_1 receptors may also inhibit Na^+ reabsorption in the CCD, perhaps by antagonizing the stimulatory effects of aldosterone as well as inhibiting the Na^+-K^+/ATPase.

In summary, renal dopamine is generated mainly in the PCT and can exert effects on more distal segments of the nephron. During salt loading, dopamine production and excretion increase in parallel with Na^+ excretion. Activation of D_1-like receptors in the PCT results in stimulation of both cAMP/PKA signaling and PLC activity, with downstream activation of the IP_3-Ca^{2+} and DAG-PKC signaling pathways resulting in inhibition of Na^+-K^+/ATPase. D_1 receptor stimulation also inhibits the Na^+-H^+ exchanger and the Na^+-K^+/ATPase. Inhibition of the Na^+ pump may also account for the natriuretic effect of dopamine in the mTAL and the CD.

Nitric Oxide

The primary action of endogenous NO [31,63,71,78–80] on renal-tubule cells is to inhibit Na^+ reabsorption, causing natriuresis and diuresis. The systemic inhibition of NOS results in Na^+ retention if the resulting hypertension and pressure natriuresis are pharmacologically prevented. The L-arginine/NO system inhibits solute and water reabsorption in most nephron segments, including PCT, TAL, and cortical and medullary CD. Supporting the notion that NO is a regulator operating in physiological conditions, the natriuresis associated with a chronic high-salt diet is accompanied by high levels of NOS activity and increases in NO production in the kidney and excretion in the urine.

All three NOS isoforms (type I or neuronal, type II or inducible, and type III or endothelial) are expressed in tubule cells. Endothelial NOS is present in PCT, TAL, and CD. Inducible NOS is found in PCT, TAL, DCT, and cortical and inner medullary CD. Neuronal NOS is limited to the TAL, macula densa, and CD. Ang II is a potent stimulant of NO production by PCT cells. In turn, NO inhibits the stimulatory action of Ang II on NaCl reabsorption.

A direct effect of NO on PCT transport is controversial, as both stimulation and inhibition of bicarbonate and fluid transport have been reported. However, there is agreement that NO inhibits both the luminal Na^+-H^+ exchanger and the basolateral Na^+-K^+/ATPase. NO may also influence ion transport by reducing cell ATP, an effect of potential importance in hypoxic injury. Very high [NO] inhibits the Na^+-K^+/ATPase via activation of PKC. Segment-dependent differences and perhaps concentration-dependent effects, since inhibition of cGMP analogs and of NO donors require large doses of blockers, may explain some contradictory findings reported in the literature.

NO is produced by eNOS in the TAL, where it inhibits NaCl reabsorption and thereby attenuates TGF at high tubule

flow rates. The inhibition of NaCl reabsorption is primarily by decreasing luminal-membrane Na^+-K^+-$2Cl^-$ cotransport, with a secondary inhibitory effect on the Na^+-H^+ exchanger. These effects result from stimulation of soluble guanylyl cyclase to form cGMP and consequent stimulation of phosphodiesterase II, reducing cAMP levels.

NOS isoforms are expressed in DCT, but there is no information on effects of NO on ion transport in this nephron segment. The CD produces NO, which inhibits NaCl reabsorption by principal cells by a mechanism independent of the Na^+-K^+/ATPase, probably involving mobilization of intracellular Ca^{2+} and inhibition of ENaC. At high concentrations, NO also impairs urine acidification by inhibiting the H^+-ATPase in intercalated cells. At low NO concentrations, a stimulatory pathway has been described that is mediated by small increases in cGMP that activate basolateral K^+ channels, hyperpolarizing the CD cell and increasing the driving force for Na^+ entry across the apical membrane. A stimulus of CD-NOS is AVP, acting via V_2 receptors. In turn, NO inhibits AVP-stimulated osmotic water permeability in the medullary CD via decreased cAMP secondary to activation of guanylyl cyclase and cGMP-dependent PKG.

In summary, the L-arginine/NO system inhibits solute and water reabsorption in most nephron segments, including PCT, TAL, and cortical and medullary CD. Ang II is a potent stimulus of NO production by PCT cells, and NO inhibits the stimulatory action of Ang II on NaCl reabsorption. A direct effect of NO on PCT transport is controversial; however, it is likely that NO inhibits both the luminal Na^+-H^+ exchanger and the basolateral Na^+/K^+/ATPase. In the TAL, NO inhibits NaCl transport and may thereby contribute to attenuation of TGF at high tubule-flow rates. The CD also produces NO, which inhibits salt transport in principal cells by a mechanism independent of the Na^+/K^+/ATPase, probably involving mobilization of intracellular Ca^{2+} and inhibition of ENaC.

Endothelin

The highest concentration of ET-1 in the body is in the renal medulla [35–37, 80–82]. CD cells secrete ET-1 across the basolateral membrane. The major stimulant is AVP, acting via V_2 receptors, but production of ET-1 is also elicited by Ang II, adrenaline, insulin, cortisol, IL-1, transforming growth factor-β, low-density lipoproteins, hypoxia, and endothelin itself. ET-1 synthesis is inhibited by atrial natriuretic peptide, NO, and prostacyclins.

The ET receptors ET_A and ET_B (both of them G-protein coupled) are found in the inner medullary collecting tubule cells where ET may exert an autocrine effect. ET provides acute negative feedback control of the AVP-induced osmotic water permeability in medullary CD. However, a chronic increase of interstitial osmolality may inhibit ET production/secretion. TAL and CD have ET_B receptors. ET inhibits NaCl transport in these nephron segments by increasing $[Ca^{2+}]_i$ and stimulating nNOS to produce NO, which in turn inhibits both Na^+/K^+/ATPase and ENaC at downstream sites, that is, the DCT and CD. ET is natriuretic by virtue of its action on ET_B receptors and NO production in the terminal CD. ET_B-receptor-deficient mice develop salt-dependent hypertension that is reversed by amiloride inhibition of ENaC channels in the distal nephron.

In summary, the main source of ET-1 in the renal medulla are the CD cells. ET production is elicited by AVP, Ang II, adrenaline, insulin, cortisol, IL-1, TGF-β, low-density lipoproteins, hypoxia, and endothelin itself. ET synthesis is inhibited by atrial natriuretic peptide, NO, and prostacyclins. ET is natriuretic by virtue of its action on ET_B receptors. TAL and CD have ET_B receptors. ET inhibits NaCl transport in these nephron segments by elevating $[Ca^{2+}]_i$ and nNOS to produce NO that inhibits both Na^+/K^+/ATPase and ENaC.

Eicosanoids

Arachidonic acid metabolites affect renal-tubule transport as well as renal hemodynamics [15,38,39,43]. Tubule segments containing cyclooxygenases (COX) are the cortical TAL, which expresses the inducible COX II, and the medullary TAL and CD, which express the constitutive COX I. Little is known about the lipoxygenase activity along the nephron. Cyt-P450 monoxygenases are present in PCT, TAL, and CD, where both EETs and 20-HETE are produced. The Cyt-P450-2 enzyme family in PCT and CD leads to preferential production of EETs in response to stimulation by Ang II, bradykinin, or ET. Increased levels of NO and CO inhibit Cyt-P450 activity and thus decrease production of EET and 20-HETE. A high-salt diet is associated with increased production of EET by PCT and CD and inhibition of NaCl transport. Agents that signal via cAMP/PKA, such as dopamine and PTH, increase 20-HETE formation in PCT. 20-HETE is the exception to the dictum that vasoconstrictors favor Na^+ retention; it inhibits NaCl reabsorption in both PCT and TAL, by PKC-mediated phosphorylation and inhibition of the α subunit of the Na^+-K^+/ATPase. Ang II stimulates 20-HETE production in the TAL by increasing $[Ca^{2+}]_i$ and stimulating PLA_2 to liberate arachidonic acid. In the TAL, 20-HETE inhibits NaCl reabsorption by acting on the luminal Na^+-K^+-$2Cl^-$ cotransporter and the basolateral Na^+-K^+/ ATPase, opposing the stimulatory action of AT_1 receptors. In addition to being stimulated by paracrine/autocrine factors, PGE_2 and 20-HETE production in TAL are triggered by activation of a luminal Ca^{2+}-sensing receptor that is coupled to $G_{\alpha q/11}$-proteins and induces PLA_2 activation and an increase in $[Ca^{2+}]_i$.

High PGE_2 in the inner medulla, produced locally by epithelial cells and interstitial cells, antagonizes vasopressin-stimulated salt reabsorption in the TAL and water reabsorption in the CD. The major tubule-cell PGE_2 receptors are EP_1 and EP_3, with highest densities found in TAL and CD. Activation of EP_1 receptors inhibits sodium reabsorption through a $G_{\alpha q/11}$-PLC system that increases $[Ca^{2+}]_i$ and stimulates PKC, inhibiting the basolateral Na^+-K^+/ATPase. EP_3 receptors stimulate a $G_{\alpha i}$-protein and reduce cAMP/PKA activity. EP_3 receptors inhibit NaCl reabsorption in the TAL by down-regulating the density of luminal-membrane Na^+-K^+-$2Cl^-$ cotransporters independently of $[Ca^{2+}]_i$, and in CD

by inhibiting ENaC. Thought to be of minor functional significance during physiological conditions, FP receptors sensitive to $PGF_{2\alpha}$ predominate along the DCT. They signal through PKC and perhaps also a PKC-independent Rho-mediated pathway.

In summary, the main effects of arachidonic acid metabolites are natriuresis and water diuresis. Tubule segments distal to the PT contain COX, leading to the production of PGE_2. Cyt-P450 monoxygenases are present in PCT, TAL, and CD, where both EETs and 20-HETE are produced. In PCT and CD, preferential production of EETs occurs in response to Ang II, bradykinin, or ET. Increased levels of NO and CO inhibit Cyt-P450 activity and thus decrease production of EET and 20-HETE. In the TAL, 20-HETE inhibits NaCl reabsorption by acting on the luminal Na^+-K^+-$2Cl^-$ cotransporter and the basolateral Na^+-K^+/ATPase. High concentrations of PGE_2 in the inner medulla, produced locally by epithelial cells and interstitial cells, antagonize vasopressin-stimulated salt reabsorption in the TAL and water reabsorption in the CD. Activation of PGE_2 EP_1 receptors inhibits Na^+ reabsorption through a $G_{\alpha q/11}$-PLC mechanism that increases $[Ca^{2+}]_i$ and stimulates PKC, inhibiting the Na^+-K^+/ATPase. EP_3 receptors inhibit NaCl reabsorption in the TAL by reducing the density of luminal Na^+-K^+-$2Cl^-$ transporters independently of $[Ca^{2+}]_i$ and in CD by inhibiting ENaC.

Bradykinin

Bradykinin [70,82] of renal origin is vasodilator and natriuretic. The enzyme kallikrein is produced and released from the distal convoluted tubule and connecting segment by exocytosis at both the apical and basolateral membranes, to the lumen and interstitial fluid, respectively. Secretion at this site makes the enzyme available at the site of substrate (kininogen) production, the principal cells of the CD. Bradykinin produced by the action of kallikrein on kininogen is present in both the lumen of the CD and the interstitial fluid. Renal bradykinin formation is normally low and is increased during sodium restriction and water deprivation. Bradykinin is inactivated by kininase II, the same enzyme as ACE. The B_2 receptors are expressed in mesangial cells, juxtaglomerular granular cells, TAL, cortical and medullary CD, and renomedullary interstitial cells. In the TAL, B_2 receptor activation inhibits NaCl reabsorption. In the CD, luminal bradykinin acts on B_2 receptors and inhibits both NaCl and water transport. The mechanism of natriuresis is the inhibition of ENaC, an effect probably mediated by PGE_2, although NO may have a role by modifying the local metabolism of atrial natriuretic peptide. Although absent during physiological conditions, tubule-cell B_1 receptors are induced during inflammation, primarily in efferent arterioles, PCT, TAL, and DCT.

In summary, bradykinin of renal origin is vasodilator and natriuretic. Bradykinin is produced by the action of kallikrein on kininogen and is present in both the lumen of the CD and the interstitial fluid. Renal bradykinin formation is normally low and increases during sodium restriction and water deprivation. In the CD, luminal bradykinin acts on B_2 receptors to inhibit NaCl and water transport.

Adenosine and ATP

Adenosine and ATP [62–65,67,83–85] are produced by renal-tubule cells and can affect their transport functions by autocrine or paracrine mechanisms. Purinergic receptors (P_1 and P_2) are responsible for these effects. P_1 receptors comprise four subtypes of which A_1 and A_2 are expressed in the collecting duct and A_1 in the thick ascending loop of Henle. When these receptors are activated by adenosine, they couple to different heterotrimeric G-proteins and have effects on Na^+ and water excretion. The P_2 receptors respond predominantly to ATP and can be P_{2x} (Ca^{2+}-permeable nonselective cation channels) and P_{2y} (G-protein-coupled receptor). The A_1 receptor block produces a reduction in Na^+ reabsorption and natriuresis. In cultures of PT cells, A_1 receptor activation increases Na^+-glucose and Na^+-phosphate cotransport at the luminal membrane and also increases $Na^+HCO_3^-$ transport at the basolateral membrane. Adenosine can be formed from cAMP secreted in the proximal tubule by an ectophosphodiesterase. The effects of adenosine are thought to be mediated by inhibition of adenylyl cyclase, because the natriuresis can be inhibited by a block of G_i-proteins with Pertussis toxin. In the TAL, activation of A_1 receptors decreases the transepithelial voltage and inhibits AVP-induced $NaHCO_3$ reabsorption, perhaps by reducing cAMP/PKA activity. These two effects suggest a reduction in salt reabsorption. In the CD, A_1 receptor activation decreases the hydraulic water permeability, increasing excretion of water in the urine. Activation of A_2 receptors also decreases the water permeability of the CD.

The PCT is a rich source of ATP that is secreted to the lumen and travels downstream to regulate transport in more distal nephron segments. Luminal P_{2x} and P_{2y} receptors in PCT and IMCD inhibit Na^+ reabsorption by Ca^{2+}-, PKC-, and arachidonic acid-dependent pathways. Activation of P_{2y} receptors in DCT and CD increases PLC activity and $[Ca^{2+}]_i$, resulting in increased luminal-membrane Cl^- permeability and inhibition of Na^+ reabsorption, at least in part because of the resulting apical-membrane depolarization. As mentioned earlier, adenosine and/or ATP may play important roles in TGF and autoregulation.

In summary, adenosine and ATP are produced by renal-tubule cells and can affect their transport functions by autocrine or paracrine mechanisms. Their effects are mediated by stimulation of the purinergic receptors P_1 and P_2. Adenosine is coupled to different heterotrimeric G-proteins, altering Na^+ and water excretion. The ATP produced in the PCT is secreted to the lumen and regulates transport in more distal nephron segments. Inhibition of Na^+ transport is mediated by both P_2 receptors types.

Gap Junctions

GAP junctions [86–88] are cell-to-cell channels that communicate epithelial and vascular smooth muscle cells (see earlier section). Cx43-formed gap junctions allow

permeation of hydrophilic molecules of up to 1 kDa. Functional regulation of Cx by phosphorylation is isoform dependent. In addition, GJ communication is modulated by membrane voltage, $[Ca^{2+}]_i$, and intracellular pH. GJs may coordinate oscillations of $[Ca^{2+}]_i$ and hence participate in intercellular signaling. Cells with constitutive higher sensitivity to a given stimulus may act as pacemakers, initiating a response and signaling neighboring cells by intercellular permeation of ions or second-messenger molecules.

Interstitial Cell–Tubule Communication

Renal interstitial cells occupy the space that surrounds the blood vessels and tubules [38,39,61]. In the renal cortex, the interstitial cells are stellate fibroblasts and lymphocyte-like cells. In the inner medulla, there are lipid-laden cells (located between thin descending limbs of Henle in a ladder-like appearance), cells similar to the fibroblasts and lymphocytes found in the cortex, and SMC-like pericytes that encircle descending vasa recta capillaries and act as sphincters to regulate medullary blood flow.

Cortical Interstitial Cells

Fibroblasts have the potential to differentiate to myofibroblasts containing α-smooth muscle actin and desmin, a process observed in response to inflammatory cytokines and thought to be important in the interstitial fibrosis of kidney diseases [89,90]. In response to hypoxia, cortical fibroblasts close to the outer medullary border produce erythropoietin, a glycopeptide hormone that stimulates erythropoiesis in the bone marrow, as well as the synthesis of hemoglobin. Little is known about the function of lymphocyte-like cells that reside in the cortical interstitium.

Medullary Interstitial Cells

Medullary interstitial cells [61,91–93] produce COX II metabolites, primarily PGE_2, in response to Ang II, AVP, ET, and bradykinin. These cells express an unusually high density of AT_1 and ET_A and ET_B receptors. The common signaling pathway involves $G_{\alpha q/11}$ activation of PLC and IP_3-induced mobilization of $[Ca^{2+}]_i$, which activates PLA_2. Locally produced PGE_2 causes relaxation of vasa recta pericytes and inhibits NaCl reabsorption in the TAL and medullary CD. In addition, PGE_2 can inhibit the ability of AVP to mobilize aquaporin-2 water channels to the luminal membrane and thus reduces the osmotic water permeability in the inner medullary CD.

Medullipins are lipids produced by medullary interstitial cells. An inactive form is released into the systemic circulation, markedly so after surgical relief from renal arterial stenosis. Medullipin becomes activated by a liver Cyt-P450 oxidase and acts as a vasodilating and natriuretic agent. It may also suppress sympathetic nerve activity. The physiological roles of medullipins, if any, are not understood.

In summary, cortical interstitial fibroblasts respond to hypoxia producing erythropoietin, a hormone that stimulates erythropoiesis in the bone marrow. Medullary interstitial cells produce COX II metabolites, primarily PGE_2, in response to Ang II, AVP, ET, and bradykinin. The common signaling pathway involves $G_{\alpha q/11}$ activation of PLC and IP_3-induced mobilization of $[Ca^{2+}]_i$, which activates PLA_2.

Conclusions

The kidney is a complex, highly sophisticated organ containing diverse cells types responsible for specialized functions, both in the renal vasculature and nephron segments. The structure of the kidney and the process of urine formation allow for cell-to-cell influences along single nephron segments that are distant in space. In addition, the proximity of parallel structures permits lateral communication between tubules, capillaries, and interstitial cells. These two kinds of cell-to-cell communication are central for functional integration at the single-nephron and whole-organ levels. Communication is mediated by paracrine and autocrine agents that act extracellularly or intracellularly by turning on signaling systems, thus eventually unifying homeostatic regulation of renal hemodynamics, glomerular filtration rate, tubule-transport processes, and urinary excretion. Endothelial cells regulate renal vascular resistance and reabsorption of salt and water by the renal tubules via a variety of messenger molecules, including nitric oxide, prostaglandins, EETs, 20-HETE, and endothelin. The vascular and epithelial cells regulate their own functions via the production of the autocrine agent 20-HETE.

Tubule cells produce dopamine, purine nucleotides, nitric oxide, prostanoids, and endothelin. These agents act locally to regulate the vasculature and transepithelial transport of salt and water. Despite a rapidly growing understanding of cell–cell signaling in the kidney, the wide variety of autocrine and paracrine systems and their actions on multiple cell types are not completely understood. Interactions with hormones and neural control systems add to this complexity. Current research is largely reductionist. Integrative studies are also needed, in particular, to discern the relative importance, cross-talk, redundancy, and compensatory effects of the various systems under physiological and pathophysiological conditions. A thorough understanding of these systems will be the foundation for rational and effective approaches to the treatment of renal diseases.

Acknowledgments

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CHAPTER 347

Prostate

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Introduction

The prostate is an exocrine gland with a heterogeneous epithelial and stromal cell composition. Located below the bladder, it surrounds the urethra. Its development, growth, and function are controlled by hormones. Androgens play a major role in this regulation. In subjects who have congenital 5α-reductase deficiency and who no longer synthesize DHT (dihydrotestosterone), the prostate no longer develops [1]. Androgen depletion induces massive apoptosis of prostatic cells. This cell death affects the epithelial cells more than it does the cells of the fibromuscular stroma [2]. Administration of testosterone to castrated animals causes an increase in the mitotic activity of the prostatic epithelial cells and restores the function of the gland. Castration early in life prevents the development of prostatic cancer and hyperplasia during ageing.

The work of Cunha and coworkers [3] showed that androgens act indirectly via the mesenchyme to ensure the growth and differentiation of the prostate. Paracrine-factor-mediated interactions between the epithelium and stroma are thus of capital importance. Not all relevant paracrine factors have been identified. The known ones include nonandrogenic steroids, peptide hormones, neuropeptides, neurotransmitters, and growth factors. All of them act via their own specific intracellular signaling pathways, but these pathways are tightly interconnected so as to elicit an integrated response ensuring coherent development and functioning of the gland.

It is noteworthy that the nature of androgen-mediated effects changes through the major periods of life: (1) During fetal life, the prostate develops first in a context where androgen levels are low but sufficient to ensure the gland's morphogenesis. (2) During adult life, prostatic growth is slight, although androgen levels are high. Their role is then to ensure the function and maintain the integrity of the prostate. (3) During aging, the prostate continues to grow despite decreasing androgen levels in the bloodstream [4].

Furthermore, androgens alone are not sufficient to control the development and function of the prostate. Pituitary hormones (prolactin, somatotropin, luteinizing hormone) and certain growth factors such as IGF-I act directly via their specific receptors present in the prostate [5,6]. These different factors induce the synthesis of proteins that are important to prostate function. Through their systemic action, these factors might be involved in resistance to androgen-deprivation-induced apoptosis. One-third of all androgen-dependent prostate tumours overexpress the androgen receptor; another third display a receptor that is neither mutated nor amplified; the remaining third have lost the receptor or express a mutated or nonfunctional receptor [7]. These differences in androgen receptivity highlight the diversity and complexity of the mechanisms involved in the uncontrolled growth of this type of cancer. The fact that these tumors become resistant to androgen-deprivation-induced apoptosis shows that within the tumor cells, survival factors also contribute to tumor progression.

If we are to understand the appearance of the various pathologies that affect the prostate in aging men, knowledge is needed about the various signals acting on the prostate and on the signaling pathways controlling the gland's function throughout the different stages of life. These signals and the intracellular mechanisms underlying their action constitute potential new targets for the development of effective prostate cancer therapies. Prostate cancer has become the number two cause of cancer deaths in Western countries, and its impact on health care, combined with that of benign prostatic hypertrophy [BPH], is considerable [8]. This chapter provides an integrated picture of the signals and signaling pathways that control the development of the prostate during fetal and adult life and during aging.

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Development of the Prostate During Fetal Life

The prostate is formed by outgrowth of the urogenital sinus epithelium (of endodermal origin) into the urogenital sinus mesenchyme (of mesodermal origin). Its morphogenesis has been remarkably described by Cunha *et al*. [3].

Rodent and human prostates are organized differently. The same applies to the branching morphogenesis of their epithelial ducts during fetal and neonatal development. The rodent prostate is organized in lobes that differ from each other both morphologically and biochemically. The lobes are named according to their anatomical position in the organ: anterior, dorsal, dorsolateral, and ventral [9]. The human prostate does not show this discrete structure. McNeal [10] has subdivided it into three zones: a central zone, a peripheral zone, and a transition zone. At least three cell types can be distinguished in the epithelial compartment of the prostate: luminal, basal, and neuroendocrine cells. The stromal compartment contains fibroblasts, muscle cells, endothelial cells, nerve cells, and blood cells.

Androgens

Androgens are required to initiate prostate development and to ensure the gland's fetal and neonatal growth [11]. The fetal period is the life stage at which prostate development is most intense. Growth and development follow the differentiation of the gonads and the establishment of the pituitary-gonadal regulatory axis. In man, the androgen receptor and 5α reductase (responsible for production of DHT) are present in the mesenchyme from the eighth week postconception. The particular distribution of the androgen receptor may be related to the branching differences observed in the various lobes of the rodent prostate during morphogenesis [12]. Androgen receptors are also found in the epithelium, but their presence there does not seem to be required for development of the gland. The work of Cunha *et al.* [13] has demonstrated that the mesenchyme expresses all of the factors required for prostate morphogenesis. Androgens act via their receptors in the mesenchyme to ensure the growth of the epithelium and differentiation of the stroma. Yet to date, there has been no confirmation of the existence of androgen-dependent mesenchyme-derived factors that alone ensure the fetal development of the prostate.

Data are incomplete and contradictory regarding the nature and mode of action of the paracrine factors mediating the action of androgens on the development of the fetal prostate. According to the types of effects they exert, these factors can be classified either as mitogenic factors, such as fibroblast growth factors (FGFs), epidermal growth factors (EGFs), and insulin-like growth factors (IGFs), or as morphogenic factors such as transforming growth factor β (TGF-β), hepatocyte growth factor (HGF), and protein PS20 [14], capable of inhibiting proliferation in the fetal prostate.

The Main Mitogenic Factors

Members of the FGF family such as FGF-7 (also called KGF for keratinocyte growth factor) and FGF-10 play important roles in prostate development during the fetal period. In the fetal prostate, expression of these two factors does not appear to be regulated by androgens.

FGF-7 is produced by fibroblasts and mesenchymal cells, but these cells do not express the receptor for this growth factor (FGF-R2iii b). Conversely, epithelial cells possess the FGF-7 receptor but do not synthesize FGF-7. FGF-7 exerts mitogenic action on the epithelium *in vitro*. FGF-7 transcripts are abundant during the development of the rat ventral prostate, and large amounts of FGF-7 are produced during periods of active growth [15]. In the rat, furthermore, FGF-7 can replace testosterone for epithelial ductal branching during morphogenesis of the ventral lobe [16]. On the other hand, FGF-7 knock-out mice show unperturbed development of the urogenital tract [17].

FGF-10 is also an important paracrine regulator of prostate development. It appears to act in the early stages of the organ's development [18]. By *in situ* hybridization, FGF-10 transcripts have been observed in the mesenchymal cells of buds undergoing branching morphogenesis of the epithelial ducts. In addition, the FGF-10 protein stimulates the development of ventral prostate explants cultured in serum-free medium. FGF-10 stimulates proliferation of the epithelium but not that of the stroma [18].

Although FGF-7 and FGF-10 show high sequence identity, they are not functionally interchangeable. Deletion of the FGF-10 gene in knock-out mice leads to the loss of several organs, including the prostate [19,20]. FGF-10 thus appears as a mesenchymal factor required for organogenesis of the prostate [21]. It has been hypothesized that FGF-10 might take over the action of FGF-7 in FGF-7 knock-out mice, thus compensating for the deletion.

IGF-I and IGF-II and their receptors have been implicated in fetal development [22]; both IGF-I and -II transcripts are present in fetal tissues [23]. We have very little information about IGF expression, IGF binding proteins, and IGF receptors in the developing prostate. Data on systemic and locally produced IGF-I are equally scant. In the adult, IGF-I and -II are present mainly in the fibromuscular stroma, whereas the type I receptor, which binds IGF-I and -II with different affinities, is present in both compartments. The type I receptor is a receptor tyrosine kinase. Arguments in favor of the involvement of IGFs in fetal development come from xenotransplant experiments [24–26]. When urogenital sinus tissue is transplanted from mice with a knocked-out IGF-I or type I receptor gene into athymic mice, the transplants show delayed growth when compared to grafts of normal fetal tissue. This shows that the IGF-I produced by the host cannot totally compensate for the lack of locally produced IGF-I with regards to the latter's effects on the fetal prostate. IGF-I is thus important for growth of the fetal epithelium, but the latter is able to differentiate in the factor's absence.

Although the epithelium of the fetal prostate is modulated by mesenchymal factors, epithelial signals are important for differentiation and proliferation of the prostatic epithelium. The product of the Sonic hedgehog (Shh) gene is a glycoprotein produced by the epithelium of the

urogenital sinus. Its overexpression in cells coincides with an increased testosterone level. The product of the Shh gene is involved in formation of the urethra and epithelial ducts [27].

Also known is the importance of the nervous system in the development of nerves and smooth muscle in fetuses with meningomyelocele. Under these conditions, there is no smooth muscle and the posterior prostate is underdeveloped. Likewise, prostate size is affected by the absence of innervations. These observations are in keeping with some very interesting observations of Kyprianou *et al.* [28] who demonstrated the role of α_1 receptor agonists in the growth of the adult prostate. Data are scant on the secretions of the neuroendocrine cells of the prostate during fetal life. What we do know is that such cells are present in the fetal prostate from the 13th week of gestation, and that they might be associated with the differentiation of the epithelium [29].

The Main Inhibitors of Proliferation

TGF-β and its isoforms (1 to 3) inhibit the proliferation of epithelial cells, alter cell differentiation, and stimulate fibroblast growth. All of the TGF-β isoforms are down-regulated by androgens. TGF-β1 has recently been shown to be capable of altering the nuclear-to-cytoplasmic distribution of androgen receptor in prostatic smooth muscle cells *in vitro* [30]. Remarkably, the effects of TGF-β on the prostate are similar to those observed after neonatal exposure of the gland to estrogens. Expression of the three TGF-β isoforms in the rodent prostate is time and cell type dependent [31,32]. Chang *et al.* [33] have shown that TGF-β1 and the RII receptor are expressed in the smooth muscle cells surrounding the epithelial ducts. TGF-β2 and TGF-β3 are present mainly in differentiating epithelial cells. The RII receptor is expressed weakly in the epithelium but more strongly in the smooth fibromuscular cells; this may reflect the fact that TGF-β-mediated effects in the prostate occur preferentially at the level of muscle cells and that TGF-β has been shown to be capable of inducing smooth muscle markers in stromal cells [34].

Activin and follistatin, which belong to the TGF-β family, have also been implicated in the branching morphogenesis of the fetal prostate. Activin A (a dimer of subunit β) and its binding protein, follistatin, have opposite effects *in vitro* on branching of the prostatic ducts. A down-regulating role of activin A on morphogenesis *in vivo* is imaginable, since activin A has been found in the epithelium and in mesenchymal aggregates at the ductal tips [35].

PS-20 has many characteristics of smooth muscle-derived inhibitor of epithelial cell proliferation. The protein was first isolated from a fetal urogenital sinus mesenchymal cell line (U4-F) and possesses growth inhibitory properties on bladder carcinoma and PC-3 cell proliferation [36]. PS-20 is localized in the smooth muscle compartment of the prostate and its sequence analysis has revealed that the protein is a member of a WAP-type serine proteinase inhibitor [14]. Details of the molecular mechanisms by which PS-20 may exert its effects are currently unknown.

Data on the involvement of EGF and of its homolog TGF-α on prostatic morphogenesis come from experiments conducted on transgenic mice with a knocked-out phospholipase Cγ gene. (Phospholipase Cγ is involved in the pathway used by EGF to regulate cell motility [37].) These mice show markedly delayed development of the prostate. Their prostatic cells are less differentiated and the ducts much less numerous. These results, like those of Xie *et al.* [38] concerning the mammary gland, show that early prostate development depends on intracellular signaling pathways involving PLCγ, known less for its role in proliferation than as a mediator of effects on cell motility.

It has been hypothesized that during aging some latent embryonic potential is reactivated, and that this is responsible for the appearance of cancer of the prostate and benign prostatic hyperplasia in elderly men. In the fetal prostate, signaling by the SSH gene product is required for expression of the gene encoding the NKX3.1 transcription factor. When this gene is deleted in mice, increased proliferation of the prostatic epithelium is observed [39]. NKX3.1 might also play a role in BPH, since Bhatia-Gaur *et al.* [40] have shown that deletion of the NKX3 gene causes epithelial hyperplasia in elderly males. Conditional loss of the NKX3.1 gene in adult mice induces prostatic intraepithelial neoplasia [41]. The gene might thus be a tumor suppressor gene.

Development of the prostate during fetal life also requires the expression of genes involved in controlling angiogenesis. Vascular endothelial growth factor (VEGF) is expressed in the human prostate from the ninth week of life. VEGF expression by cultured fibroblasts is increased by androgens [42], showing that androgen-dependent genes are important in angiogenesis during the early development of the prostate. The extracellular matrix plays an interesting role in angiogenesis. In the matrix, plasminogen and its activators are activated, and this leads to production of powerful inhibitors of angiogenesis. Elfman *et al.* [43] have recently shown that the amino-terminal peptide of the urokinase plasminogen activator inhibits branching morphogenesis of the ventral prostate in mice. This activity is related to that of angiostatin, an active plasminogen fragment obtained by limited proteolytic cleavage of the molecule. Interestingly, angiostatin-like peptides have been produced through the action of PSA, the prostate-specific antigen, a kallikrein-like enzyme [44].

The mesenchyme also produces other inhibitors of epithelial proliferation during prostate development. Bone morphogenetic protein 4 (BMP-4) is expressed from the 14th day of gestation. As the fetus develops, this expression gradually becomes limited to the mesenchyme. *In vitro*, BMP-4 inhibits epithelial proliferation and duct branching in a dose-dependent manner [45].

Last, fucosyl transferase-1 has also been identified as a modulator of epithelial proliferation during fetal development [46] and of branching morphogenesis *in vitro*. Fucosyl transferase is an enzyme present at the surfaces of epithelial cells. Its involvement demonstrates the importance of cell–cell interactions in the fetal development of the prostate.

Estrogens

The developing prostate is also sensitive to the action of estrogens and endocrine disruptors. Two estrogen receptors, α and β, are present in the prostates of rodents and humans alike. In rodents the α receptor is located exclusively on fibroblasts and smooth muscle cells. Expression of the β receptor is low during fetal life, but it increases after birth as epithelial cells differentiate into lumino-epithelial cells. Furthermore, an increasing gradient of β receptor concentration has been observed from the proximal part of the ducts to the distal part. It has been hypothesized that the β receptor might play an important role in epithelial differentiation in the course of prostate development [47,48].

During morphogenesis of the rat or mouse prostate, large doses of endogenous or exogenous estrogens induce permanent changes in prostatic growth. In the mouse, these estrogen-induced effects are dose dependent. High estrogen doses reduce the size of the adult prostate, whereas low doses increase it. These low doses of estrogen also favor a larger number of epithelial ducts and an increased level of androgen receptor [49]. When a rat is treated during the neonatal period with high doses of estrogen, it shows in adult life a highly abnormal ventral prostate, with numerous hyperplasias and dysplasias, despite the delayed prostatic growth observed after treatment [50]. The prostatic lesions observed may result from aberrant cell–cell interactions and defective cell–cell communications [51] and from epithelial differentiation defects involving reduced expression of the β receptor. Chang and coworkers [52] have recently shown that estrogen exposure during the neonatal period in the rat perturbs the TGF-β signaling pathways and thereby blocks $p21^{cip1/WAF1}$-mediated epithelial differentiation and stops proliferation of the fibroblasts surrounding the prostatic ducts.

The Adult Prostate

In the adult prostate, the daily renewal of 1–2% of the cells is compensated by cell death due to apoptosis. This cell homeostasis results from a balance between the proliferation-enhancing effects of certain signals (growth factors, neuropeptides, etc.) on the one hand, and proliferation-inhibiting and apoptosis-inducing effects on the other. Androgens modulate the pathways that stimulate proliferation and they antagonize apoptosis. These proliferative signals act univocally or interfere with other signaling pathways.

Factors Involved in Controlling Proliferation

The main mitogenic signals exerting their effects on the adult prostate are EGF/TGF-α and basic FGF (bFGF). These two growth factors account for 80% of all proliferation-stimulating effects. Several FGF isoforms are also expressed during adult life by the prostatic epithelium, but their role in proliferation and differentiation seems less important than that of bFGF. Epithelial and stromal cells are not the only cells to produce bFGF—so do macrophages and endothelial cells [53]. The mesenchyme is the main target of bFGF *in vivo*. No action of bFGF on the epithelium had been demonstrated *in vivo*, but bFGF does stimulate the proliferation of epithelial cells in culture [54]. The prostate contains high levels of EGF. EGF, heparin-binding EGF, and TGF-α are all secreted by prostatic stromal cells on which they exert their main mitogenic effects. EGF is also secreted by human epithelial cancer cell lines and perhaps by normal epithelial cells, at low levels. The selective response of adult prostatic epithelial cells to the paracrine action of TGF-α rather than to the autocrine action of EGF had led to the suggestion that the EGF receptor might be located on the basolateral surface of the epithelial cells. Tight junctions would prevent EGF from reaching the receptors [37]. The mitogenic effects are mediated by transactivation of the EGF receptor, requiring cleavage of the ligand by extracellular matrix metalloproteinases [55].

In the adult, most of the mitogenic signals occurring in the prostate act through receptor tyrosine kinases and the corresponding transduction pathways. Other signals, released by neuroendocrine cells (such as bombesin, somatostatin, bradykinin) or by the endothelium (such as endothelin-1) exert their proliferation-enhancing activity via G-protein-coupled receptors (GPCRs) and the MAP kinase pathway (and, in particular, via ERK, the extracellular signal regulated kinase). This pathway is an intracellular point of convergence of mitotic signals acting via GPCRs and signals acting via receptor tyrosine kinases. In this context, Putz *et al.* [56] have shown that blocking of the EGFR inhibits not only the action of EGF on prostatic cells, but also that of IGF-I and PKA activators.

The androgen receptor whose expression is constant in the adult prostate undergoes ligand-independent transactivation via phosphorylations induced by receptor tyrosine kinases or serine/threonine kinases and via protein kinases A and C [57]. Culig *et al*. [58] have shown that IGF-I, FGF-7, and EGF activate transcription mediated by androgens and their receptors and that the antiandrogen bicalutamide inhibits this effect. The CREB protein, involved in transducing messages transiting via cyclic AMP and PKA, also intervenes in the activation of transcription by the androgen receptor [59,60].

IGF family factors exert major effects on the regulation of growth in general. IGF-I and IGF-II are synthesized mainly by the liver in response to growth hormone stimulation. They stimulate cell proliferation and differentiation and inhibit apoptosis. Recent interest in the IGF family in relation to the prostate stems from *in vivo* observations. Transgenic mice overexpressing the bovine growth hormone display oversized prostates. The effect is attributed to increased systemic levels of IGF-I [61]. Transgenic mice with knocked-out IGF-I and type I receptor genes have underdeveloped prostates [62]. When rats are treated systemically with IGF-I, the treatment stimulates prostatic growth [6]. Young untreated acromegaly patients have an overdeveloped prostate compared to healthy patients [63].

IGFs are also produced locally by many tissues. In the prostate, they have an autocrine/paracrine action via the type I receptor tyrosine kinase. The type II receptor is not involved in intracellular signaling. Its role would appear to be related to the uptake and degradation of IGF-II. IGF availability in prostatic cells is modulated by carrier proteins (called IGF binding proteins or IGF-BPs), at least seven of which have been well characterized [64]. IGF-BPs affect IGF availability in a cell-type-specific manner. IGF-BP3 binds more than 90% of the circulating IGFs. At prostate level, it can be cleaved *in situ* by prostate-specific antigen (PSA), whose proteolytic activity is kallikrein-like. The effect of this proteolytic action is to make IGF-I available to its type I receptor and to favor IGF-mediated mitogenic effects [65]. Moreover, TGF-β1 is believed to exert its antiproliferative action by stimulating IGF-BP3.

IGF-BP5 is bound to the extracellular matrix. It potentiates the action of IGFs on DNA synthesis. Gregory *et al.* [66] have shown in the xenograft CWR22 model that androgens can regulate the expression of IGF-BP5 but not that of other IGF-BPs. High levels of IGF-BP5 would thus enable prostatic cells to recruit more IGF-I, and this would favor cell proliferation in the presence of androgens. On the other hand, production of IGF-I and its receptor is regulated by androgens: Nickerson *et al*. [67] have shown that they are both overexpressed during androgen-deprivation-induced apoptosis.

In transgenic mice overexpressing bovine growth hormone (GH), the observed increase in prostate weight might also result from the direct action of the hormone on the prostate. Bovine GH has no prolactin activity in rodents and a specific growth hormone receptor is present in the prostate. This receptor mediates effects that are at least partially androgen independent [5]. Growth hormone receptors are also expressed more strongly by LNCAP and PC-3 cells than by normal prostatic cells. This implies that growth hormone might be involved in the development of prostate cancer. This hypothesis is supported by the work of Jungwirth *et al.* [68], who demonstrated *in vivo* a major inhibitory effect of a somatostatin analog on xenografted prostatic tumor cell lines.

In this context, it is well established that prolactin can also affect the proliferation and differentiation of prostatic cells [69] via activation of specific receptors [70]. The prostates of transgenic mice overexpressing prolactin weigh 20 times as much as those of normal animals [61]. These effects are attributed in part to the action of prolactin on testicular steroidogenesis and to its major role in Leydig cell proliferation. Because the prolactin receptor is also present in the prostate, the hormone may also act directly to stimulate transcription of certain genes via activation of Stat5 [71]. Some of these effects may be androgen independent [72]. Prolactin is also produced by the prostate [73] and can thus exert paracrine/autocrine effects within the organ. Untergasser *et al*. [74] have shown that this local network of interactions is not involved in prostatic pathologies. The relative importance of prolactin's endocrine and paracrine/autocrine effects could doubtless best be evaluated in transgenic models. Prolactin and prolactin receptor knock-out mice have been produced [75,76] but the effects of these deletions on the prostate have not yet been assessed.

Of the gonadotropins playing a major role in reproduction, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), only LH has receptors in the prostate [77]. These G-protein-coupled receptors acting via cyclic AMP are functional, since binding of the hormone causes the accumulation of certain mRNAs [78]. Because these effects have been observed on prostatic explants cultured in androgen-free medium, LH might act on the prostate in an androgen-independent manner. In addition, Tao *et al.* [78a] have shown that LH/hCG is expressed in BPH. The study of LH signaling in the prostate thus opens interesting prospects for further investigation.

Factors Involved in Apoptosis

In the adult prostate, androgen deprivation leads to apoptosis becoming a major, macroscopic process. In this phenomenon, the cells concerned no longer proliferate, but remain at stage G_0 or engage in a defective cell cycle. In the presence of androgens, cells enter apoptosis in response to various stimuli such as glucocorticoids, cytokines, and cell surface death signal transmitting receptors [79,80] or to physical factors such as hypothermia and ionizing radiation [81].

The main role of TGF-β1 in the adult prostate is to inhibit the gland's growth by controlling cell proliferation and inducing epithelial cell apoptosis [82]. TGF-β1 also inhibits fibroblast growth. Bretland *et al.* [83] have shown on stromal cells derived from prostatic adenomas (BPH) that the nature of the effect observed depends on the TGF-β concentration used. It is generally accepted that TGF-β plays its inhibitory role via induction of the cyclin-dependent kinase (CDK) inhibitors $P15^{INK}$ and $P21^{CIP}$ [84]. This induction results in cell growth arrest. Induction of $P15^{INK}$ and $P21^{CIP}$ is mediated by Smad2 and Smad3 [85]. In androgen-deprivation-induced prostatic apoptosis, TGF-β plays an inducing role that has been described at length [82,86,87]. Most interestingly, Bruckheimer and Kyprianou [88] have recently shown that dihydrotestosterone, at physiological concentrations, stimulates TGF-β-induced apoptosis. These results provide a molecular basis for androgen priming of prostate cancer cells in order to obtain maximal apoptosis in antiandrogen treatment of the prostate. It is noteworthy in this context that TGF-β1 has been implicated in determining the nuclear or cytoplasmic localization of the androgen receptor [89]. By favoring the receptor's cytoplasmic location, TGF-β might inhibit the cell response to androgens. Moreover, Reddy *et al.* [90] have shown that clusterin interacts with the type II TGF-β receptor at the level of its intracytoplasmic enzymatic domain. Clusterin is the protein most strongly overexpressed in androgen-dependent apoptosis, and its intracellular localization also depends on TGF-β [91]. These results suggest a regulatory role for clusterin in prostatic cell apoptosis.

In androgen-dependent prostate regression, the FAS ligand and its receptor also appear to be involved. The FAS ligand is expressed by epithelial cells, and this expression is increased

in the prostate after castration [92]. In mutant mice with an altered FAS antigen, moreover, prostatic tumor transplants regress less after castration than grafts implanted in the wild type [93]. Binding of the FAS ligand to the FAS receptor leads to recruitment of the FADD death domain protein. This results in the activation of caspases-8 and -10 via the RIP protein, and in initiation of apoptosis by these proteins. TNFα also induces prostatic apoptosis by binding to its type I membrane receptor. The complex formed recruits TRADD, which in turn recruits FADD. From this association arises an apoptosis-activating pathway between FAS and TNFα.

The TNF-α-related-apoptosis-inducing ligand (TRAIL, also called AP0-2L) [94,95] and APO-3L are other members of the TNFα family. TRAIL can induce apoptosis of several human prostatic cell lines: ALVA-31, DV-145, PC-3, JCA-1, and TSV-Pr1 [96]. It has been shown that TRAIL can trigger apoptosis of normal prostatic epithelial cells within 6 hr of treatment. Hence, TRAIL might participate in cell homeostasis in the prostatic epithelium [97]. TRAIL is a type II transmembrane protein. It exerts its apoptotic action via the DR-4 and DR-6 receptors [98]. The intensity of this action is modulated by decoy receptors lacking the death domain and conferring resistance to the action of TRAIL. Expression of osteoprotegerin (OPG), a decoy receptor of TRAIL, by prostate cancer cells could confer a survival advantage on these cells [99]. TRAIL-triggered apoptosis, like FAS-triggered apoptosis, involves FADD recruitment and activation of the caspase pathway. Although TRAIL expression is ubiquitous, this factor inhibits tumor growth *in vivo* without causing any toxic effect in rodents or nonhuman primates. This makes it, unlike FAS-L, an interesting molecule for treating many types of tumors [100,101].

Because cell renewal occurs at a low rate in the adult prostate, we can assume that apoptosis is counteracted by the action of various survival factors. The many messages that activate receptor tyrosine kinases or GPCRs in the prostate may induce this type of effect via activation of the protein kinase B/Akt [102] and also via the transcription factor NFχB [102]. The inhibitory action of NFχB on apoptosis is controversial, but it might result from expression of the genes involved in oxidative stress [103]. Other signaling pathways are used to activate resistance to apoptosis. Prolactin delays androgen-dependent apoptosis in cultured explants and it can replace testosterone as an agent maintaining rat prostatic explants in long-term culture [104]. In these explant cultures, prolactin acts on the survival of prostatic epithelial cells by activating Janus kinases and Stat5a/5b [105]. This transduction pathway is prostate specific and might be used specifically to maintain the viability of androgen-deprived prostatic epithelial cells. Other pathways such as the B/AKT kinase pathway have also been proposed as mediating the antiapoptotic effects of PRL [106]. In the prostatic epithelium, PRL receptors are located preferentially on the apical side of the cells. This argues in favor of a paracrine/autocrine action of the hormone in this organ [107]. Prolactin also mediates the inflammatory effects observed in the dorsolateral lobe of Wistar rats treated with estradiol [108] and in Noble rats treated with a mixture of testosterone and estradiol [109]. On the other hand, hyperprolactinemia induced by sulpiride injection causes an increase in size and inflammation of the lateral lobe of the prostate, associated with Bcl-2 overexpression. This overexpression might lead to deficient apoptosis at the level of this lobe [110]. The pituitary gland secretes, depending on the physiological circumstances, a phosphorylated variant of prolactin, which acts as an antagonist at receptor level [111]. A nonhydrolyzable variant of the unphosphorylated molecule has been shown to delay effectively *in vivo* the androgen-independent growth of DU145 cells in nude mice [112]. These results have raised interest in the prolactin signaling pathway as a potential target for new therapies for androgen-independent prostate cancer.

Several publications have shown the importance of Bcl-2 in resistance to apoptosis. Very recently, Banerjee *et al.* [113] showed that Bcl-2 is differentially expressed in the different lobes of the rodent prostate. Its overexpression is age dependent and it correlates with cell survival in the absence of androgens. Bcl-2 and Bcl-xL are known to inhibit the proapoptotic effects of medicinal treatments and radiotherapy on various cancers. It has been shown that Bcl-2 protects against apoptosis induced by the FAS ligand and TNF [114]. Likewise, Bcl-2 has recently been shown to inhibit TRAIL-induced apoptosis in three human prostatic cell lines [115]. The link between Bcl-2 and prostatic cancer is well established. It has recently been shown by immunohistochemistry that Bcl-2, Bcl-x2, and Mcl-1 increase as prostate cancer progresses [116]. It is generally accepted that the main site of the antiapoptotic action of Bcl-2 is the mitochondrion [117], but it has been reported that Bcl-2 could be a chaperone interacting with certain procaspases to prevent their activation [118]. Bax, in this context, would exert an apoptosis-inducing effect by binding to Bcl-2-procaspase complexes. This competition between Bax and Bcl-2 would probably explain why the Bax/Bcl-2 ratio is high at the onset of androgen-deprivation-induced apoptosis [119].

It is known that the neuroendocrine cells of the prostate resist androgen-dependent apoptosis and do not express Bcl-2. This gives them a privileged role in progression of androgen-resistant prostatic cancer. It has recently been shown that the neuroendocrine cells of the prostate express survivin [120]. Survivin is a member of the recently discovered apoptosis inhibitor family [117]. Survivin expression in prostatic cancer cells has recently been demonstrated and resistance to apoptosis of these cells could be due in part to inhibitors of apoptosis proteins (IAP) expression. It has recently been shown that three of the most commonly used prostate cancer cell lines (DU-145, 2LNCap, and PC-3) expressed IAPs and survivin [121], suggesting that these inhibitors may make an important contribution to apoptotic resistance in patients with prostate cancer.

P75 neurotrophin receptor ($P75^{NTR}$) has been suggested to be a candidate tumor suppressor gene in human prostate [122]. Indeed, $P75^{NTR}$ is a low-affinity receptor for NGF that is expressed to varying degrees in epithelial cells [123–125]. In these cells, $P75^{NTR}$ inhibits cell proliferation

and promotes apoptosis [126]. Loss of expression of $P75^{NTR}$ in prostate cancer cells appears to block a potential programmed cell death pathway facilitating cell survival [122].

Because expression of 75^{NTR} is lost in prostate cancer, NGF-mediated growth of cancer cells occurs via the high-affinity Trk receptor (Tropomyosin receptor kinase), which is expressed in nonmetastatic prostate cancer and in cancer cell lines derived from metastases [127]. Although the Trk receptor was first identified as a colon cancer oncogene, Trk mutations within the human prostate cancer have never been found [128]. Thus Trk signaling pathways might be important in prostate cancer development.

The autonomous nervous system, which ensures the maintenance of the gland's structure and controls its secretions [129], has recently been implicated in the regulation of apoptosis in the prostate. Kyprianou *et al.* [28] have shown that α_1 adrenergic receptors are involved in controlling apoptosis and its induction by α_1 antagonists. These authors have also demonstrated a proapoptotic effect of Doxazosin and Terazosin on prostatic cells. These results suggest that α adrenergic antagonists have therapeutic potential in the treatment of BPH and cancer of the prostate.

The Prostate during Aging

Steroid Hormones during Aging

During aging, the prostate can be affected by two major pathologies: BPH and prostate cancer. Among the various species, only man spontaneously develops prostate cancer in old age. BPH and cancer develop from two different zones of the human prostate: the transition zone for BPH and the peripheral zone for prostate cancer. BPH is due mainly to proliferation of the fibromuscular stroma, whereas prostatic cancer develops in the epithelium. Causal relationships between these two diseases have not been clearly established. The development of prostatic cancer only rarely results from a malignant evolution of BPH. The cellular and biochemical mechanisms involved in initiating these two pathologies are different. Men as young as 30 may show intraprostatic tumor foci, whereas the first signs of BPH often appear after the age of 40. Yet these two diseases progress in an identical steroid context characterized by slowly decreasing bloodstream androgen levels and increasing estrogen levels.

Krieg *et al*. [130] have given a good description of the androgen context of the prostate during aging. They have shown that the accumulation of estrogens in the gland is an age-dependent phenomenon. Both 17β-estradiol and estrone increase in the stroma with age, whereas their concentrations in the epithelium remain constant. The dihydrotestosterone level varies in opposite fashion, decreasing in the epithelium and remaining constant in the stroma. What's more, it has been shown that in the transition zone of the human prostate, that is, the tissue where BPH preferentially develops, the estrogen-to-androgen ratio increases with age [131]. Given the action of androgens and estrogens on the development of the fibromuscular stroma, the role of these steroids seems fundamental in the development of prostatic pathologies [132].

During aging, expression of the androgen receptor remains constant in the normal prostate and in BPH [133]. Both the androgen receptor and 5α reductase are expressed mainly in the stroma. The epithelium also expresses the receptor but much less strongly. In the hyperplastic epithelium, the androgen receptor is expressed more abundantly in the luminal cells than in the basal cells. In the stroma, the receptor is present on the smooth muscle cells and fibroblasts. Increased androgen and prolactin levels resulting from knock out of the aromatase gene (ArKO) in transgenic mice lead to prostatic hyperplasia. Yet testosterone supplementation in elderly men seems to have no effect on prostatic growth. In the ArKO mouse model, the absence of estrogens does not lead to any cancerous development of the prostate [134]. These results show that both androgens and estrogens are required for the development of prostate cancer.

Estrogens exert their effects by binding to two types of receptors, Erα and Erβ, which are differentially expressed in the adult prostate. Erα is stromal and Erβ is present in the glandular epithelium of the rat prostate [48]. In the adult rat, there is an increasing gradient of Erβ expression from the proximal part of the ducts to the distal part. Estrogens are produced by peripheral aromatization of testosterone by aromatase. This enzyme is present in the adult prostate but very little is known about its activity during aging. On the other hand, aromatase is present in two androgen-independent human cell lines, PC3 and DU-145 [135], where it might mediate the effects of testosterone. In man, circulating estrogen levels increase with age. This increase is due to increased aromatization in the adrenal gland after the age of 50. The increased estrogen levels inside the prostate may be due to the increased serum levels of these steroids at the andropause. Because of its stromal localization, the Erα has been implicated in the preferential development of the stroma. Likewise, the epithelial localization of Erβ suggests that it might be involved in the development of prostatic cancer. Yet Erβ has also been implicated in BPH as a result of observations on transgenic mice. Erβ knock-out mice develop prostatic hyperplasia late in life [136]. Erβ might thus play a protective role during abnormal growth of the gland by exerting an antiproliferative action. More detailed studies of the prostate, however, failed to corroborate these initial results [137,138]. The reason for such a discrepancy is still unclear. In hyperplasia caused by estrogen treatment during fetal or neonatal life, the level of Erβ is reduced in adult life. It is important to establish the role of the β receptor and its interactions with Erα. In the prostate, the effects mediated by these two receptors appear to be mutually antagonistic. Paech *et al*. [139] have shown that tamoxifen and genistein exert agonistic effects on Erα but antagonistic effects on Erβ.

It is interesting to note that some *in vitro* studies show that estrogens can bind to androgen receptors. This was observed on LNCaP cells, which are androgen sensitive and possess a mutated androgen receptor [140]. It is believed, however, that estrogens act essentially via coactivators, to

modulate transcription of androgen-controlled genes. ARA70 is an example of a coactivating protein [141]. It has been shown in DU-145 cells transfected with the gene encoding the androgen receptor that 17β-estradiol binds to this receptor and activates transcription in the presence of ARA70 [142]. This estrogen-mediated transcriptional activation is specific to the androgen receptor and does not require the presence of an estrogen receptor. This result clearly shows the importance of the joint action of estrogens and androgens in regulating gene expression in the prostate. The action of estradiol on the transcription of androgen-dependent genes supports the idea of using antiestrogens like diethylstilbestrol (DES) to treat pathologies of the prostate.

Prostatic cancer evolves from androgen dependence to stages where it requires for its progression only local factors or factors that deregulate the intracellular mechanisms that make the gland's growth androgen dependent. This progression of prostatic cancer usually occurs in man after the age of 50, although prostatic tumor foci may be present in the gland around the age of 30. When cancer is diagnosed at a sufficiently early stage, the androgen receptor is functional and sometimes amplified at both the gene and protein levels. This greater androgen receptivity of prostate tumors may explain the development, growth, and progression of androgen-dependent prostate cancer: The process would be triggered by transcriptional activation of androgen-dependent genes. PSA is the prototype of these androgen-regulated factors and is used in the early diagnosis of prostate cancer. Yet PSA levels remain very high in androgen-resistant prostatic cancer after androgen depletion. There is an apparent paradox here, because it is generally accepted that the androgen receptor is nonfunctional in prostate cancer, once the cancer has become hormone resistant. Yet it has been demonstrated that androgen receptor expression also increases in some androgen-resistant and metastasizing tumors [143,144]. Compensatory sensitivity to androgens has also been proposed to explain progression of androgen-resistant prostate cancer. This concept stems from observations on LNCaP cells cultured in androgen-free medium. Cells appearing in these cultures express three times more androgen receptor than cells cultured in the presence of DHT or testosterone. The overproduced receptor is functional, because it responds to androgen stimulation. The mitogenic action of androgens can also be amplified in androgen-sensitive tumors by mechanisms involving coactivators of steroid hormone receptors [145]. These coactivators are important to the assembly of the RNA polymerase II preinitiation complex to the translation start site. *In vivo* studies suggest that overexpression of these coactivators associated with that of the steroid hormone receptors may play a role in cancer development by enabling the receptors to activate their target genes independently of their ligand.

It has been shown *in vitro* that in addition to ARA70, other factors can up-regulate androgen receptor activity. Brady *et al*. [146] have shown that the Tip 60 protein is a coactivator of androgen-receptor-controlled transcription. The ARA54 factor, whose gene has been cloned by Kang *et al*. [147], is also a protein binding to the androgen receptor and modulating its activity. Some *in vivo* studies have shown that certain coactivators can be overexpressed or mutated in hormone-dependent cancers. In an *in vivo* model where the SRC-1 gene was inactivated (SCR-1 stands for steroid receptor coactivator-1), Xu *et al*. [148] have shown that the product of this gene is involved in the growth of the prostate and other hormone-dependent organs. The P-300/CBP coactivator is mutated in prostate cancer. It may be involved in controlling the expression of P53, whose role in the induction of apoptosis is well known [149].

The cAMP Signaling Pathway

As mentioned earlier, cross-talk between steroid hormone receptors and receptor tyrosine kinases, Ser/Thr kinases, and GPCRs may explain the progression of prostatic pathologies. IGF-I, KGF (FGF-7), and EGF can activate androgen-receptor-mediated transcription [58]. The CREB protein also intervenes in androgen-receptor-mediated effects [150]. On the other hand, it has also been shown that EGF, IGF-I, and cyclic AMP (cAMP) activate the signaling pathway of the α receptor via its AF-1 domain [151]. Estradiol can also cause rapid accumulation of cAMP in the prostate, through action at the level of the plasma membrane [152]. In addition, binding of oestradiol to sex hormone binding globulin (SHBG) can cause an increase in the intracellular level of cAMP. SHBG is the carrier protein for sex steroids in the bloodstream. When not bound to its ligand, SHBG binds to membrane receptors at the surface of prostatic cells. When estradiol binds to SHBG, the receptor–SHBG complex is activated, and this causes an increase in the intracellular level of cAMP and activation of PKA. This leads to increased expression of PSA in prostatic cells, specific to PKA activation. This alternative pathway for estrogen action might be involved in controlling the effects of male and female sex hormones on the prostate. It is noteworthy that SHBG can bind testosterone, DHT, or estradiol, albeit with different affinities. Binding of one of these steroid hormones to SHBG might thus lead to local competition at the level of prostate cells [153].

Classically, high cAMP levels activate protein kinase A (PKA) and the latter in turn can activate several signaling pathways. In the prostate, cAMP notably increases the activity of MAP kinases [154]. Furthermore, Nazareth *et al.* [154] have shown that PKA is responsible for activation of the N-terminal region of the androgen receptor, involved in DNA binding. PKC has also been implicated in androgen receptor modulation. De Ruiter and coworkers [155] have shown in transient transfection experiments performed on Chinese hamster ovary (CHO) cells that PKC acts in synergy with the activated androgen receptor. Activation by PKC, however, fails to induce ligand-independent androgen receptor activation.

Last, an increased cAMP level can also result, *in vivo*, from the action of several neuropeptides or hormones acting via adenylate-cyclase-coupled GPCRs. The corresponding receptors are present in the prostate [5] and some of them

are more abundant in BPH and prostate cancer [157,158]. These ligands can act systemically like the pituitary hormones or in paracrine/autocrine fashion like certain ligands produced by endothelial and neuroendocrine cells.

Growth Factor Signaling

Growth factors and their receptors are involved in the gland's normal growth. Several of them are proto-oncogenes; changes in their expression lead to the development of prostate cancer and to its uncontrolled growth. This is the case of EGF and TGF-α, which act via the same receptor and whose expression, according to some investigators, is altered in prostatic cancer [159], although other authors say it is not [160]. EGF is mitogenic toward prostatic epithelial cancer cells, and its receptor is present at their surface both *in vivo* and *in vitro* [161]. EGF and its receptor exert pleiotropic effects on prostatic cancer cells, stimulating their proliferation and increasing their invasiveness.

Another mechanism involves, for example, up-regulation of a receptor. HER-2/Neu, HER-3, and HER-4 are proto-oncogenes belonging to the EGF receptor family. HER-2 is expressed differentially in the normal prostate and in cancerous tissue; HER-2/Neu is not expressed by the normal epithelium but most epithelial cancer cells express it. HER-2/Neu induces proliferation of these cells and increases their invasiveness. It has also been implicated in progression of androgen-dependent cancer, as a coactivator of the androgen signaling pathway. Furthermore, HER-2/Neu-dependent activation of the androgen receptor has been shown to involve the MAP kinase pathway [162]. In the absence of androgens, HER-2/Neu increases the growth and survival of prostatic cancer cells by stimulating the PI3K/AKT pathway. AKT transactivates the androgen receptor by binding to it specifically and phosphorylating its residues Ser213 and Ser791 [163]. These results provide a molecular mechanism that would explain the role of Her-2/Neu in the progression of androgen-independent prostatic cancer, where overexpression and genetic amplification have been reported [164]. Yet other studies indicate that HER-2/Neu overexpression is relatively rare in this type of cancer. Heregulin binds to the HER-3 receptor, an isoform of the EGFR family, and activates HER-2, HER-3, and HER-4 by inducing formation of heterodimers between the different receptors of the family. A naturally secreted soluble form of HER-3 (p-85s HER-3) has been identified and recently been shown to inhibit activation of HER-2, HER-3, and HER-4 [165]. Identification of this natural inhibitor opens up prospects for its use in treating tumors whose growth is heregulin dependent.

Tumor progression is also influenced by TGF-β1, its isoforms, and other, more distant members of the same family, inhibin α, activin, follistatin, and certain BMPs. *In vivo*, TGF-β1 increases tumor growth and metastasis. The mechanism prevailing in this family appears to be down-regulation of the type I and II TGF receptors in tumor cells. At the same time, TGF-β1 is overexpressed in the same tumor cells. The result is a strong antiproliferative effect on the neighboring healthy cells. This autocrine/paracrine mechanism favors tumor progression at the expense of the host's cells. The involvement of this mechanism in tumor growth is confirmed by the observation that cancer cells in which TGF-β RII expression is restored undergo apoptosis when transplanted into a nude mouse. BMP family proteins induce bone formation *in vivo* and have been implicated in the development of prostate cancer bone metastases. Several BMPs are expressed by normal epithelial tissue, including BMP-2, -3, and -4. BMP-6 seems *in vivo* to be most directly involved in advanced prostatic cancer [166]. Its expression correlates with the Gleason score used to stage prostatic tumors [167].

FGF-2 (basic FGF) affects cell proliferation by an autocrine mechanism of cancer cells. It also stimulates angiogenesis and this favors the appearance of metastases. The capacity of FGF-2 to stimulate metastase formation reflects its capacity to regulate turnover of the extracellular matrix by modulating protease expression and collagen and fibronectin synthesis.

Another way to modify the growth of prostatic tumors is illustrated by the action of IGFs. These growth factors bind to seven different binding proteins. Located in the extracellular matrix, these proteins recruit to the matrix both circulating and locally produced IGFs. IGF-BP5 seems to be the only androgen-regulated BP, whence its role in the proliferation of tumor cells in the presence of androgens. However, overexpression of the inhibitory IGF-BP4 has been shown to delay the onset of prostate tumor formation *in vivo* [168] IGF-I is also a powerful mitogen for prostatic tumor cells. *In vitro*, IGF-I stimulates growth of PC3 and DU-145 cells, and an antisense oligonucleotide corresponding to the IGF-I receptor inhibits this growth [169,170]. On the other hand, evidence demonstrates that synergism exists between IGF-I and EGF at low androgen concentrations [171].

Last, growth factors have angiogenic properties. VEGF, a glycoprotein [172], which might be produced by the neuroendocrine or cancer cells, seems to be involved in neovascularization. Hypoxia may be the mechanism controlling VEGF synthesis [173]. It has recently been shown that testosterone itself controls the blood flow to the prostate [174] and that in castration-induced apoptosis, the blood flow first diminishes with the onset of apoptosis. This observation may explain the increase in VEGF enabling reconstruction of the vascular network during testosterone supplementation. Testosterone might also stimulate VEGF synthesis and, hence, the development of metastases.

Conclusions

Prostatic cancer is a heterogeneous disease. This applies both to its causes and to the mechanisms that facilitate its progression. Several molecular changes occur during this progression, giving the cancer cells a selective advantage over normal cells. Although tumor progression occurs in the context of androgen independence, the changes occurring in the tumor are only partially independent from the

signaling pathways involving the androgen receptor. There is cross-talk between the intracellular signaling pathways used by the androgen receptor and those used by other steroid hormones or by various growth factors. A therapeutic consequence is that in a patient having undergone total androgen suppression, the expression of certain androgen-regulated genes may be maintained. It is important to identify the genes whose regulation occurs partially via the signaling pathway involving the (possibly mutated) androgen receptor, in order to develop effective therapies for androgen-independent prostatic cancer. It is equally interesting to identify those genes that, in the normal prostate, are repressed by androgens. These genes might encode survival factors enabling cancer cells to elude therapies based on androgen deprivation.

Prostate cancer, like benign prostatic hyperplasia, develops also in a context where androgen levels are decreasing in the bloodstream and estrogen levels are rising. It would certainly be interesting to identify the genes regulated by estrogens via the α and β receptors in the normal and cancerous prostate. At present, treatment of hormone refractory prostatic cancer with the antiestrogen tamoxifen must be considered preliminary [175].

Tumor progression toward androgen-resistant stages is accompanied at the cell level by changes involving the acquisition or overproduction of growth factors and their receptors, deregulation of the local synthesis of binding proteins, and overproduction of angiogenic and survival factors. All of these mechanisms use highly interconnected signaling pathways. By identifying the common parts of these pathways, it should be possible to target simultaneously several mechanisms leading to androgen independence. Various experimental approaches have led to identifying the MAP kinase and ERK pathways as potential targets [176]. Another potentially interesting approach would be to reintroduce apoptosis into prostatic tumor cells by gene therapies targeting the caspase pathway, and particularly caspases 3 and 7 [177].

The use of prodrugs activated by the PSA is certainly another possibility [178], as is the use of peptides with arginine-glycine-aspartic acid [179] structures or nonpeptidic molecules [180]. Prostate cancer control might also involve systemic factors such as prolactin and the various partners of the growth hormone/IGF axis. The use of antagonists of these messengers, of inhibitors of their synthesis, and of enzymes promoting the degradation of their binding proteins might be studied at the level of both endocrine and paracrine/autocrine regulatory mechanisms. Other targets for therapy certainly remain to be discovered.

It is important to realize that progress in this field will depend on learning about cell signaling mechanisms in both the normal and the pathological prostate.

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CHAPTER 348

Retrograde Signaling in the Nervous System: Dorsal Root Reflexes

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Cell-to-Cell Signaling in the Nervous System

Anterograde Signaling

The nervous system is designed for rapid cell-to-cell communication, which allows for quick adjustments in behavior in response to environmental imperatives. Some of the special features of nerve cells that provide for rapid communications between neurons and other nerve cells or effectors, such as muscle or gland cells, include nerve impulse propagation along the axons of neurons and synaptic transmission between neurons and other cells.

The great Spanish neuroanatomist, Santiago Ramón y Cajal, proposed the "law of dynamic polarization" of the nervous system [1]. This "law" states that nerve impulses propagate in dendrites toward the neuronal cell body and in axons away from the cell body. However, Sherrington [2] demonstrated that nerve impulses can propagate in either direction along axons and that it is the synapses that channel information flow in a forward direction (for example, from sensory input to motor output) through the nervous system [3]. Synapses serve as rectifiers. This is generally true whether the synapses are of the chemical or the electrical variety [4,5]. Neural signals are conveyed from a neuron to another cell in one direction across the synapse. Nerve impulses in the postsynaptic cell do not normally have a substantial effect on the presynaptic cell.

Several additional corrections to Cajal's concept are also necessary. Information flow in dendrites is generally in the form of synaptic currents, which affect the membrane potential at the action potential trigger zone in the axon hillock, where a nerve impulse may be triggered. The action potential then conducts distally along the axon, but also backward (antidromically) into the dendrites [6].

Dorsal root ganglion cells, the primary afferent sensory neurons of the spinal cord, do not have dendrites. Instead, they have a bifurcating axon that ends in sense organs in the periphery and in synapses within the spinal cord or caudal medulla on upstream neurons that are involved in motor responses or in the transmission of sensory information to higher levels of the nervous system. The peripheral axon normally conducts nerve impulses evoked in response to sensory stimulation toward the cell body of the dorsal root ganglion cell, and the central axon conducts nerve impulses away from the cell body and toward the synapses in the CNS (Fig. 1, topmost primary afferent neuron).

Retrograde Signaling

In some instances, chemical communication occurs between post- and presynaptic elements of a synapse, although this generally does not result in nerve impulse propagation in the reverse direction to the normal. For example, in the process of long-term potentiation, as studied in slices of the hippocampus, one of the factors that enhances synaptic transmission may be the generation of nitric oxide (NO) in the postsynaptic cell and the retrograde diffusion of the NO

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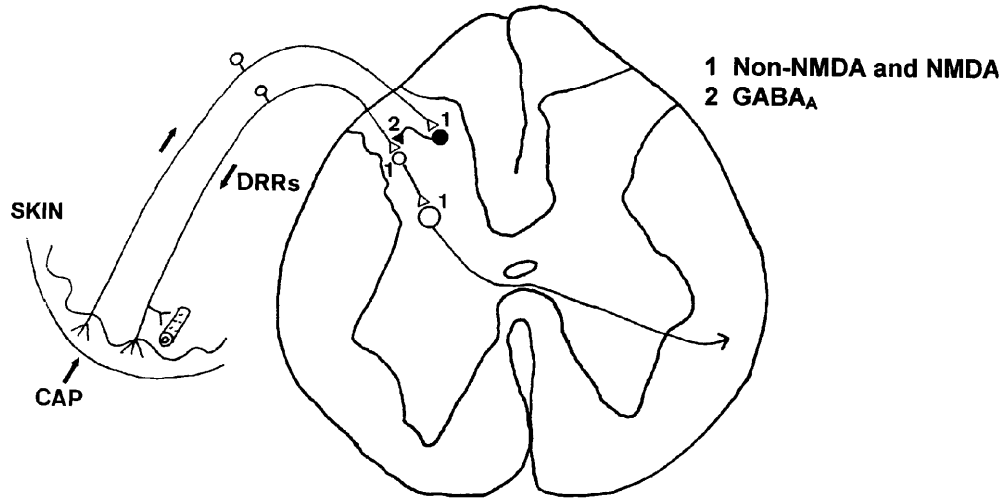


Figure 1 The upper left arrow in the diagram shows the normal direction of nerve impulse conduction in a primary afferent neuron. Conduction is from peripheral receptor endings, in this case in the skin, into the spinal cord. Also shown is a circuit involving a GABAergic inhibitory interneuron (shown in black) that releases GABA onto the synaptic terminals in the dorsal horn of another primary afferent neuron. Propagation of dorsal root reflexes (DRRs) in another primary afferent neuron toward the periphery is indicated by the lower right arrow. The receptor types that are involved in transmission within the dorsal horn circuit include non-NMDA and NMDA glutamate receptors and $GABA_A$ receptors. (Reprinted with permission from [38].)

across the synapse. Activation of intracellular signaling pathways in the presynaptic terminals leads to enhanced neurotransmitter release and stronger synaptic excitation [7].

At the level of neural circuits, there is often an arrangement of neurons that allows a postsynaptic neuron to provide feedback inhibition or excitation within a circuit. For example, activation of motor neurons in the spinal cord can lead not only to contraction of the skeletal muscle fibers supplied by the motor neurons, but also to inhibition of the same and related motor neurons. This inhibition involves the propagation of nerve impulses along recurrent collaterals of the motor axons and activation of inhibitory interneurons (called Renshaw cells after their discoverer), which in turn cause postsynaptic inhibition of the motor neurons on which the axons of the Renshaw cells synapse [4].

Neurogenic Inflammation

Antidromic Vasodilation

The neurotransmitter released at synapses in the spinal cord by most dorsal root ganglion cells is glutamate [8]. However, many small caliber primary afferent fibers, such as nociceptors, also contain and release peptides, such as substance P and/or calcitonin gene-related peptide [9]. Transmitter release is triggered by the invasion of synaptic terminals by nerve impulses, which cause the opening of voltage-gated calcium channels, Ca^{2+} influx into the presynaptic terminals, and exocytosis of synaptic vesicles that contain neurotransmitter [4,8]. Note, however, that this neurotransmitter release mechanism is present not only in the central terminals of dorsal root ganglion cells within the spinal cord but also in the terminals of these sensory neurons in the periphery [10]. This implies that the propagation of nerve impulses in the antidromic direction in sensory neurons will result in the release of transmitter substances in peripheral tissue. What would be the consequence of such an event?

Bayliss [11] stimulated the distal stump of a cut dorsal root electrically so that nerve impulses were conducted toward the periphery. The antidromically propagated action potentials in the sensory axons resulted in vasodilation in the skin. *Antidromic vasodilation* produced in this manner is accompanied by plasma extravasation, which leads to neurogenic edema [12,13]. For antidromic vasodilation and neurogenic edema both to occur, the electrical stimuli have to activate unmyelinated nociceptive sensory axons (C-fibers), although finely myelinated (Aδ) fibers can contribute to antidromic vasodilation [14–16]. Treatment of neonatal rats with capsaicin prevents antidromic vasodilation, indicating that the sensory fibers involved are capsaicin sensitive [13,17]. The vasodilation is attributable chiefly to the release of calcitonin gene-related peptide, although release of substance P probably also contributes, and the neurogenic edema to substance P release [10].

Inflammation

Inflammation is characterized in classical medical terminology by the occurrence of rubor, calor, tumor, and dolor. Inflammation in the skin often depends on an "efferent function" of its "afferent innervation." If inflammation is prevented when the skin is denervated, this type of inflammation is termed *neurogenic inflammation.*

Observations such as those of Bayliss [11] led to the proposal by Thomas Lewis [18] that inflammation of the skin

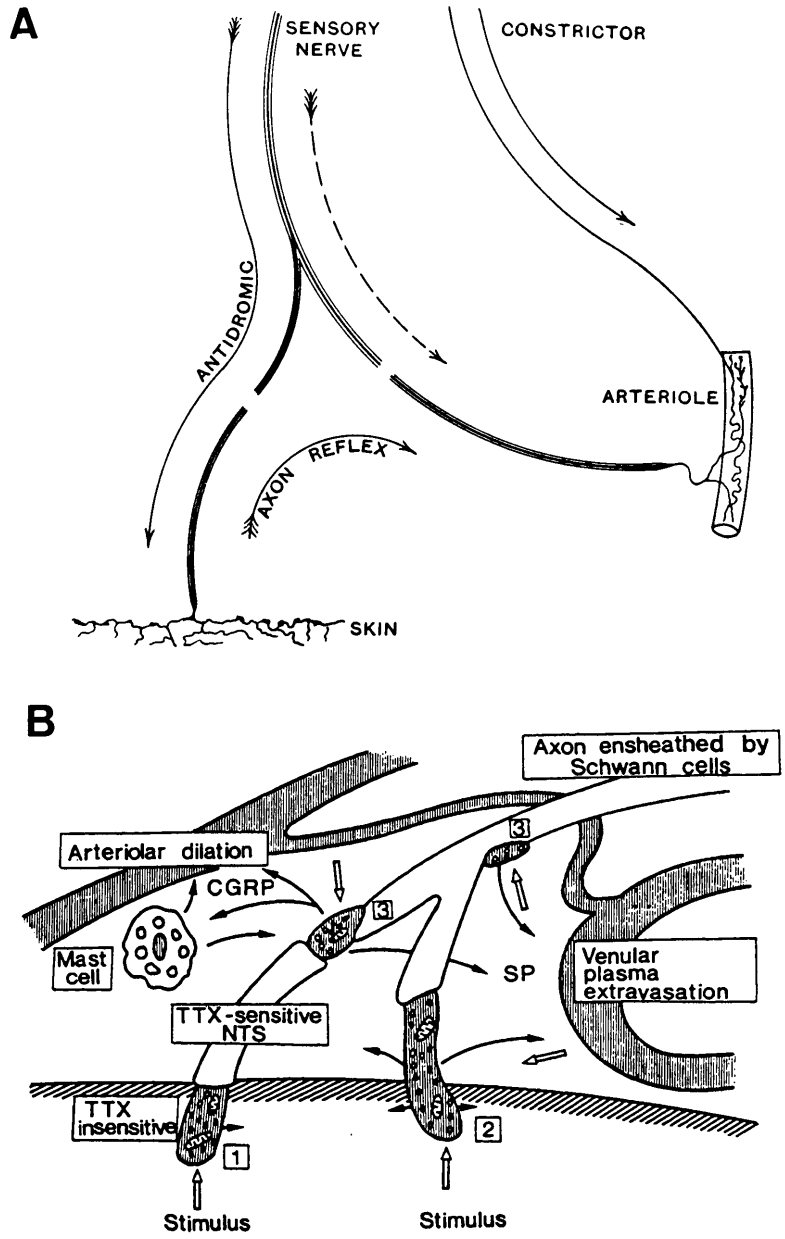


Figure 2 (A) The axon reflex as conceived by Lewis [18]. A sensory axon has terminals in the ski and projects to the spinal cord. However, it gives off a side branch that has endings near an arteriole. Activation of the sensory endings causes a nerve impulse to propagate as an axon reflex through the side branch, where a vasodilator substance is released on the arteriole. Antidromic activation of the same sensory axon by electrical stimulation of the dorsal root through which it enters the spinal cord also causes an axon reflex action on the arteriole. (B) Magnified view of a conceptualization similar to that of (A). A nerve terminal is shown with several sites for the release of the peptides, calcitonin gene-related peptide (CGRP) and substance P (SP). Stimuli applied to the nerve terminals can cause direct release of the peptides without the requirement for action potential generation (i.e., release still occurs in the presence of tetrodotoxin). However, release from the axon away from the terminals depends on propagation of an action potential (axon reflex). The CGRP causes vasodilation (and also stimulates a mast cell), and the SP produces plasma extravasation from a precapillary venule. (Part A reprinted with permission from [17]. Part B reprinted with permission from CRC Press, Boca Raton, Florida.)

is initiated by release of substances from nerve terminals, and that a damaging stimulus could cause not only direct release of substances from the affected nerve terminals but also from branches of these nerve terminals by propagation of an *axon reflex* (Fig. 2A). Although Lewis thought that histamine might be the chemical substance that produced inflammation, as mentioned, it is now believed that calcitonin gene-related peptide is the most important agent producing

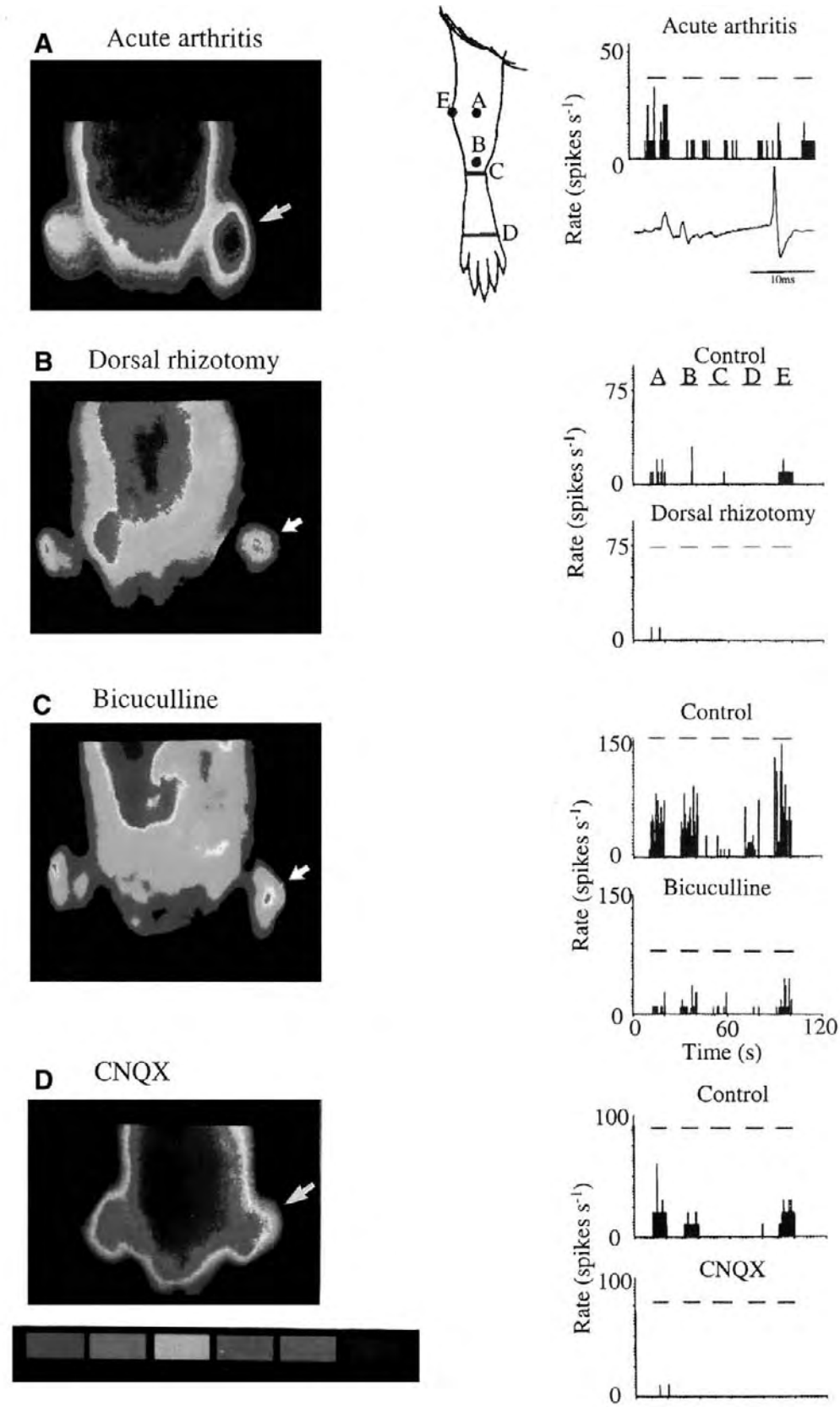


Figure 3 The temperature of the body surfaces of rats was monitored by use of a thermograph. The abdomen and knees of each rat were placed in contact with the thermograph, which displayed the temperature of just these parts of the body surface. An arrow by each thermograph illustrated indicates the knee that had been injected with kaolin and carrageenan to initiate inflammation. The amount of swelling and the temperatures of knee joints on the two sides can be judged by their size and color. The temperature scale at the bottom indicates that the highest

vasodilation and that substance P is responsible for neurogenic edema (Fig. 2B). Other substances that are involved in the progression of inflammation are released by injured cells, including platelets and immune cells, or are synthesized from plasma proteins. Some of these substances include not only histamine, but also serotonin, bradykinin, prostaglandins, and cytokines. A lowered pH and raised temperature are other important factors.

Dorsal Root Reflexes as Retrograde Signals

A form of retrograde signaling that occurs in the spinal cord and brain stem under certain conditions involves the triggering of *dorsal root reflexes.* It is unclear if these represent a physiological or a pathological process. Nevertheless, dorsal root reflexes that occur in certain types of primary afferent neurons can have dramatic peripheral effects that contribute to neurogenic inflammation [19].

Dorsal Root Reflexes and Primary Afferent Depolarization

Dorsal root reflexes are action potentials that are initiated in the axon terminals of dorsal root ganglion cells within the spinal cord. Dorsal root reflexes are triggered by dorsal root potentials [20,21], which are depolarizations that can be large enough to reach threshold in the primary afferent endings [22,23]. The dorsal root reflexes then propagate retrogradely (antidromically) back out the dorsal root and peripheral nerve to the sensory terminals in peripheral tissue (Fig. 1, DRRs) [24,25]. A depolarizing dorsal root potential (or *primary afferent depolarization*) normally results in a form of presynaptic inhibition [4]. However, when dorsal root reflexes occur, enhanced synaptic transmission is seen centrally [26,27], as well as peripheral release of transmitters. Primary afferent depolarization results from the activation of GABAergic interneurons in the spinal cord, and release of GABA causes depolarization of primary afferent terminals through an action on $GABA_A$ receptors (Fig. 1) [28]. This depolarization results from an efflux of Cl^- through Cl^- channels ([29]; also see review by Willis [19]).

Most investigators have assumed that dorsal root reflexes do not occur under normal conditions. However, recently dorsal root reflexes have been recorded from proprioceptive afferents during locomotion under relatively physiological conditions [30]. It was suggested that these nerve impulses might help determine the membrane excitability of the sensory terminals and hence the intensity of sensory discharges during certain phases of the step cycle. However, when dorsal root reflexes occur in fine afferent fibers, especially in C and Aδ nociceptors, peripheral events occur that resemble those that are produced by stimulation of a dorsal root.

Experimental Arthritis

Injection of the irritants kaolin and carrageenan into the knee joint in an experimental animal will result in the development of acute arthritis [31,32]. This arthritis is characterized by swelling of the joint (neurogenic edema) and by an increase in joint temperature (due to vasodilation). As the arthritis develops, dorsal root reflexes can be observed both in joint nerves and in filaments of dorsal root [33–35]. Part of the neurogenic inflammation of the knee joint can be attributed to a direct action of the irritants on the nerve terminals in the joint, which presumably causes the release of active substances from the terminals (see Fig. 2B). However, half of the swelling and part of the elevation in temperature can be prevented when dorsal root reflexes in the joint afferents are blocked by spinal cord administration of either CNQX or bicuculline (Fig. 3) [34,36,37]. Thus, part of the neurogenic inflammation is attributable to dorsal root reflexes.

Acute Inflammation of the Skin by Capsaicin

Similarly, when capsaicin is injected into the skin, a local reaction occurs near the injection site that is probably due to a direct effect of the capsaicin on peripheral endings of cutaneous nerve fibers. However, vasodilation and neurogenic edema also occur in the surrounding area of skin. The vasodilation or "flare" spreads at least 3 cm from the injection site [38]. However, in rats, the peripheral arborization of the terminals of C fibers is only 1–6 mm in diameter [16], and so the extent of the flare cannot be explained on the basis of axon reflexes. Intradermal injection of capsaicin results in powerful dorsal root reflexes that can be demonstrated in fine primary afferent fibers, including both C and Aδ fibers, but not in the large Aβ fibers (Fig. 4) [39].

If the appropriate peripheral nerves or dorsal roots are cut before the capsaicin injection, the flare is blocked (Fig. 5A) [38]. This surgery would prevent the spread of dorsal root reflexes into the nerve endings, but it should not interfere

Figure 3, *cont'd* temperature is signified (counterintuitively) by blue and the lowest by red. Note that there is a large difference between the size and temperature of the inflamed knee and the contralateral knee of an untreated, arthritic rat (A) but that the knees are nearly symmetrical when the dorsal roots were cut prior to the knee injection (B) or when the animals were pretreated by spinal cord administration of bicuculline (C) or CNQX (D). Therefore, cutting the dorsal roots or administering drugs to the spinal cord that block $GABA_A$ or glutamate receptors prevented much of the inflammation of the knee. The drawing of the rat hind limb at the top center shows the sites stimulated to evoke the dorsal root reflexes (A–E) shown in the right panels. In each of these panels, a peristimulus time histogram shows the dorsal root reflexes that resulted from stimulation at sites A–E. The trace below the uppermost panel illustrates dorsal root reflexes recorded from the medial articular nerve on the injected side in an untreated, arthritic rat. The other panels show the dorsal root reflexes recorded before and after dorsal rhizotomy or spinal administration of bicuculline or CNQX. (Reprinted with permission from [40].)

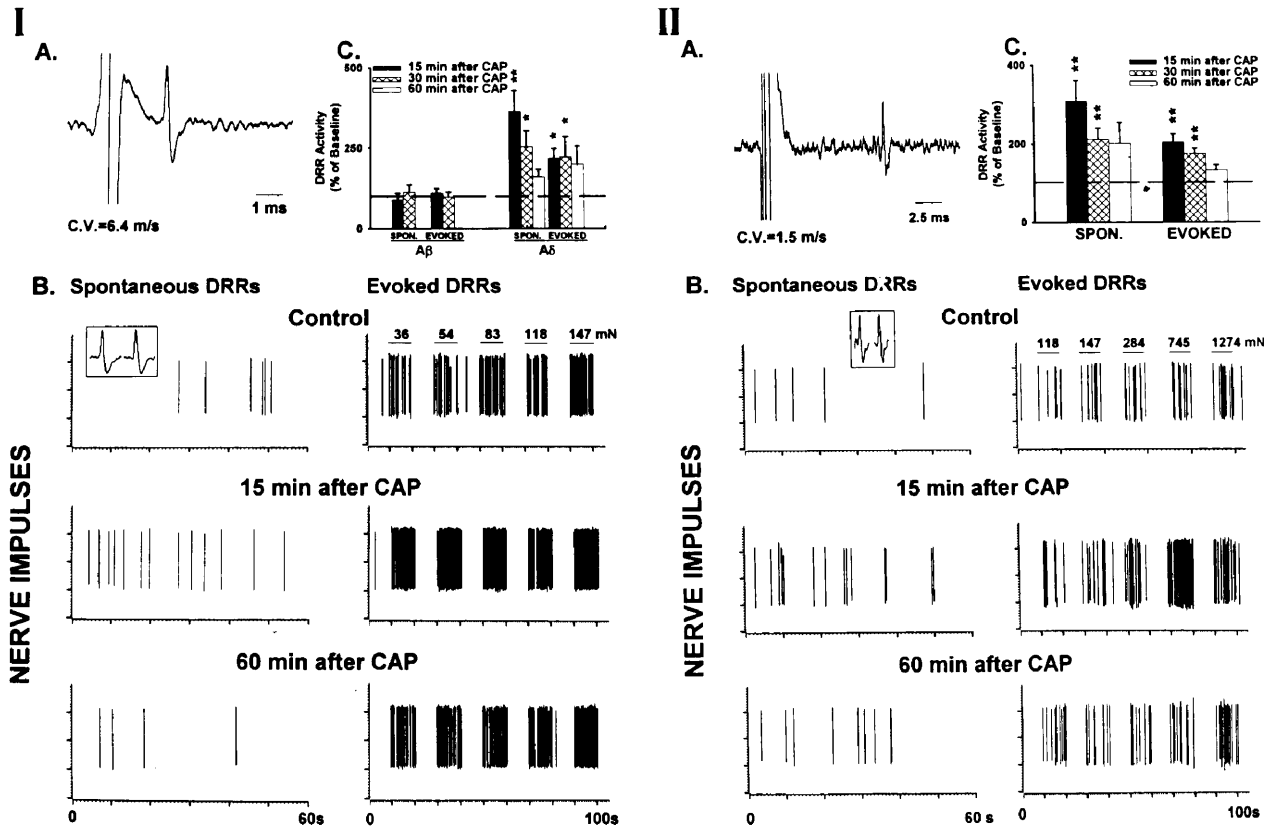


Figure 4 Changes in spontaneous and evoked dorsal root reflex activity before and after intradermal injection of capsaicin. (I) The recordings in (A) and (B) were from an Aδ fiber in a dorsal root filament (cut distally; other filaments of the dorsal root and adjacent dorsal roots were left intact). The conduction velocity of the axon was determined to be 6.4 m/sec based on the latency of the action potential following stimulation of the dorsal rootlet near the spinal cord in (A) and the measured conduction distance. The summary bar graphs in (C) show the increases in both the spontaneous and evoked dorsal root reflexes in Aδ ($n=11$) and the absence of such changes in Aβ fibers ($n=6$). (II) The responses of a C fiber recorded in the same way and summary histograms for recordings from C fibers ($n=10$). (Reprinted with permission from [39].)

with "axon reflexes." The flare can also be prevented by spinal cord administration of antagonist drugs that block $GABA_A$ receptors (bicuculline) or receptors for excitatory amino acids (CNQX, AP7), as shown in Fig. 5B. This can be explained by prevention of primary afferent depolarization by blocking responses of $GABA_A$ receptors and by prevention of the activation of GABAergic interneurons by sensory volleys that enter the spinal cord from the skin and release glutamate to excite these interneurons. The neurogenic edema that occurs following capsaicin injection is also blocked by these surgical and pharmacological interventions, as are the dorsal root reflexes evoked by the injection [38].

Conclusions

Neurons are designed to communicate rapidly with other neurons and with effectors so that behavior can be adjusted quickly. Intercellular communication in the nervous system involves synaptic transmission between individual elements of a neural network. Propagation of information in such a network is generally unidirectional. However, instances are known of retrograde signaling. What may prove to be an important peripheral mechanism is the production of dorsal root reflexes triggered in primary afferent nociceptive neurons during inflammation. Dorsal root reflexes depend on a synaptic process known as primary afferent depolarization, which results from the release of γ-aminobutyric acid onto the terminals of the afferent fibers in the spinal cord and an action on $GABA_A$ receptors. Normally, the depolarization is subthreshold, but when it is large enough to exceed threshold for the afferent endings, nerve impulses are triggered, and these propagate retrogradely to the periphery. Damage causes an increase in the probability of dorsal root reflexes. The peripheral terminals of the nociceptive afferents then release neurotransmitters, including substance P and calcitonin gene-related peptide, which cause vasodilation and neurogenic edema. Experiments in which dorsal root reflexes are blocked by spinal cord administration of drugs that prevent GABAergic transmission interfere with the development of inflammation in the knee joint and in the skin, supporting the hypothesis that the retrogradely transmitted dorsal root reflexes contribute to neurogenic inflammation.

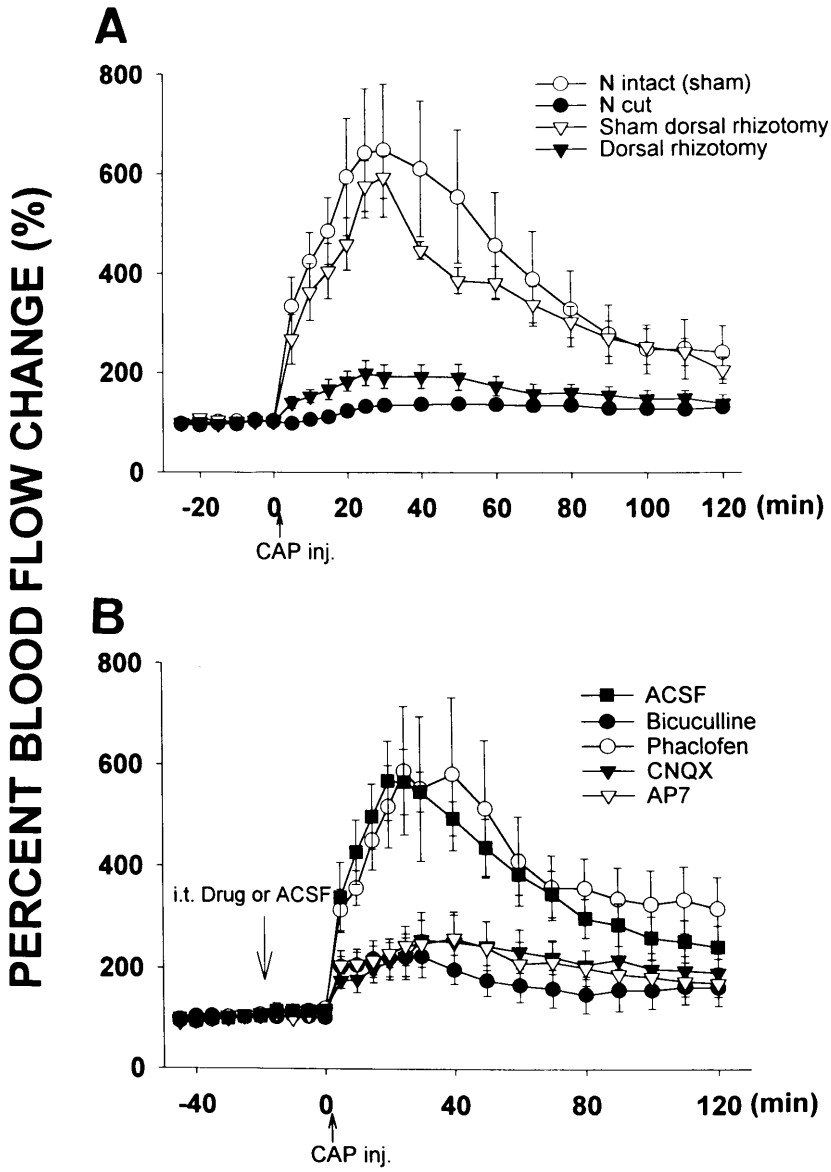


Figure 5 Blood flow changes following intradermal injection of capsaicin. Blood flow was measured by a laser Doppler blood flow probe placed on the plantar skin of the foot 15–20 mm distal to the site of capsaicin injection. The blood flow after capsaicin is presented as a percentage of that recorded before the injection. The experiments in (A) involved surgical procedures, whereas those shown in (B) involved pretreatment with an intrathecal infusion of a drug. Control groups had either sham surgery or intrathecal administration of artificial cerebrospinal fluid (ACSF). (A) shows that cutting the appropriate peripheral nerves or performing dorsal rhizotomies prevented most of the blood flow change induced by capsaicin injection. (B) shows that intrathecal pretreatment with the $GABA_A$ receptor antagonist, bicuculline, the non-NMDA glutamate receptor antagonist, CNQX, or the NMDA receptor antagonist, AP7, reduced the blood flow change, whereas the $GABA_B$ receptors antagonist, phaclofen, did not. (Reprinted with permission from [38].)

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CHAPTER 349

Cytokines and Cytokine Receptors Regulating Cell Survival, Proliferation, and Differentiation in Hematopoiesis

Fiona J. Pixley and E. Richard Stanley

General Aspects of Hematopoiesis

Blood contains red cells, megakaryocytes, lymphocytes, monocytes, and the various types of granulocytes. All mature blood cell types turn over rapidly, requiring active synthesis of large numbers of new cells to maintain a steady state. Differentiated blood cells are all ultimately derived from a small pool of undifferentiated, pluripotent hematopoietic stem cells. This process, involving extensive cell proliferation and differentiation, is known as *Hematopoiesis* and begins in the embryonic yolk sac (primitive hematopoiesis) before maturing into definitive hematopoiesis in the fetal liver and adult bone marrow. Hematopoiesis is regulated by a large number of cytokines that are present in the microenvironment. Specific subsets of these cytokines influence each step in the process. This chapter reviews our current knowledge of the biology of adult hematopoiesis with an emphasis on the cytokines and their target signaling pathways important in steady-state maintenance of nonlymphoid cell lineages. Due to page limitations, only selected papers or comprehensive reviews are referenced.

Till and McCulloch [1] introduced the concept of a hematopoietic stem cell with the capacity to (1) self-replicate, (2) proliferate to produce many cell progeny, and (3) differentiate to generate all the mature blood cell types. The pool of such stem cells, which are normally quiescent or cycling slowly, represents only 10^{-5} of the total nucleated bone marrow cells in the mouse. Upon division, a stem cell gives rise to an indistinguishable daughter cell, and a daughter cell proliferates extensively and differentiates to give rise to common myeloid and lymphoid progenitor cells, which, as they proliferate and differentiate, progressively develop a more restricted capacity for differentiation, eventually giving rise to cells that are capable of forming only one mature blood or lymphoid cell type [2] (Fig. 1).

Many different cytokines in the microenvironment of the bone marrow stimulate the development of cells of different lineages. These growth factors may be circulating or bound to either the surface of their producing cells or to the extracellular matrix. Under the influence of specific cytokines, progenitor cells can also proliferate and differentiate in semisolid culture to form macroscopic colonies of differentiated cells, hence the term *colony-stimulating factor* (CSF) for the responsible growth factor [3]. The analysis of mice with targeted inactivations of the genes encoding most of these cytokines and their receptors has greatly increased our understanding of the biology of hematopoiesis. The dominant role of cytokines in the regulation of hematopoiesis has led to their rapid clinical application in maintaining normal hematopoiesis in the face of a variety of pathological conditions.

Hematopoietic Cytokines

The hematopoietic cytokines are glycoproteins, which are either constitutively present in the circulation (e.g., CSF-1, SCF, FL, G-CSF, EPO, and TPO) or induced to appear in

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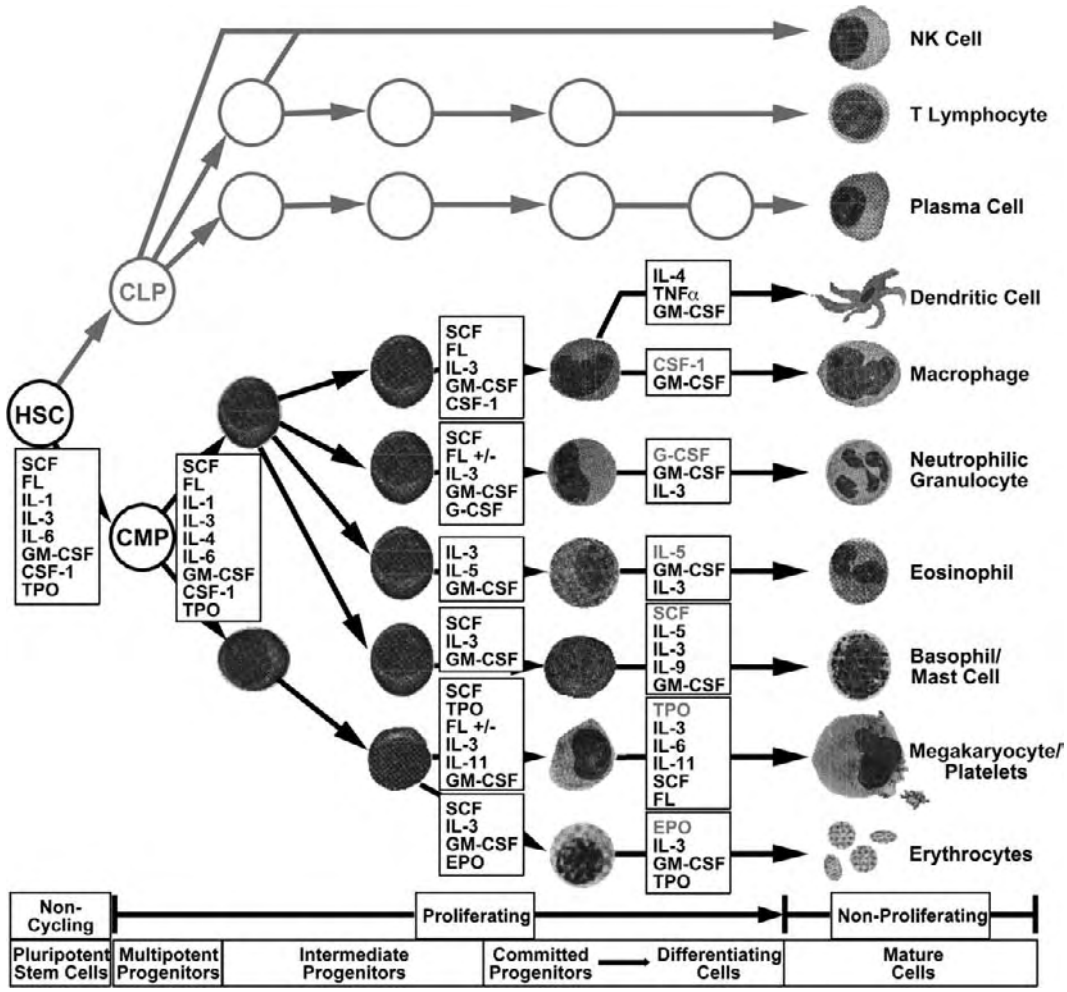


Figure 1 Hematopoietic cells, indicating the points of regulation by hematopoietic cytokines. The primary cytokines regulating proliferation and differentiation of committed progenitors of individual lineages are colored in red. Abbreviations: HSC, hematopoietic stem cell; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; SCF, stem cell factor; FL, flt ligand; IL, interleukin; GM-CSF, granulocyte/macrophage colony-stimulating factor; CSF-1, colony-stimulating factor 1; G-CSF, granulocyte-CSF; TPO, thrombopoietin; EPO, erythropoietin; TNF, tumor necrosis factor. (A color representation of this figure is available on the CD version of the *Handbook of Cell Signaling*.)

response to infection or inflammation (e.g., GM-CSF, IL-3, IL-5, IL-6, and IL-11), and their concentrations may be increased in response to specific triggering conditions such as EPO, which is increased in response to hypoxia and/or anemia (Table I).

Primitive multipotent hematopoietic cells coexpress different lineage-specific cytokine receptors at low levels require a combination of cytokines (e.g., SCF, IL-1, IL-3, IL-6, GM-CSF, and CSF-1) for lineage commitment. As these cells differentiate, they lose receptors for some cytokines (e.g., SCF or IL-3), while increasing expression of receptors for the late-acting cytokines (e.g., CSF-1 or EPO). When they reach the stage of committed progenitor cell, their further proliferation and differentiation is along one particular lineage and is regulated by one or more late-acting cytokines. Within specific lineages, the most primitive cells respond by both proliferating and differentiating (e.g., committed macrophage progenitors → monoblasts → promonocutes → monocytes → macrophages), whereas differentiating, nondividing cells (e.g., peritoneal macrophages) require the cytokines for survival, activation, and function. CSF-1 and GM-CSF, for example, prime mature cells for neutrophil and macrophage activation by other agents [3]. Despite an apparent overlap in target cell specificity of several cytokines, their functions are largely nonredundant as indicated by the distinct hematopoietic phenotypes of cytokine or receptor nullizygous mice (Table I). Transcription factors such as GATA-1 and PU.1, which regulate cytokine receptor expression, are also important in hematopoietic cell commitment.

The "permissive" model of hematopoietic cell regulation by cytokines is one in which the growth factor does not have a role in multipotent progenitor cell commitment but simply allows the survival and proliferation of committed cells. In contrast, the "instructive" model posits that specific cytokines direct multipotent progenitors to become committed to a specific lineage. It is uncertain whether cytokine regulation of differentiation is simply permissive or instructive since there is good evidence for both mechanisms. Both may be

Table I Hematopoietic Cytokines

Cytokine	Sources of cytokine- or cytokine-receptor	Primary Hematopoietic Phenotype deficient mice[a]
SCF	Bone marrow stroma, Fibroblasts, Placenta, Others	Severe macrocytic anemia
FL	Ubiquitous	NK cell and dendritic cell deficiencies
CSF-1	Endothelial cells, Fibroblasts, Uterine epithelium, Macrophages	Osteopetrosis
EPO	Kidney proximal tubular cells, Liver	Severe anemia
G-CSF	Activated bone marrow stroma, Macrophages, Fibroblasts, Endothelial cells	Neutropenia
TPO	Hepatocytes, Endothelial cells Fibroblasts	Thrombocytopenia
IL-3	Activated T cells	Reduced delayed hypersensitivity
GM-CSF	Bone marrow stroma, Activated T cells, Endothelial cells, Fibroblasts, Macrophages	Pulmonary alveolar proteinosis
IL-5	Activated helper T cells	Eosinophil deficiency
IL-2	Activated T cells	Autoimmune disease
IL-15	Ubiquitous, increased by activation	Lymphopenic, deficient in NK, NK-T, CD8+ cells and gδT cells
IL-7	Fetal liver, bone marrow and thymic, stromal cells, lymphoid cells, others	Reduces T and B cells
IL-4	TN2 and NK1.1+ T cells, mast cells, basophils and eosinophils	TN2 deficient
IL-9	TN2 cells, mast cells and eosinophils	Pulmonary mastocytosis and goblet cell hyperplasia
IL-6	Ubiquitous, in response to inflammatory stimuli	Reduced T cells, IgG & IgA responses, impaired neutrophil/macrophage function
IL-11	Ubiquitous, in response to inflammatory stimuli	Embryonic Lethal
LIF	Monocytes, bone marrow stroma	None reported

[a]References for these cytokine and cytokine receptor-deficient mice, except for the IL-9 deficient mouse [17] are listed in [3].

utilized depending on the receptors and commitment steps involved [3].

The phenomenon of synergism between predominantly late-acting, lineage-restricted cytokines, such as CSF-1, EPO, and G-CSF, with predominantly early-acting cytokines such as SCF, in stimulating the proliferation and differentiation of primitive multipotent cells provides a mechanism for coupling the changes in levels of the late-acting cytokine. These late-acting cytokines are tightly regulated by the primary stimuli, to the channeling of multipotent cells into a lineage in order to satisfy the demand for differentiated cells. The mechanisms underlying synergism between cytokines in the regulation of primitive hematopoietic cell proliferation can occur directly at the level of the receptors for the synergizing cytokines or be due to synergistic effects between postreceptor signal transduction pathways [3].

Signaling through Cytokine Receptors

General

Hematopoietic cytokine action on target cells is mediated by specific, high-affinity, cell-surface receptors that signal for progenitor cell survival, proliferation, and differentiation, and mature cell survival and activation. Cytokine receptors can be classified according to the presence of either intrinsic or associated tyrosine kinase activity, the structure of the extracellular domain (ECD), and the requirement for common shared receptor subunits (Fig. 2). The end result of any cytokine binding to its cognate receptor is the phosphorylation of particular receptor intracellular domain (ICD) tyrosine residues that act as binding sites for downstream signaling molecules. Different activated receptor cytoplasmic

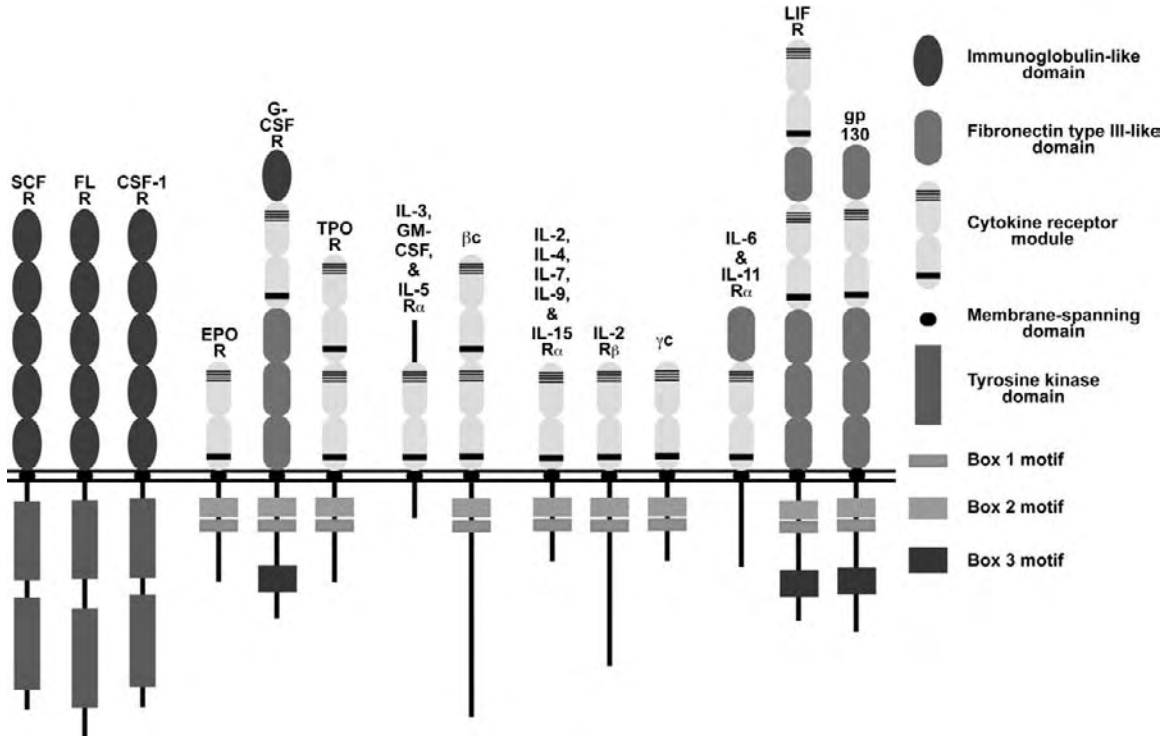


Figure 2 Schematic of selected high-affinity hematopoietic cytokine receptors showing the similarity between those containing intrinsic tyrosine kinase domains (left) and the modular nature of the cytokine receptor family that are preassociated with cytoplasmic tyrosine kinases.

domains often bind a common signaling molecule or family of signaling molecules. Targeted downstream pathways include those regulating gene transcription, protein translation, actin cytoskeletal remodeling, and cell adhesion and motility [3–5].

Tyrosine Kinase Receptors

The three hematopoietic cytokines signaling through tyrosine kinase receptors, stem cell factor (SCF), flt3 ligand (FL), and colony-stimulating factor 1 (CSF-1), are members of a family of homodimeric cytokines that share some sequence and structural similarity. SCF and CSF-1 have been shown to have effects on nonhematopoietic as well as hematopoietic cells.

The SCF, CSF-1, and FL receptors, all members of the PDGF receptor family, possess ECDs comprised of five heavily glycosylated immunoglobulin-like repeats, a transmembrane domain, and an intracellular tyrosine kinase domain that is interrupted by a kinase insert domain. Binding of their cognate bivalent ligands by this class of receptors stabilizes their noncovalent dimerization, permitting receptor activation and trans-tyrosine phosphorylation of one ICD by the other. The receptor phosphotyrosines act as "docking sites" for *src* homology region 2 (SH2) and other phosphotyrosine binding domains of signaling and adaptor proteins that bind the receptor and may themselves become tyrosine phosphorylated (Fig. 3A). Many of the signaling pathways activated by these receptors, including the MAP kinase (MAPK) cascade, the JAK/STAT pathway, Src family members, and PI3-kinase, are shared. Two of the receptors, SCFR and CSF-1R, are encoded by the protooncogenes c-*kit* and c-*fms*, respectively. The v-*kit* and v-*fms* oncogenes are present in feline sarcomatous retroviruses and contain mutations of the normal cellular genes that render their encoded receptors constitutively active in the absence of cytokines.

SCF AND THE SCF RECEPTOR (SCFR)

SCF influences development in the three different lineages involving pigmentation, hematopoiesis, and fertility. It is widely expressed, as both secreted and cell surface forms, during embryogenesis and in a variety of adult tissues, including bone marrow stromal cells, fibroblasts, and endothelial cells, as well as the yolk sac and placenta. Cells expressing the SCFR are frequently contiguous with SCF-expressing cells and include germ cells, interstitial cells of Cajal in the gut, melanocytes, and early hematopoietic cells. Hematopoetic cell SCFR expression is low in very primitive multipotent progenitors, highest in committed progenitors, and decreases as cells mature. Because pluripotent stems cells do not appear to express the SCFR and can survive *in vitro* in the absence of SCF, it may not act on the earliest hematopoietic precursors. Although SCF has a broad spectrum of activity on hematopoietic cells, it has little activity alone but acts synergistically with many of the hematopoietic cytokines, especially IL-6 and IL-3, to increase the numbers of precursors of most, if not

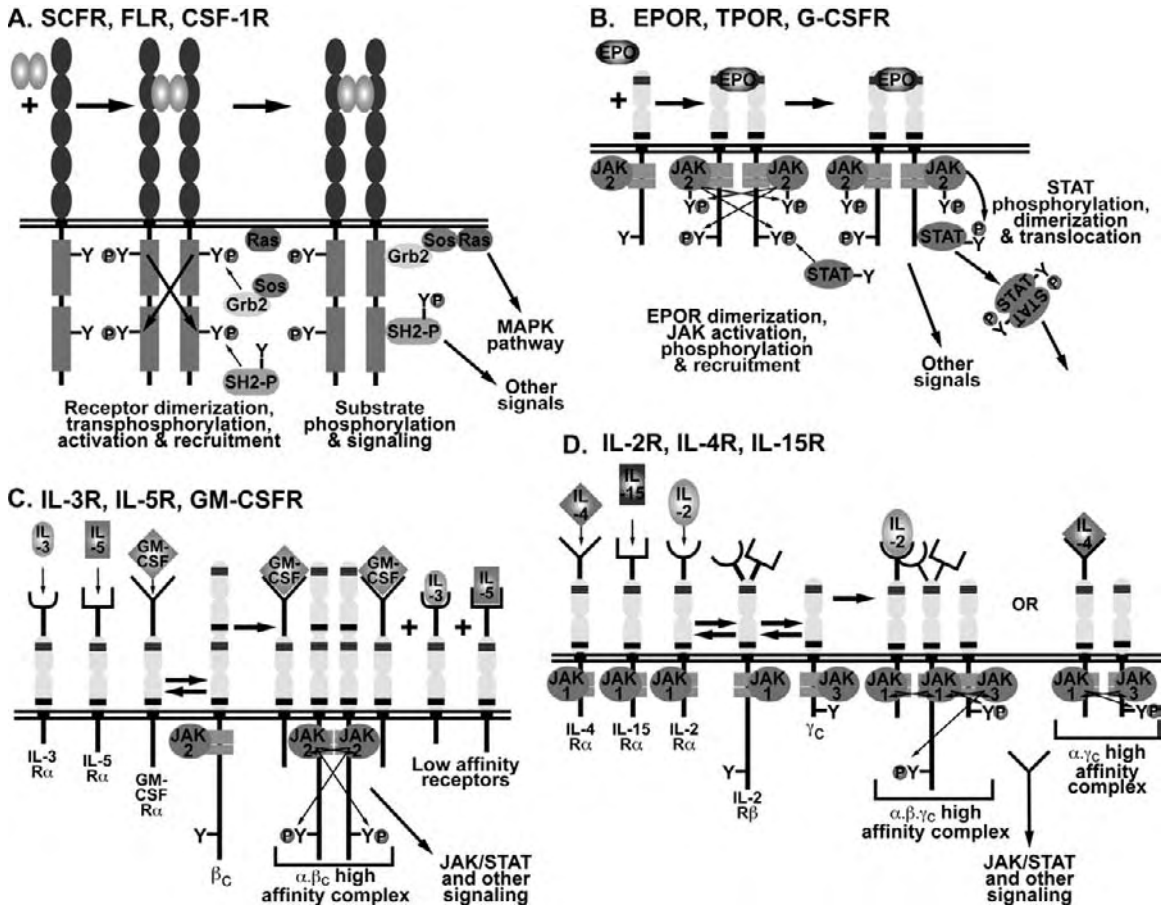


Figure 3 Models for the activation and signaling of hematopoietic growth factor receptors. (A) Homodimeric receptors possessing intrinsic tyrosine kinase domains; SH2-P, SH2 domain- or PTB domain-containing signaling molecules. (B) Homodimeric cytokine family receptors. (C) Multimeric cytokine receptors sharing the common β subunit, βc, illustrating the concept of cross-competition between cytokines for the β subunit that is necessary for the formation of the high-affinity signaling complex. GM-CSF is shown forming the high-affinity complex. (D) Receptors sharing the common γ subunit, γc, form a high-affinity $\alpha \cdot \gamma c$ receptor complex (e.g., IL-4) or a high-affinity $\alpha \cdot \beta \cdot \gamma c$ receptor complex utilizing a second signaling subunit, IL-2β, (e.g., IL-2, IL-15).

all, lineages. Spontaneously occuring SCF-null *Sl/Sl* mice are embryonic lethals, but a partially functional Sl^d allele allows embryonic survival, yielding severely anemic and mast-cell-deficient mice, indicating that the major effects of SCF are on erythropoiesis and mast cell development. In combination with EPO, SCF enhances the number of erythroid precursor cells generated from primitive multipotent cells and allows precursors to respond to levels of EPO that are too low to elicit a response in the absence of SCF. Mast cells and their progenitors require SCF throughout the differentiation of the lineage from early precursors to mature, primed tissue mast cells. There is also strong evidence of biologic activity for SCF on the megakaryocyte, granulocyte/macrophage, and lymphoid lineages, yet the effects of the absence of SCF on their development is minimal, implying some redundancy in cytokine action on these lineages [6,7].

FL AND FLT3

FL, which occurs largely as a cell-surface, noncovalently associated homodimer, regulates the proliferation of primitive hematopoietic cells. It is widely expressed while hematopoietic expression of its cognate receptor, flt3, is predominantly restricted to the progenitor/stem cell compartment. Like SCF, FL alone cannot stimulate the proliferation of its primitive hematopoietic target cells, but rather synergizes with other hematopoietic cytokines. In contrast to SCF, however, there is little or no effect of FL on erythroid, or megakaryocyte progenitor cells, because flt3-deficient mice have no defects in red cell, megakaryocyte, or platelet production. In combination with GM-CSF, TNF, and IL-4, FL enhances the production of dendritic cells, and dendritic cell numbers are reduced in FL-deficient mice [7].

CSF-1 AND THE CSF-1 RECEPTOR (CSF-1R)

CSF-1 regulates the survival, differentiation, and function of cells of the mononuclear phagocytic (monocyte/macrophage) lineage and the function of cells of the female reproductive tract. Mature forms of the disulfide-linked CSF-1 homodimer include a secreted glycoprotein, a secreted proteoglycan, and a cell-surface glycoprotein. CSF-1 is synthesized by a variety of cell types, including fibroblasts, endothelial cells, bone marrow stromal cells, osteoblasts,

keratinocytes, astrocytes, myoblasts, and breast and uterine epithelial cells. Circulating CSF-1 is elevated in response to bacterial, viral, and parasitic infections and is primarily cleared by Kupffer cells, so the number of sinusoidally located macrophages determines the concentration of the cytokine responsible for their production, a simple feedback control. CSF-1 homodimer binding by the CSF-1R results in the formation or stabilization and activation of a receptor dimer. After activation and tyrosine phosphorylation of signaling molecules, most of the receptor–ligand complexes are internalized, ubiquitinated, and destroyed.

The osteopetrotic (*Csf-1^{op}/Csf-1^{op}*) mouse, which possesses an inactivating mutation in the CSF-1 gene, exhibits impaired bone resorption associated with a paucity of osteoclasts, no incisors, poor fertility, a lower body weight, a shortened life span, and deficiencies in blood monocytes and macrophages in most tissues, indicating that CSF-1 is the primary regulator of mononuclear phagocytes. Restoration of circulating CSF-1 in newborn *Csf-1^{op}/Csf-1^{op}* mice cures their osteopetrosis, monocytopenia, and some but not all of the tissue macrophage populations, demonstrating additional local regulation by CSF-1. CSF-1 regulates the development of macrophages with trophic and scavenger (i.e., physiological) functions, whereas the development of macrophages involved in inflammatory and immunologic (i.e., pathological) functions such as lymph node and thymic macrophages apparently depends on other cytokines. CSF-1 synergizes with other cytokines (e.g., IL-1, SCF, IL-3, IL-6) to stimulate the proliferation and differentiation of multipotent cells to committed macrophage progenitors that respond to CSF-1 alone [8,9].

Cytokine Signaling by Homodimerization of a Single, Non-Tyrosine Kinase Receptor Polypeptide Chain

Erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), and thrombopoietin (TPO) signal by initiating homodimerization of their cognate non-tyrosine kinase receptors, although additional receptor subunits may be involved. These receptors belong to a large family of receptors, the cytokine receptor family, defined by the presence of an ECD cytokine receptor module (Fig. 2). These modules contain conserved short amino acid sequence elements, particularly two pairs of conserved cysteine residues near the amino terminal end of the motif and a Trp-Ser-X-Trp-Ser (WSXWS) sequence, the function of which is not clear, near the transmembrane domain. The EPOR, G-CSFR, and TPOR each contain conserved motifs in their ICDs that mediate constitutive association with members of the cytosolic Janus kinase (JAK) family (Figs. 2 and 3B). The associated JAKs (JAK2 with EPOR and TPOR; JAK1 with G-CSFR) are activated by formation of the ligand–receptor complex, leading to their tyrosine phosphorylation and to tyrosine phosphorylation of the signaling subunit. This creates docking sites for molecules with protein tyrosine binding (PTB) or SH2 domains, and to phosphorylation and activation of these recruited signaling molecules. Recruited molecules include the signal transducers and activators of transcription (STATs), additional JAKs, and other cytosolic tyrosine kinases and SH2 domain-containing protein tyrosine phosphatases. The MAPK pathway and PI3 kinase are also activated in response to these cytokines.

EPO AND THE EPO RECEPTOR (EPOR)

Epo is the primary regulator of erythropoiesis and is synthesized primarily by the proximal convoluted tubules of the kidney in response to hypoxia. Expression and homodimerization of the EPOR is necessary and sufficient for cell responsiveness to EPO although, to facilitate the synergistic interaction of EPO and SCF, it functionally and physically interacts with the SCFR and possibly also with the IL-3R β chain. EPOR expression, which is correlated with the proliferative and differentiation effects of EPO in erythroid lineage cells, increases from BFU-E to CFU-E and erythroblasts (Fig. 1) before decreasing again during the terminal stages of differentiation. EPO also stimulates the release of maturing normoblasts from the bone marrow and increases the amount of hemoglobin synthesized per erythrocyte [10].

G-CSF AND THE G-CSF RECEPTOR (G-CSFR)

G-CSF, which is a monomer that shares sequence homology with IL-6, is synthesized by a variety of cell types, including stromal cells, fibroblasts, and endothelial cells, usually in response to inflammatory stimuli such as lipopolysaccharide (LPS), tumor necrosis factor (TNF), and IL-1. The large ECD of its receptor contains one immunoglobulin and three fibronectin domains as well as the cytokine receptor module, and it binds G-CSF with high affinity in a 2 : 2 stoichiometry. G-CSF is the physiological regulator of neutrophil production, stimulating the proliferation and differentiation of committed neutrophil progenitor cells without affecting other granulocytic lineages, and its levels are increased during infection. It also synergizes with IL-3 or SCF to stimulate the proliferation and differentiation of primitive multipotent hematopoietic progenitor cells. However, mice lacking G-CSF or its receptor, while neutropenic, possess some nature neutrophils and have only a mild reduction in committed granulocyte-macrophage progenitor cells, suggesting that G-CSF is not necessary for neutrophil lineage committment. It also enhances the survival of mature neutrophils and may prime their functional responses [3].

TPO AND THE TPO RECEPTOR (TPOR)

Structurally, TPO can be divided into two domains, an amino terminal portion with EPO homology and a carboxy terminal domain that is widely species divergent and lacks homology with other known proteins. It is synthesized primarily by hepatocytes, endothelial cells, fibroblasts, and the proximal tubule cells of the kidney. The TPOR gene, *c-mpl*, was originally identified as the cellular counterpart of *v-mpl*, the oncogene carried by the murine myeloproliferative leukemia virus, and is restricted in its expression to megakaryocytes, platelets, and primitive hematopoietic cells. TPO affects all aspects of megakaryocyte and platelet development, including stimulation of megakaryocyte progenitor cell

proliferation and differentiation, and stimulation of platelet release. It has also been shown to stimulate proliferation of hematopoietic stem cells. Mice lacking either TPO or TPOR possess an identical phenotype and exhibit an 80–90% decrease in platelets with no effect on numbers of other differentiated blood cells. However, despite their selective thrombocytopenia, both mutant mice also exhibit a 60% reduction in multipotent and committed myeloid progenitors and are deficient in stem cells capable of long-term repopulation [11].

Cytokines Signaling through Receptors with a Common β subunit

The three cytokines, interleukin-3 (IL-3), granulocyte/macrophage CSF (GM-SCF), and interleukin-5 (IL-5), which signal through receptors sharing a common β subunit, are members of a subfamily of cytokines that appear to share a common ancestry despite their lack of homology at the amino acid level. Their genes have a similar structure and map closely together on chromosome 5. The cytokines signal through receptors comprised of a cytokine-specific α chain, which alone exhibits low affinity for the cytokine, and a larger, shared β chain, βc, that can interact with any of the three α chain–cytokine complexes to generate a specific, high-affinity complex (Fig. 3C). Ligand-induced disulfide bonding between an α and β subunit and dimerization of the βc subunit are required for signaling, producing a complex consisting of two receptor α chains, two βc chains, and two ligand molecules. The shared βc leads to competition between IL-3, GM-CSF, and IL-5, although the biological significance of this is not well understood. Their α subunit ECD cytokine receptor module structures are similar to that of EPOR (Fig. 2), and the divergent ICDs are required for proper receptor function. The 120-KDa β subunit contains two ECD cytokine receptor motifs and a long cytoplasmic tail that is required for proliferative signaling. Box 1 and box 2 motifs in the ICD contain the docking sites necessary for the association of JAK2 and recruitment of other signaling molecules to the activated receptor resulting in rapid tyrosine phosphorylation of several cellular proteins, including the β subunit of the receptor itself (Fig. 3C) [12].

IL-3 AND THE IL-3 RECEPTOR (IL-3R)

IL-3 is a secreted monomeric glycoprotein that is synthesized almost exclusively by T cells in response to antigen stimulation. The IL-3R consists of a unique IL-3-specific, low-affinity α subunit and the βc subunit. However, a duplication of the βc gene has occurred in the mouse to produce βIL-3, which interacts with IL-3Rα to create an additional low-affinity receptor for IL-3. IL-3 is a pleiotropic hematopoietic cytokine supporting the proliferation and differentiation of both primitive multipotent progenitor cells and committed myeloid progenitors. Working alone, it stimulates primitive hematopoietic cells to form multilineage colonies, comprised of neutrophils, basophils, eosnophils, monocytes, and megakaryocytes. In combination with SCF, IL-1, and CSF-1, IL-3 stimulates the proliferation and differentiation of even more primitive precursors and, in concert with other late-acting hematopoietic cytokines, it stimulates multipotent cells to specific lineage commitment. For example, the combination of IL-3 and CSF-1 allows the proliferation of primitive cells that do not respond to CSF-1 alone to give rise to committed, CSF-1R-expressing macrophage progenitors. Once committed, progenitor cells and their progeny lose expression of IL-3R and their ability to respond to IL-3. Since IL-3 synthesis is highly restricted and regulated and IL-3-deficient mice display only a diminished delayed hypersensitivity with no obvious steady-state hematopoietic phenotype, it seems to be important only during hematopoietic demand [3].

GM-CSF AND THE GM-CSF RECEPTOR (GM-CSFR)

GM-CSF, a monomeric glycoprotein, is constitutively synthesized by macrophages, endothelial cells, and fibroblasts and inducibly expressed in a variety of cells, especially T cells. The GM-CSFR α subunit has three alternative transcripts used to produce either the main form, a soluble form, and an alternative membrane-spanning subunit with an elongated C terminus. All are functional, but their relative physiological significance is not yet well understood. GM-CSF has been shown to have a broad range of biological effects, acting on both progenitor cells and mature, terminally differentiated cells. The survival, proliferation, and differentiation of all stages of cells in the neutrophil, macrophage, and eosinophil lineages are supported by GM-CSF and it appears to be important in the activation and enhancement of function of mature cells in these lineages. However, at most stages of development, each lineage also requires the presence of the other lineage-specific cytokines, G-CSF, CSF-1, and IL-5. The antigen-presenting dendritic cells appear to be derived from myeloid lineage and possibly also from lymphoid lineage cells. GM-CSF, with IL-4 and TNFα, stimulates myeloid-derived predendritic cells to differentiate into mature dendritic cells. GM-CSF also synergizes with EPO and TPO on primitive hematopoietic cells to generate erythroid and megakaryocytic progeny, respectively, and *in vivo* GM-CSF administration increases the number of circulating neutrophils, monocytes, and eosinophils and the number of tissue-fixed macrophages. Nevertheless, since GM-CSF-deficient mice have normal granulocyte and macrophage production in both steady-state and stressed conditions, GM-CSF apparently does not play an important role in blood cell production [13].

IL-5 AND THE IL-5 RECEPTOR (IL-5R)

Biologically active IL-5 is a dimer that is secreted predominantly by antigen-stimulated T lymphocytes, but also by NK cells, mast cells, B cells, eosinophils, and endothelial cells. However, only eosinophils and basophils, precursors of both lineages, and some B cells express both subunits of the IL-5R, so IL-5 has a restricted biological activity. It is the primary late-acting cytokine for eosinophil proliferation and differentiation, for mature eosinophil survival and activation, and *in vivo*, in the development of eosinophilia. Complete abrogation of the development of eosinophilia secondary to parasitic infections is observed in IL-5-deficient mice.

In combination with GM-CSF, IL-3, and IL-4, IL-5 stimulates the survival, proliferation, and differentiation of the basophil-mast cell lineage [14].

Cytokines Signaling through Receptors with a Common γ Subunit

Five hematopoietic cytokines, IL-2, IL-4, IL-7, IL-9, and IL-15, signal through a high-affinity receptor comprised of a cytokine-specific α chain and a common γ chain (γc), in a manner similar to IL-3, GM-CSF, and IL-5 and their common βc, in that the cytokine binds the α subunit with low affinity and requires further binding of the γc for high-affinity α·γc complex signaling (Fig. 3D). Additionally, IL-2R and IL-15R, which probably have a common ancestry and constitute a separate cytokine receptor subfamily, also share a common β subunit, IL-2Rβ (Fig. 2), which can directly bind both ligands and, thus replace the α chain but is most usually included in a high-affinity α·β·γc complex (Fig. 3D). The signaling pathways described for all of these receptors are similar and, because the ICDs of each receptor lack catalytic activity, involve associated JAKs. Upon ligand binding and assembly of the high-affinity receptor–ligand complex, JAK1, constitutively associated with the α and β subunits, and JAK3, constitutively associated with the γc subunit, become activated and transphosphorylated. The JAKs phosphorylate the receptor chains, leading to recruitment and phosphorylation of phosphotyrosine-associating signaling intermediates, including STATs 1, 3, 5A, 5B, and 6 that dimerize and move to the nucleus to direct transcription. The MAPK pathway is activated via Shc interactions with the α or β subunit and PI3 kinase activation is brought about by recruitment of the Src-family kinases and/or insulin receptor substrates 1 and 2 to the receptors. Spontaneous mutations in the genes for γc and JAK3 produce, respectively, an X-linked and an autosomal severe combined immunodeficiency (SCID) syndrome in man, and mice lacking either γc or JAK3 are also severely immunodeficient, indicating the importance of these shared signaling pathways in immune function. Because the primary role of IL-2, IL-7, and IL-15 is regulation of the development and/or function of lymphoid lineage cells, only a very brief summary of their actions is outlined, while IL-4 and IL-9 have more pleiotropic effects and will be covered in more detail [15].

IL-2, IL-7, IL-15, AND THEIR RECEPTORS

IL-2 stimulates both a prompt T-cell immune response to antigens and a subsequent, rapid dampening of this response. Because IL-2-deficient mice die from autoimmune disorders yet mount a normal response to infections, the primary role of IL-2 appears to be the promotion of apoptosis of excess activated T cells. IL-15 exhibits considerable functional overlap with IL-2 in their regulation of innate immunity, through the two shared receptor subunits, IL-2Rβ and γc. However, in contrast to IL-2/IL-2Rα, IL-15Rα alone binds IL-15 with high affinity and IL-15R signaling has distinct effects on activated T cells. Rather than promoting apoptosis of excess T cells as does IL-2, IL-15 stimulates the survival of memory T cells for the secondary immune response. IL-7 plays a critical role in the early development of both B and T cells in mice and of T cells only in man. Deletion of the gene for either IL-7 or IL-7R produces severe B- and T-cell lymphopenia.

IL-4 AND THE IL-4 RECEPTOR (IL-4R)

IL-4 is a pleiotropic cytokine that is secreted by T_H2 and NK1.1+T cells, basophils, mast cells, and eosinophils. The high affinity IL-4R is widely expressed in hematopoietic and nonhematopoietic cells and its expression is upregulated by IL-4 itself. In hematopoietic cells, the IL-4R consists of the IL-4Rα and γc subunits and requires JAK3 for STAT6 activation. Nonhematopoietic signaling by IL-4 utilizes the IL-4Rα subunit in combination with the IL-13R rather than γc. Consequently, IL-4 can act on many cell types and can modulate cytokine production by a variety of tissues. Its primary role, however, is in the regulation of T helper cell differentiation to T_H2 cells and in B-cell Ig switching. Apart from its effects on lymphocytes, IL-4 inhibits CSF-1-induced macrophage colony formation and megakaryocyte colony formation, but enhances G-CSF-induced granulocyte colony formation and IL-3-induced basophil, mast cell, and eosinophil generation. Along with GM-CSF and TNFα, IL-4 induces the differentiation of myeloid lineage cells into dendritic cells. It also regulates the production of inflammatory mediators in a pattern consistent with its anti-inflammatory role.

IL-9 AND THE IL-9 RECEPTOR (IL-9R)

IL-9 is a pleiotropic growth factor that appears to be important in the pathogenesis of asthma. It is predominantly secreted by T cells, especially T_H2 cells, but also by mast cells and eosinophils, particularly those derived from asthmatic subjects. IL-9R is expressed by T and B cells, eosinophils, and neutrophils and IL-9 has been shown to stimulate early T-cell development, B-cell Ig production, including IgE, mast cell survival, proliferation, and cytokine release as well as up-regulation of eosinophil IL-5R expression. Hence, IL-9 influences many aspects of the inflammatory process that underlies asthma.

Cytokines Signaling Through a Common gp 130 Subunit

At least three hematopoietic cytokines, IL-6, IL-11, and leukaemia inhibitory factor (LIF), and three nonhematopoeitic cytokines, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1), comprise the IL-6-type cytokine group. All share at least one subunit, gp130, in their receptor complexes and, as a result, functional overlap is frequent. IL-6R and IL-11R each contain a unique, cytokine-specific α chain, whereas the LIFR-specific subunit is much larger (Fig. 2) and also is more promiscuous because it is incorporated in receptor complexes for OSM, CNTF, and CT-1. The signaling complexes for this group of cytokines also vary in their stoichiometry. Il-6, IL-6Rα, and gp 130 bind together in a 2 : 2 : 2 molecular ratio to produce a high-affinity hexameric complex. A similar situation exists for the

IL-11R while LIF complexes in a 1 : 1 : 1 stoichiometry with LIFR and gp130. Gp130 signal transduction is mediated via activation of the constitutively associated JAKs, JAK1, JAK2, and TYK-2, which activate STAT1, STAT3, and STAT5 in turn. The MAPK pathway is also activated through SHP-2 binding to a juxtamembrane phosphotyrosine on gp130. Gp130-deficient mice die *in utero* due to severe hematopoietic and cardiac problems [16].

IL-6 AND THE IL-6 RECEPTOR (IL-6R)

IL-6 is a multifunctional cytokine produced by both lymphoid and nonlymphoid cells and its receptor is also expressed on a wide range of cells. In hematopoiesis, IL-6 acts synergistically with a number of cytokines, including SCF, IL-3, FL, CSF-1, and TPO, to stimulate primitive multipotential hematopoietic cell proliferation, myelopoiesis, and megakaryocyte production as well as lymphopoiesis. IL-6-deficient mice have reduced primitive multipotent progenitors, megakaryocyte progenitors, and neutrophils, and they are severely defective in their responses to tissue damage or infection.

IL-11 AND THE IL-11 RECEPTOR (IL-11R)

IL-11 is similar to IL-6 in many respects apart from its shared receptor subunit. Both IL-11 and its receptor are widely expressed in hematopoietic and nonhematopoietic tissues. Its expression is induced by pro-inflammatory and anti-inflammatory cytokines, and hematopoietic cell targets include those of the myeloid, erythroid, and megakaryocytic lineages. Although the effects of IL-11 on hematopoiesis are very similar to those of IL-6 and largely synergistic, adult IL-11Rα nullizygous mice are hematopoietically normal, highlighting the significant redundancy in this family of cytokines.

LIF AND THE LIF RECEPTOR (LIFR)

LIF is also multifunctional and is produced by monocytes and stromal cells in response to activating stimuli such as IL-1β and LPS. The hematopoietic effects of LIF are similar to those of IL-6 and IL-11 and, as for IL-11, LIF-deficient mice have no obvious hematopoietic phenotype.

Conclusions

The maintenance of the normal complement of hematopoietic cells requires the complex interaction of a large number of cytokine signaling pathways, some of which are redundant while others are critically important. Moreover, this complexity is increased by the actions of a number of other cytokines that also regulate hematopoiesis but primarily act elsewhere and which are therefore not covered in this review. They include IL-1 and IL-18, which do not signal through cytokine receptor motif-containing receptors, IFNγ, TGFβ, TNFα, and TNFβ, which commonly inhibit rather than promote hematopoiesis, several chemokines, and the mammalian Notch paralogs and their ligands, which regulate primitive hematopoietic cells.

In an extension of their physiological roles, some hematopoietic cytokines are used extensively in the clinical setting, usually to correct deficiencies of specific hematopoietic lineages such as EPO for anemia, TPO for thrombocytopenia, and GM-CSF and G-CSF for granulocytopenias.

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CHAPTER 350

Regulation of Bartlett Endogenous Stem Cells in the Adult Mammalian Brain: Promoting Neuronal Repair

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Adult Neurogenesis Revealed

A central dogma of neuroscience for greater than a century was that the brain was a static nonrenewable organ. The centerpiece of this dogma was that no new neurons were produced in the central nervous system (CNS) following development. Following a histological examination of the developing CNS, investigators as famous as Ramón y Cajal [1,2] and His [3] concluded that owing to its complex architecture and elaborate connections, the idea of the brain changing at all after its formation could not be seriously entertained. While reports describing mitotic figures in the cerebrum of the adolescent rat [4] and lining the walls of the lateral ventricles [5,6] surfaced in the early 1900s, these early reports of mitotic activity were basically overlooked and rarely cited owing to the prevailing hegemony and the crude nature of the detection methods used.

It was not until the late 1950s with the introduction of [^{3}H]-thymidine autoradiography that a serious challenge was mounted to the dogma. Smart [7] was the first to apply this technique to study proliferation in the adult brain and while he reported that neurogenesis and gliogenesis occurred in the 3-day-old postnatal mouse, he was unable to detect evidence for such an event in the adult. It was not until several years later that Altman [8] discovered putative adult neurogenesis through a series of experiments designed to test the kinetics of glial cell proliferation following brain trauma. However, even though Altman [9–14] continually reported [^{3}H]-thymidine autoradiographic evidence for new neurons in the neocortex, dentate gyrus, and olfactory bulb of adult rats, a lack of definitive immunocytochemical markers allowed this also to be dismissed. Indeed, it took the better part of another two decades before two teams—Michael Kaplan and colleagues [15,16] working on adult rats and Fernando Nottebohm and colleagues [17–21] investigating the seasonal production of learned song in birds—succeeded in unambiguously demonstrating production of new neurons in the adult brain. Although these studies documented neurogenesis in the adult CNS, they suggested this phenomenon was localized to two distinct brain regions: the olfactory bulb and the hippocampus.

Another major step was combining BrdU labeling techniques with specific monoclonal antibodies that recognized cell-type-specific molecules [22], which ultimately enabled the unambiguous identification of newly generated cell types. This technological achievement had three immediate effects: It provided indisputable evidence of the incidence and location of neurogenesis and gliogenesis in the adult CNS, it facilitated the stereological estimation of the total number of specific cells being generated in any given region at any given time, and it engendered a more detailed search for the precursor cell population that continued to give rise to new cells in the adult brain. It was against this background that the race to identify and isolate and the cell responsible for producing these neurons in adult brain began.

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Isolation and Culture of Neural Stem Cells

Traditionally, stem cells were thought to be located only in tissues where differentiated cells were most susceptible to loss and the need for replacement great, such as the skin [23], intestinal epithelia [24], and the blood [25]. Indeed, the best known example of an adult stem cell is the hematopoietic stem cell (HSC), which is found in the bone marrow and is ultimately responsible for the generation of all blood cell types throughout the life of the animal [25–27]. Because the adult CNS was thought not to exhibit a significant amount of neuronal death and to have no regenerative capacity, the existence of neural stem cells seemed both unlikely and unnecessary. However, in 1992 two independent groups successfully demonstrated the presence of putative stem cells in the adult mammalian CNS.

In our study, cells from various regions of the adult mouse forebrain were dissociated and cultured at high density in the presence of serum and basic fibroblast growth factor (FGF-2), allowed to proliferate, and then differentiated into neurons using a conditioned medium from an astrocyte-like cell line (Ast-1) [28]. The FGF-2 system was ultimately refined to show that single precursors could be grown *in vitro* to give rise to clonal progeny [29]. Reynolds and Weiss [30] employed epidermal growth factor (EGF) in a serum-free culture system. which resulted in the death of the majority of cell types harvested from the periventricular (PV) region within 3 days of culture, but allowed a small population (<0.01%) of the starting population to enter a period of active proliferation, even at very low cell densities. By using such a system, they were able to demonstrate that a single adult CNS cell could proliferate to form a ball of undifferentiated cells they termed a *neurosphere*, which in turn could be dissociated to form numerous secondary spheres, or induced to differentiate, generating the three major cell types of the CNS. This was a major finding because it demonstrated for the first time that the precursor cell exhibited the three cardinal attributes of a bona fide stem cell: proliferation, self-renewal, and the ability to give rise to a number of differentiated, functional progeny [24,31].

Just as the advent of [^{3}H]-thymidine autoradiography, BrdU immunocytochemistry, and hybridoma technologies facilitated a more detailed understanding of adult neurogenesis, the neurosphere culture system provided investigators with a valuable tool for assaying neural stem cell activity. Although the neurospheres provided the means to begin to study neural differentiation and search for stem cell activity in different parts of the nervous system, it told us nothing about the identity, number, or factor regulation of the stem cell itself. The neurosphere assay did, however, provide a means to begin to identify and purify this stem cell using this assay. Surprisingly, it was almost another 10 years before this was achieved.

Because no specific stem cell markers were available, we began the purification process in 1995 using a totally empirical approach. Single cells harvested from the PV region of adult mice were examined for their ability to bind a number of fluorescently tagged ligands or antibodies using a fluorescent activated cell sorter (FACS). Populations with different binding properties were sorted and then assayed for stem cell activity using the neurosphere assay. After examining more than 500 different ligands and antibodies, we were able to identify a small, distinct population of adult stem cells whose cell surface was characterized by the expression of minimal or undetectable levels of peanut agglutinin receptors $(PNA)^{lo}$ and mCD24 (also known as heat-stable antigen, $HSA)^{lo}$. This $PNA^{lo}HSA^{lo}$ subpopulation (Fig. 1A, lower boxed region), a discrete population representing $0.27 \pm 0.07\%$ of the unsorted population, was comprised almost exclusively of NSCs. When cultured under clonal conditions, in which one $PNA^{lo}HSA^{lo}$ cell was plated per well, approximately 80% of the cells (1:1.28) gave rise to neurospheres [32]. Furthermore, the $PNA^{lo}HSA^{lo}$ population contained 63.2% of the overall NSC activity, strongly suggesting that the vast majority of NSCs in the periventricular region of adult mice were of this phenotype.

Although FACS analysis suggested that all the putative NSCs were similar, a more precise characterization of the differentiation and self-renewal capacity of the $PNA^{lo}HSA^{lo}$ cells was undertaken to confirm that bone fide neural stem cells had been purified [32]. To determine this, clonally derived neurospheres generated from individual $PNA^{lo}HSA^{lo}$ cells were differentiated by growth factor withdrawal and the addition of serum, and then assessed by immunocytochemistry (ICC) for the presence of neurons and glia. In all cases, differentiated clonally derived spheres (a total of 201 in $n=3$ experiments) contained glial fibrillary acidic protein positive (GFAP+ve) astrocytes, β-tubulin type III positive (β-tubulin+ve) neurons, and O4 positive (O4+ve) oligodendrocytes. To assay for self-renewal capacity, clonally derived spheres were once again generated from freshly isolated $PNA^{lo}HSA^{lo}$ NSCs, then after 7 days *in vitro*, each primary sphere was dissociated into a single cell suspension and plated into individual 96-well plates. In all cases (348 spheres across $n=3$ experiments) secondary spheres were formed from each dissociated primary sphere. These clonal experiments demonstrated that each cell could proliferate, self-renew, and give rise to a family of differentiated progeny, the hallmark characteristics of a neural stem cells [24, 33]. To determine whether neurospheres derived from sorted cells retained these properties when passaged, three clonally derived cultures from individual $PNA^{lo}HSA^{lo}$ cells were generated and passaged every 5–7 days *in vitro* for 3 months. When individual spheres from each culture were transferred to differentiating conditions (116 spheres in total), GFAP+ve, O4+ve, and β-tubulin+ve cells were present in every sphere examined. Furthermore, in 100% of the cases, individual passaged spheres retained the ability to give rise to more than one subsequent sphere, suggesting that they have all of the characteristic *in vitro* properties previously described for NSCs [30,34,35].

Recently, several different neural cell types have been purported to be capable of behaving as neural stem cells under a variety of conditions *in vitro* [26,36–40]. That is,

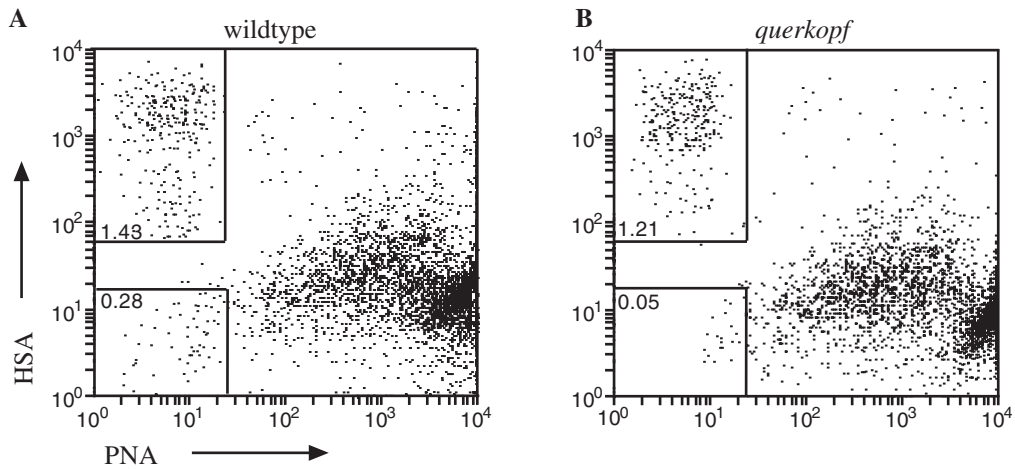


Figure 1 Querkopf mice are deficient in PNA^{lo}HSA^{lo}NSCs. Viable CNS cells were harvested from individual adult *querkopf* mutant mice and wildtype littermates and sorted for PNA and HSA binding. Flow cytometric analysis revealed similar sorting profiles between all mice sampled, except for the PNA^{lo}HSA^{lo} NSC populations, which showed an approximately 6 fold diminution (mean 6.17 + 1.36 SEM) in the percentage of cells of this phenotype in the mutant *querkopf* compared to wildtype littermates. Of note, was the dramatic loss of the ultra-low PNAHSA population. Values in boxed regions represent the % of total viable cell number.

they have shown the ability to generate clusters of cells *in vitro*, which are reminiscent of multipotent neurospheres. Unfortunately, it is unclear whether the formation of such clusters represents bona fide neurospheres (i.e., contain multipotent NSCs), because high cell densities and protracted times in culture makes such an interpretation difficult. We sought to investigate whether subpopulations additional to the PNA^{lo}HSA^{lo} in the PV contained latent stem cell activity (as measured by neurosphere formation) by culturing sorted cells in serum free media (NS-A) supplemented with growth factor(s) other than EGF and bFGF. Thus, cells were harvested from adult CBA mice and sorted into PNA^{lo}HSA^{lo}, PNA^{lo}HSA$^{mid\text{-}hi}$, PNA$^{mid\text{-}hi}$HSA^{lo}, and PNA$^{mid\text{-}hi}$HSA$^{mid\text{-}hi}$ fractions. Each fraction was cultured for a total of 21 days *in vitro* at equivalent densities, with new media added every 7 days *in vitro*. The factors and respective concentrations employed were PDGF alone (10 ng/ml), LIF alone (10,25, 50 ng/ml), BMP-2 alone (25,50,100 ng/ml), growth hormone alone (50,100,500 ng/ml), stem cell factor (200 ng/ml) alone, and in combination with IL-3 (2 ng/ml), IL-6 (20 ng/ml), and G-CSF (20 ng/ml). This latter combination of factors was employed to assay for hematopoietic stem cells. In all of the preceding cases, after three independent rounds of experimentation, in the absence of EGF or bFGF costimulation, no latent stem cell activity was detected in any of the various subpopulations. Furthermore, we also followed culture conditions described by Kondo and Raff [40] to specifically screen for oligodendrocyte precursor cell (OPC) activity, but failed to observe the formation of neurospheres in any of the sort fractions. Of note in this regard, the PNA^{lo}HSA^{lo} cells do not share the phenotype of OPCs because they are negative for the A2B5 antigen, which is present on all OPCs [40–42].

The fact that we assayed for latent stem cell activity (sphere formation) in other subpopulations using a variety of growth factors and had not detected spheres up to 3 weeks after stimulation suggested that we had not missed a population of stem cells, which reinforced our original observation that there is a single predominant stem cell type in the PV region. This is a very important point, because the periventricular region not only contains the highest frequency of EGF- or FGF-2-responsive NSCs in the CNS [30,43–47], but, more importantly, the NSCs from this region of the adult telencephalon have been shown to have the capacity to generate neurons and glia under a variety of in both the normal and diseased animals [48–51]. It is these stem cells that continue to generate neuroblasts, which migrate along the rostral migratory stream and ultimately differentiate into new neurons in the olfactory bulb throughout adulthood [48–50,52], underlining their functional significance in neuronal production under normal physiological conditions. It is also these stem cells that have been shown to generate neurons in the hippocampus and cortex after injury [53,54].

We sought to verify that the NSC population we had identified corresponded to the functional stem cell population. We did this by examining the stem cell pool of *querkopf* mutant mice [55], which show significant lack of neurons in the region of the brain that continually acquires new neurons in the postnatal and adult animal: the olfactory bulb. We reasoned that if PNA^{lo}HSA^{lo} NSCs generated new neurons in the olfactory bulb under normal conditions, the neuronal deficiency in *querkopf* may be reflected by a selective deficiency in the PNA^{lo}HSA^{lo} stem cell population. Examination of the FACS dot-plot profiles revealed this to be the case: sorting profiles from mutant and wild-type mice were indistinguishable, except for the size of the PNA^{lo}HSA^{lo} compartment, where mutant mice exhibited an approximate sixfold reduction in cells of this phenotype. Indeed, closer examination of the PNA^{lo}HSA^{lo} region (lower boxed area; Fig. 1B)

revealed a loss of an as yet unrecognized PNAHSA ultra-lo population. Whether this is indeed a distinct population of cells has yet to be determined, because the reduction in $PNA^{lo}HSA^{lo}$ cell numbers did not affect the frequency of stem cell activity contained within this compartment, when compared to littermate controls. Because no change in stem cell activity was detected in other sorted populations, these results cannot be explained by a shift in stem cell phenotype. Furthermore, the studies of Thomas *et al.* [55] showed that there was no change in the proliferation rate or cell death rate observed in these mice during development and postnatally, further supporting the conclusion that the main defect is the lack of stem cell activity. Preliminary experiments have since revealed that when multipotent neurospheres are generated and differentiated from *querkopf* mice, they exhibit a significant reduction in the percentage of neurons produced *in vitro* as compared to littermate controls, providing further support for an underlying stem cell defect. Taken together, the results discussed demonstrate a high degree of correlation between the size of the $PNA^{lo}HSA^{lo}$ population and neuronal production, suggesting that this population acts as a functional stem cell *in situ* generating new neurons under normal physiological conditions.

Recently, two publications appeared in *Science* [56,57] claiming that bone marrow cells had the ability to generate new neurons *in vivo*. The strategy those researchers had employed to purify stem cells from the bone marrow was based on the selective exclusion of the DNA-binding dye Hoechst 33342 [58,59]. Sorting based on Hoechst 33342, a dye that specifically but reversibly joins with DNA [60,61], seems to rely on the differential ability of cells to exclude the dye, and in murine bone marrow defines a small (0.07%) homogeneous population, termed SP (side population) cells. This SP population, which extrudes the dye very efficiently, expresses low or undetectable levels of CD34, lacks markers of mature blood cell types (i.e., Lin^{-}), and provides long-term hematopoietic reconstitution in lethally irradiated mice [59], suggesting it represents a bona fide hematopoietic stem cell population. Because it has been suggested that the HSC and the NSC represent the same cell in different parts of the body, and the Hoechst 33342 technique has been used to isolate nonhematopoietic (muscle and epidermis) stem cell types [58,62], we sought to determine if these two populations were similar by performing further immunocytochemical examination of the $PNA^{lo}HSA^{lo}$ NSCs and by seeing if Hoechst exclusion could reliably predict neural stem cell activity.

Accordingly, PV cells were harvested from adult mice and processed so as to isolate the $PNA^{lo}HSA^{lo}$ population and finally incubated in media containing 5 μg/ml Hoechst 33342 for 60 min at 37°C before sorting occurred. As shown in Fig. 2, two discrete regions of PNA/HSA labeled periventricular cells (Figs. 2A and 2C), were analyzed for the distribution of the Hoechst 33342 staining patterns (Figs. 2B and 2D). The Hoechst staining profile of cells within the $PNA^{lo}HSA^{lo}$ compartment (Fig. 2B) revealed that the vast majority (approximately 90%) of the $PNA^{lo}HSA^{lo}$ cells were brightly labeled, while the majority of cells in the $PNA^{lo}HSA^{mid\text{-}hi}$ compartment were Hoechst low (Fig. 2D). Culturing of the Hoechst low population shown in Fig. 2D under conditions known to generate neurospheres did not increase NSC frequency more than simply selecting for $PNA^{lo}HSA^{mid\text{-}hi}$ cells. To further identify which populations of cells excluded the dye and confirm that Hoechst 33342 labeling was ineffective to enrich for neural stem cell activity, PNA/HSA labeled periventricular cells were first analyzed on the basis of Hoechst staining. As illustrated in Fig. 2F, three distinct populations (Hoechst-bright, Hoechst-mid, and Hoechst-low) of PV cells can be distinguished on the basis of Hoechst 33342 staining. Examination of the distribution of PNA/HSA labeled cells within the Hoechst low population revealed that the majority (86.6%) of Hoechst 33342 low cells were contained within the $PNA^{lo}HSA^{mid\text{-}hi}$ compartment (Fig. 2E). Thus, in contrast to previously published work demonstrating that HSC activity is unambiguously contained within a specific Hoechst "low" compartment, Hoechst 33342 labeling could not be used to enrich for NSC activity.

Furthermore immunocytochemical analysis of $PNA^{lo}HSA^{lo}$ cells revealed no expression of cell-surface antigens associated with endothelial and hematopoietic stem cells. NSCs were negative for the following markers:

1. CD90.2, which reacts with the Thy 1.2 alloantigen on thymocytes and peripheral T cells [63];
2. CD31, also known as PECAM-1 (platelet endothelial cell adhesion molecule), which is expressed constitutively on adult endothelial cells and weakly on peripheral platelets and leukocytes [64–66];
3. CD117, which reacts with the mouse c-Kit receptor and is expressed on virtually all hematopoietic progenitor cells [67,68];
4. CD135, which reacts with Flk-2/Flt-3, a receptor protein tyrosine kinase closely related to c-Kit, and is expressed in hematopoietic stem cells and also primitive progenitor cells [69,70];
5. CD34, a cell-surface protein of unknown function that has in the past been used to positively select for long-term repopulating HSCs [26,27];
6. CD45, a cell-surface tyrosine phosphatase present on all nucleated hematopoietic cells including HSCs, but not found on any nonhematopoietic cell types [71]; and
7. Ly-6A/E, also known as Sca-1, which is expressed on multipotent, long term-engrafting, HSCs in mice [26,27].

Thus, the data strongly suggest that the $PNA^{lo}HSA^{lo}$ NSC is unrelated to the bone marrow-derived stem cell.

Regulation of Stem Cell Differentiation into Neuron

During the past decade an increasing number of studies have been directed toward understanding the growth factor regulation of nervous system development [72–75]. Of interest here is that many of the factors that play critical roles during early neural development are thought to modulate

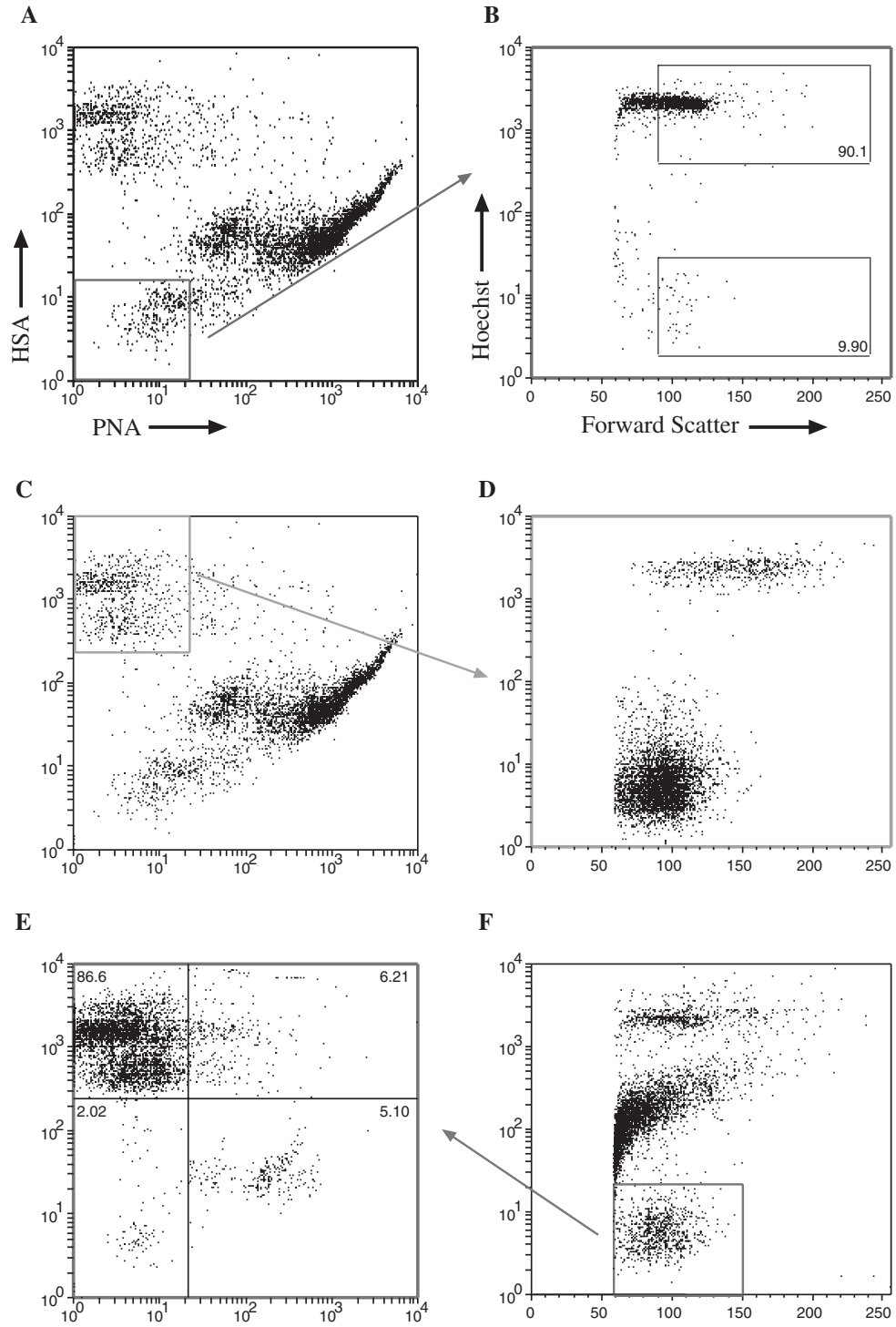


Figure 2 Hoechst staining profile of PNA/HSA labeled periventricular cells. Two populations [$PNA^{lo}HSA^{lo}$(A) and $PNA^{lo}HSA^{mid\text{-}hi}$(C)] were analyzed from the same suspension of PNA/HSA labeled periventricular cells to determine the distribution of Hoechst 33342 stained cells within each compartment. Approximately 90% of cells within the $PNA^{lo}HSA^{lo}$ compartment (box, A) were brightly labeled with the DNA-binding dye Hoechst 33342 (upper box, B). In comparison, the majority of cells within the $PNA^{lo}HSA^{mid\text{-}hi}$ compartment (box, C) were not Hoechst bright (D). Panel (E) illustrates the Hoechst staining pattern of a population of freshly isolated >7μm periventricular cells. When the Hoechst low compartment (box, F) was analyzed for the distribution and percentage of PNA/HSA labeled cells, the majority of cells (86.6%) were located in the $PNA^{lo}HSA^{mid\text{-}hi}$ compartment (E). Numerical values represent the % of cells located within that region (B) or quadrant (E) from individual representative experiments. (A color representation of this figure is available on the CD version of the *Handbook of Cell Signaling*.)

various aspects of adult neurogenesis, including extrinsic cues such as cytokines signaling through the leukemia inhibitory factor (LIF) receptor complex, such as LIF and ciliary neurotrophic factor (CNTF) [37,76] and growth factors such as members of the FGF family [74], the bone morphogenic protein (BMP) family [77], and platelet-derived growth factor (PDGF) [36,78] as well as intrinsic cues, including neurogenic basic helix–loop–helix (bHLH) genes such as *NeuroD* and *Neurogenin* [79–83].

While CNTF, interleukin-6 (IL-6), oncostatin-M, and LIF all signal through the LIF receptor and its associated receptor subunit gp130, they exhibit a wide range of effects on many different cell types. For example, in the developing CNS, both CNTF and LIF have been shown to play a vital role in the differentiation of neural precursors into astrocytes [36–38, 84–87] or oligodendrocytes [85,86,88–91]. Somewhat paradoxically, LIF and CNTF have also been shown to promote neurogenesis under some conditions [76,85,92–95]. To determine its role on neural stem cells, Shimazaki and colleagues [96] analyzed the number of multipotent neurospheres that could be derived from LIFR-deficient (heterozygous) and wild-type mice. They report that both the number of NSCs and the number of tyrosine hydroxylase (TH) positive neurons in the olfactory bulb (implied progeny of NSCs) were significantly reduced [96], suggesting the importance of the LIFR in NSC regulation. Of interest though, when the authors investigated the number of spheres that could be generated from E14 $LIFR^{-/-}$ mice versus wild-type controls, they found no significant difference. However, when assayed for the ability to continue to generate spheres upon passaging (i.e., self-renewal) they found that unlike wild-type controls, cultures derived from $LIFR^{-/-}$ mice could not be expanded beyond seven passages, suggesting that the LIFR played a more specific role in the long-term maintenance of stem cells in culture. Consistent with this hypothesis, the authors report a 37% reduction in the number of sphere-producing cells harvested from adult $LIFB^{+/-}$ versus wild-type mice. In addition, when CNTF, which also signals through the LIFR/gp130 complex, was injected into the lateral ventricle of adult mice for 6 days, these authors report a 24% increase in the number of EGF-responsive sphere forming cells, confirming the importance of the LIFR, but also suggesting that CNTF may play a role in regulation of the NSC. Given that CNTF and its specific receptor subunit are expressed in the developing CNS and, most interestingly, in the periventricular region of the adult forebrain [97,98], and that it has been shown in the past to enhance the survival of a variety of neural cell types [75], these results are not totally unexpected. Having confirmed that LIF and CNTF are indeed candidate molecules for the maintenance of NSCs, the challenge now is to more clearly define the role(s) and mechanism of action of each cytokine that signals through the LIFR in regulating the activity of the NSCs.

Although we know that the interaction between external influences such as cytokines and internal cues causes the differential expression of neurogenic genes and hence regulates neural stem cell fate [99–101], what is less understood is how a particular cell (a stem cell, for example) can process and integrate the variety of simultaneous external signals it receives (which in turn active different signal transduction pathways) and ultimately achieve an appropriate biological outcome (i.e., differentiation into a neuron). One possible explanation is that under some circumstances, neural stem cells have the capacity to overcome signals that normally inhibit neuronal differentiation. In the case of LIF and CNTF, which both signal through the LIF receptor and employ the JAK/STAT pathway [102], work in our lab suggests that the differential regulation of JAK/STAT signaling via suppressor of cytokine signaling (SOCS) molecules is important in determining the differentiation of NSCs into neurons versus astrocytes.

SOCS molecules are key regulators of LIF receptor signaling and are able to inhibit the JAK/STAT pathway downstream of cytokines such as IL-6 or LIF [103–105]. At least eight members of the SOCS family (SOCS1–7 and CIS) [106] are differentially expressed in a variety of tissues and can be up-regulated by a range of cytokines and growth factors. Recently we have shown that SOCS1, SOCS2, and SOCS3 are expressed throughout neural development, with SOCS2 being the most highly expressed.

A most surprising finding was that astrocytes expressed SOCS1 and SOCS3, whereas neurons expressed SOCS2 exclusively. This pattern of expression was not altered by treatment with a variety of cytokines, although cytokines regulated the level of individual SOCS gene expression. Our observation that SOCS2 was restricted to the neuronal lineage was reinforced by its *in situ* expression pattern, which showed SOCS2 expression in neurons of the developing cortex and hippocampus [107]. Both the *in situ* observations and the Northern analysis [107] showed that the level of SOCS2 mRNA is significantly up-regulated as neurons are generated in the developing forebrain and down-regulated postnatally. Surprisingly, SOCS2 was not expressed in the adult multipotent neural stem cells isolated by FACS, but was expressed in progenitors and neurons but not astrocytes, suggesting a role for SOCS2 in the regulation of neurogenesis.

Results from experiments in which SOCS2 was overexpressed or deleted in neural cells support this concept. Overexpression of SOCS2 in neurospheres resulted in significant induction of neuronal differentiation. Deletion of SOCS2 expression from CNS neural precursors resulted in decreased numbers of neurons being generated. *In vitro*, the neural stem cells from $SOCS2^{-/-}$ mice generated significantly fewer neurons but more astrocytes compared to stem cells from wild-type mice, a result that was also confirmed in neuroepithelial cells from E10 mice. Because no difference was seen in the total number of cells generated in culture and the total number of differentiated cells was not affected, the results support the idea that SOCS2 plays a role in neurogenesis by promoting neuronal and inhibiting glial cell fate and not in cell survival or proliferation. The *in vivo* findings supported the *in vitro* observations.

We found a 30% reduction in the number of NeuN positive neurons across all layers of the cortex in adult SOCS2$^{-/-}$ mice compared to wild-type mice, but found no reduction in the total cell density in the mutant cortex, indicating that non-neuronal cells, glia, had been generated in proportionally higher numbers, similar to our observations *in vitro*. This decrease of the neuron-to-glia ratio is similar to that observed *in vitro* for neural precursor cells differentiated in the presence of LIF or CNTF, in which the neuron-to-glia ratio is also decreased [37,87]. Thus, expression of SOCS2 appears to promote the ability of a precursor cell to differentiate into a neuron and inhibit its differentiation into an astrocyte. Conversely, a lack of SOCS2 expression appears to favor astrocyte differentiation.

Because the level of SOCS2 expression appears to regulate neural precursor cell differentiation, the key questions are what regulates SOCS2 expression and what does SOCS2 regulate? Growth factors that signal through the LIF receptor complex appeared to be the most likely candidates for both regulating and being regulated by SOCS2. We have shown that they up-regulate SOCS2 expression in neuroepithelial cells and SOCS2 has been shown, albeit weakly, to regulate downstream signaling by LIF in some cells [108]. Furthermore, LIF and related molecules have been shown to exert effects on neural precursors similar to that observed here with changes in SOCS2 expression levels: either an increase in neuronal differentiation [76] or promotion of astrocyte differentiation [37,38,87]. Addition of LIF to neurosphere cultures profoundly increased the number of astrocytes while inhibiting neuron differentiation; however, this effect was the same in both SOCS2$^{-/-}$ and wild-type mice. Thus, it appears that SOCS2 is not a major regulator of biological signaling through the LIF receptor complex in neural precursor cells. This finding is in accordance with SOCS2$^{-/-}$ mice whose phenotype of gigantism appears to be due to dysregulation of GH and/or IGF1 signaling [109]. GH now also appears to be a major regulator of neuronal differentiation. Although a role for GH in neural stem cell differentiation has not previously been described, GH is expressed in whole fetal rat brain during neural development, from E10, with a peak of expression before birth [110]. Moreover, GH production has been shown in cultured brain cells [111], and somatostatin is produced by embryonic cortical neurons [112]. Together with our demonstration here that one form of the GH receptor is expressed as early as E10 in the mouse brain, these studies further support a role for GH, modulated by SOCS2, in negatively regulating neuronal differentiation of neural precursor cells.

How Does SOCS2 Regulate Neuronal Differentiation?

The main role of SOCS2 appears to be regulation of STAT5 activation downstream of GH signaling. SOCS2$^{-/-}$ mice exhibit an enlarged growth phenotype consistent with hyperresponsiveness to GH signaling [109]. The overgrowth phenotype of the same line of SOCS2$^{-/-}$ mice used in this study requires STAT5b for expression of the phenotype [113]. In addition, both STAT5a and STAT5b activation is prolonged in cells from these mice in response to GH due to their inability to effectively down-regulate the activation [113]. Regulation of STAT5 activation by SOCS2 has also been demonstrated in overexpression studies [114,115]. Although the precise mechanism by which SOCS2 regulates STAT5 activation remains to be determined, it appears to involve SOCS2 competitively binding to the STAT5 binding sites on the GH receptor and thus inhibiting STAT5 activation [115,116].

Although SOCS2 regulates STAT5 activation, how this translated to regulation of neurogenesis was unclear. One possibility was that GH inhibits expression of neurogenic genes, such as *Neurogenin*, similar to our recent demonstration that members of the FGF family regulate *Notch* and *Delta* expression to inhibit neuronal differentiation [101]. Expression of SOCS2 would therefore be required to overcome GH inhibition of neurogenic gene expression. The level of expression of neurogenic genes is important in determining neuronal versus glial cell fate. Neural progenitor cells from Neurogenin-2 (Ngn2)/Mash1 double knock-outs showed decreased neuronal and increased astrocyte differentiation of cortical progenitor cells [83], whereas overexpression of Neurogenin-1 (Ngn1) promoted neurogenesis and inhibited gliogenesis by competing with the JAK/STAT pathway for the transcriptional coactivators CBP/p300 as well as directly inhibiting STAT activation [83].

Given that SOCS2 appears to act by regulating STAT5 signaling, it may as a consequence regulate the availability of the JAK/STAT pathway to compete with Ngn1 for CBP/p300. We found not only that the levels of Ngn1 were decreased in SOCS2-cells compared to wild-type cells *in vitro* and *in vivo*, but also that addition of GH acutely decreased Ngn1 levels in wild-type cells *in vitro* by 50–60%, to a level comparable to that in SOCS2$^{-/-}$ cells. This thus provides a potential mechanism for the decreased neurogenesis and increased gliogenesis observed in the SOCS2$^{-/-}$ cells. However, unlike SOCS2-mice, Ngn1 null mice do not have a robust cortical phenotype. This is presumably due to compensation by other bHLH genes, such as Ngn2 and Mash1, and double knock-outs are required before a phenotype is observed [82,117]. Therefore, it is probable that SOCS2 does not affect Ngn1 alone but also affects other bHLH genes, although this remains to be determined.

Thus, our data suggest a hypothesis whereby SOCS2, via its regulation of GH signaling, regulates levels of Ngn1 expression and thus the ability of a neural stem cell to differentiate into a neuron or an astrocyte. Although we have shown that SOCS2 regulates the biological effects of GH signaling in neural precursor cells, we have also shown that only LIF and related cytokines, not GH, regulate SOCS2 expression in these cells. We suggest therefore that LIF-like molecules inhibit a precursor's response to GH by up-regulation of SOCS2 expression, while at the same time promoting astrocyte differentiation by activation of STAT3 [38], a process that is possibly regulated by SOCS3 as part

of a normal negative feedback loop for LIF signaling [108]. SOCS2 would, therefore, be acting as an integrator of multiple signal transduction pathways and its level of expression would be important in determining final biological outcome of signaling by multiple stimuli.

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Index

A

AA′ loop of Apo2L/TRAIL, **1:**307
ABC transporter group, **1:**117
Abelson cytoplasmic tyrosine kinase, **1:**422
Abl
  c-, **3:**249–250
  C-terminal region of, **3:**249–250
  cytoplasmic signaling function of, **3:**252
  description of, **3:**249
  domains of, **3:**249–250
  kinase domain binding proteins and, **3:**251–252
  N-terminal region of, **3:**249
  nuclear signaling function of, **3:**252–254
  oncogenic potential of, **3:**249
  platelet-derived growth factor and, **3:**252
  proline-rich motif binding proteins and, **3:**252
  proteins interacting with, **3:**250–252
  SH2 binding proteins and, **3:**251
  SH3 binding proteins and, **3:**250–251
  signal transduction, **3:**252–254
  tyrosine kinase, **3:**254
Abp1, **3:**414
Acetylcholine, **2:**119
Acetylcholine receptors, nicotinic
  α subunit, **1:**224
  binding sites, **1:**224–225
  channel, **1:**225
  cytoplasmic domain, **1:**226
  description of, **1:**204–205
  discovery of, **1:**223
  extracellular domain of, **1:**224
  function of, **1:**223
  homopentameric, **1:**226
  neuronal-type, **1:**223
  quaternary structures, **1:**224
  secondary structures, **1:**224
  structure of, **1:**223–226
  subunit composition, **1:**223
  tertiary structures, **1:**224
Acetyl-CoA, **3:**353
Acetylsalicylic acid, **2:**283
Acid sphingomyelinase, CD40 signaling effects on, **1:**320
ACP1, **1:**733
Actin
  cdc42 GTPase module effects on, **2:**735
  lipid rafts effect on assembly of, **2:**212
  PPI-actin binding effects on protein regulation, **2:**212–213
Actin comets, **2:**210–211
Actin cytoskeleton
  cdc42's role in, **2:**701, **2:**716
  disruptions of, **3:**411
  dynamin and, **3:**413–414
  in growth cones, **2:**878
  polarity of, **2:**735
  rho GTPases and
    cell migration, **2:**701–702, **2:**851
    description of, **2:**701, **3:**294
    remodeling, **3:**414–416
    signaling, **2:**702–703
  in yeast, **3:**411–413
Actin polymerization
  mechanisms of, **2:**210
  phosphatidylinositol phosphate 5-kinase overexpression effects, **2:**210–211
  stimulation of, **2:**209–211
Actin regulatory proteins, **2:**768
α-Actinin, **1:**463, **2:**212, **2:**317
Actin-related protein 3, **1:**296
Activated cdc42 kinase
  description of, **2:**745
  mechanism of, **2:**746
Activated transcription factor 6
  BiP inhibition of, **3:**315
  description of, **3:**279
  unfolded protein response transcriptional activation and, **3:**313
Activating protein 1
  characteristics of, **3:**550
  description of, **3:**99, **3:**490–491
  dexamethasone effects, **3:**491
  DNA-binding domain of, **3:**99
  Fos, **3:**99–100

Activating region 1, **2:**532
Activation loop
  autoinhibition of, in insulin receptors, **1:**300–302
  protein kinase
    description of, **1:**443, **1:**541
    PKC, **1:**552
Activators of G-protein signaling, **2:**567
Activin, **3:**472, **3:**593
Activin βB, **2:**800, **2:**802
Activin receptor, **2:**800
Activin receptor-like kinases, **3:**502
Actomyosin, **2:**850–851
ActR-II_{ec}, **1:**291
Acylprotein thioesterase 1, **2:**676
Adaptor proteins, **1:**381–382, **1:**393, **1:**399, **1:**464, **2:**7, **2:**842, **2:**863
ADD-1/SREBP-1c, **3:**42
Adenomatous polyposis coli, **2:**408, **2:**411, **2:**893, **3:**161, **3:**405
Adenophostin A, **2:**37
Adenosine triphosphate
  calcium release and, **2:**46
  cyclic nucleotide-dependent protein kinase inhibitors, **2:**488–491
  inositol 1,4,5-trisphosphate receptors and, **2:**42
  renal production of, **3:**586
  tissue transglutaminase binding, **2:**722
Adenovirus E4orf4 protein, **2:**410
Adenylyl cyclases
  AC1, **2:**422, **2:**424
  AC2, **2:**423
  AC3, **2:**422
  AC4, **2:**423
  AC5, **2:**423
  AC6, **2:**423
  AC7, **2:**423
  AC8, **2:**423
  AC9, **2:**423
  Bacillus anthracis, **2:**422
  C1 domain, **2:**420–421
  C2 domain, **2:**420–421
  calmodulin activation of, **2:**422
  cAMP modulation by, **2:**535
  catalytic core of, **2:**422
  description of, **2:**419
  domain structure of, **2:**420
  forskolin-induced activation of, **2:**422
  function of, **2:**419–422
  G protein subunit regulation of, **2:**563, **2:**586, **2:**640
  G-protein-coupled receptor activation, **2:**422
  GSα and, **2:**578
  inhibition of, **2:**422–423
  isoforms, **2:**419, **2:**423–424
  membrane-bound, **2:**419, **2:**421
  P site inhibitors, **2:**422
  Paramecium, **2:**535–536
  physiology of, **2:**423–424
  posttranslational modification of, **2:**422
  regulation of, **2:**422–423
  soluble, **2:**423
  structure of, **2:**419–422
  T-cell activation and, **3:**550
  Trypanosoma brucei, **2:**422, **2:**540
Adherens junctions, **1:**72–73
Adipocyte differentiation
  description of, **3:**40–41
  negative regulation of, **3:**42
  tumor necrosis factor α effects on, **3:**43
Adipose tissue development
  in vitro studies of, **3:**40–41
  PPARγ's role in, **3:**39–40
  3T3-L1 preadipocytes, **3:**40–41
  transcriptional networks in, **3:**40–42
ADP-ribosylation, mono
  bacterial toxin-induced, **2:**614–615
  cellular, **2:**616
  cycle of, **2:**614
  description of, **2:**613–614
  endogenous, **2:**615–616
  substrates, **2:**616
Adrenalin, **1:**2, **3:**376
β-Adrenergic receptor kinase, **1:**182
Adrenocorticotropic hormone, **3:**379
Adventitial cells, **3:**457
AF-1, **3:**25, **3:**36, **3:**62
AF-2, **3:**25, **3:**36, **3:**62
AF-2 helix, **3:**22–23
Affinity chromatography
  elution step of, **2:**303
  protein–protein interactions studied using, **2:**302–303
AG 490, **1:**452–453
AG 1433, **1:**456
AGC kinases
  description of, **1:**515
  hydrophobic motif of, **1:**516, **2:**196
  PDK1 activation of, **1:**515, **2:**195–197
Aging
  caloric restriction and, **3:**371
  FOXO transcription factors and, **3:**88
  prostate gland and
    cAMP signaling pathway, **3:**598–599
    description of, **3:**591
    growth factor signaling, **3:**599
    steroid hormones, **3:**597–598
  retrograde regulation and, **3:**371
Agonist binding
  glutamate receptors, **1:**219–221
  G-protein coupled receptors, **1:**182, **1:**184, **1:**188
AGS-3, **2:**573
AICAR, **1:**531
AKAP. *See* A-kinase anchoring proteins
A-kinase anchoring proteins
  AKAP78, **2:**390
  AKAP79, **1:**632, **2:**385, **2:**390, **2:**399
  AKAP350/CG-NAP, **2:**385
  characteristics of, **1:**613, **1:**617, **2:**189, **2:**326, **2:**330–331, **2:**383
  discovery of, **2:**390
  functions of, **2:**383
  mAKAP, **2:**386
  phosphodiesterases and, **2:**433
  protein kinase A subcellular targeting by
    AKAP15/18α, **2:**379–380
    AKAP75/79/150, **2:**379
    cellular functions of, **2:**380

description of, **2:**377, **2:**390, **2:**596, **3:**384
determinants, **2:**378–379
domains of, **2:**378–379
hydrophobic interactions, **2:**378–379
structure, **2:**378–379
targeting domains, **2:**379–380
signaling complexes
AKAP350/CG-NAP, **2:**385
cAMP signaling units, **2:**385–386
description of, **2:**383, **2:**459
G-protein signaling through, **2:**384–385
kinase/phosphatase, **2:**385, **2:**459
schematic diagram of, **2:**384
tumor suppression by, **2:**596
Akt
activation of, **3:**83
description of, **1:**446
inhibitors of, **1:**457–458, **2:**742
phosphoinositide 3-kinase and, **3:**568–569
Alloreactivity, **1:**66–67
Altered peptide ligands
definition of, **1:**80–81
T-cell receptor/pMHC and, **1:**65, **1:**67, **2:**341
Alternative splicing
apoptosis and, **3:**331–332
cdk-related kinases in, **3:**333
cell cycle and, **3:**332–333
description of, **3:**331
Aluminum fluoride, **2:**576, **2:**658, **2:**724
Alzheimer's disease, **2:**91
Amino acids
chemokine structure, **1:**149–150
cyclooxygenase-1, **2:**265
cyclooxygenase-2, **2:**265
g-protein coupled receptors, **2:**565
insulin effects on uptake of, **3:**307
low-molecular-weight protein tyrosine phosphatases, **1:**734
mTOR regulation by, **1:**529–530, **3:**300–301
phosphatidylinositol transfer proteins, **2:**227
PPP family, **1:**597–598
protein synthesis regulated by, **3:**300
protein tyrosine phosphatases, **1:**654, **1:**662, **1:**677
translational control by
description of, **3:**299
GCN system, **3:**299–300
TOR signaling pathway, **3:**300–302
α-Amino-3-hydroxy-5-methylisoxazole 4-propionic acid, **2:**329
Amlexanox, **3:**396
AMPA receptors, **2:**385, **2:**397, **2:**399–400
AMP-activated protein kinase
description of, **1:**531, **1:**535
medical uses of, **1:**536
mRNA turnover and, **3:**287
mTOR, **3:**301
regulation of, **1:**535–536
structure of, **1:**535
α-subunits, **1:**535
Amphiphysin I, **3:**414
Amphiphysin II, **3:**414
Anaphase promoting complex, **1:**697, **2:**408, **3:**403
Anaphase promoting complex/cyclosome, **3:**131
Anchor cells, **2:**810
Anchoring inhibitor peptides, **2:**380
Anchoring proteins
A-kinase
AKAP78, **2:**390
AKAP79, **1:**632, **2:**385, **2:**390, **2:**399
AKAP350/CG-NAP, **2:**385
characteristics of, **1:**613, **1:**617, **2:**189, **2:**326, **2:**330–331, **2:**383
discovery of, **2:**390
functions of, **2:**383
mAKAP, **2:**386
phosphodiesterases and, **2:**433
protein kinase A subcellular targeting by
AKAP15/18α, **2:**379–380
AKAP75/79/150, **2:**379
cellular functions of, **2:**380
description of, **2:**377, **2:**390, **2:**596, **3:**384
determinants, **2:**378–379
domains of, **2:**378–379
hydrophobic interactions, **2:**378–379
structure, **2:**378–379
targeting domains, **2:**379–380
signaling complexes
AKAP350/CG-NAP, **2:**385
cAMP signaling units, **2:**385–386
description of, **2:**383, **2:**459
G-protein signaling through, **2:**384–385
kinase/phosphatase, **2:**385, **2:**459
schematic diagram of, **2:**384
tumor suppression by, **2:**596
protein kinase C regulation by, **1:**553, **2:**189
Androgen receptor, **3:**35
Androgen(s)
prostate gland development and, **3:**592
steroid receptors of, **3:**23
Ang-1
angiogenesis and, **2:**850–851, **3:**58
COUP-TFII regulation of, **3:**58
endothelial cell survival and, **2:**852
Ang-2, **2:**851, **3:**456, **3:**578, **3:**582
Ang-2 receptors, **3:**582
Angiogenesis
Ang-1's role in, **2:**850–851, **3:**58
cells involved in, **3:**456, **3:**458–459
coordination of, **3:**456–460
COUP-TFII effects on, **3:**58–59
definition of, **3:**57, **3:**455
description of, **1:**455, **2:**849
endothelial cell survival during, **2:**851–852
endothelial cell–mural cell interactions, **3:**460
extracellular matrix degradation, **3:**456
G-protein coupled receptor promotion of, **2:**590–591
initiators of, **3:**455–456
overview of, **2:**855–856
PPARγ inhibition of, **3:**58
proangiogenic factors, **3:**456, **3:**459
smooth muscle cells in, **3:**456
sphingosine 1-phosphate, **2:**590–591
transcription factors involved in, **3:**58
vascular endothelial growth factor and, **2:**849, **2:**856, **3:**57–58, **3:**456, **3:**537, **3:**599
vessel-specific requirements in, **3:**456

Angiogenic factors, **3:**456, **3:**459
Angiopoietins
  Ang-1
    angiogenesis and, **2:**850–851, **3:**58
    COUP-TFII regulation of, **3:**58
    endothelial cell survival and, **2:**852
  Ang-2, **2:**851, **3:**456, **3:**578, **3:**582
  definition of, **2:**849
  signaling pathways activated by, **2:**849
Angiotensin II. *see* Ang-2
Ania-6, **3:**333
Ania-6a, **3:**333
Anions, **1:**216
Ankyrin-rich transmembrane protein, **2:**842
Annexins
  affinity of, **2:**101
  calcium channels and, **2:**101–102
  definition of, **2:**101
  5, **2:**101–102
  6, **2:**102, **2:**392
  summary of, **2:**102–103
Antibodies
  architecture of, **1:**33–34
  for coprecipitating interacting proteins, **2:**297
  description of, **1:**45
  Fab fragment conformational changes
    description of, **1:**34
    main-chain rearrangements, **1:**34–35
    side-chain rearrangements, **1:**34–35
    V_L-V_H rearrangements, **1:**36
  heavy chains of, **1:**39, **1:**45
  historical discoveries, **1:**33
  immunoglobulin G, **1:**33–34
  light chains of, **1:**39
  structure of, **1:**39, **1:**45
  V(D)J recombination, **1:**33
Antibody–antigen interface
  energetic maps of, **1:**41
  Fab fragment conformational changes effect on, **1:**36
  overview of, **1:**39–40
  studies of, **1:**39–40
  thermodynamic mapping of, **1:**40–42
Antidromic vasodilation, **3:**608
Antigen recognition response element, **3:**335
Antigen-presenting cells
  altered peptide ligands, **2:**341
  CD40's role, **1:**319
  description of, **1:**73
  function of, **2:**339
  major histocompatibility complex molecules expressed on, **1:**355
  mutations in, **1:**623
  T-cell junction with, **1:**79, **1:**358
Antigen(s), **1:**33
Antigen-specific T-cell receptors, **3:**546
Anti-Müllerian hormone, **3:**537
Aortic arch, **3:**467–468
AP-1. *see* Activating protein 1
AP180, **2:**156–157
Apaf-1, **3:**434
Apo2L/TRAIL
  AA′ loop of, **1:**307
  structure of, **1:**305–306
Apo2L/TRAIL–DR5 complex
  CRD3 orientation in, **1:**306–307
  crystal structure of, **1:**306
  description of, **1:**305–306
Apoptosis
  Akt targets in, **3:**568
  alternative splicing and, **3:**331–332
  bcl-2, **3:**435–437, **3:**596
  cadherins, **2:**895
  calpain-induced, **2:**109
  caspase involvement in, **2:**351–352
  cdc42's role in, **2:**716
  CD95-mediated, **1:**315
  cell cycle vs., **3:**437
  ceramide-induced, **2:**257
  death receptors. *see* Death receptors
  definition of, **3:**485
  description of, **1:**466, **3:**431–432
  features of, **3:**431, **3:**485–486
  follicle development and, **3:**537
  FOXO transcription factor regulation of, **3:**86–87
  function of, **2:**351
  histone phosphorylation and, **3:**94–95
  inhibitors of
    antagonists, **2:**354
    baculovirus domains, **2:**354
    caspase regulation by, **2:**353
    description of, **2:**353
    X-linked, **2:**353
  mitochondria's role in, **3:**434–435
  neuronal, **3:**485–486, **3:**489
  neurotrophin regulation of, **3:**488–489
  NF-κB and, **3:**433
  nuclear Abl kinase promotion of, **3:**254
  outer membrane permeabilization during, **3:**435
  $p75^{NTR}$ and, **3:**488
  prostate gland, **3:**595–597
  R5 subunits and, **2:**411
  radiation-induced, **3:**214, **3:**216
  schematic diagram of, **2:**352
Apoptosome, **3:**434
Arabidopsis thaliana
  leucine-rich repeat receptor protein kinases in, **1:**579–581
  PP2C genes in, **1:**638
Arachidonic acid
  description of, **2:**265
  metabolites, **3:**577–578
  phospholipase A_2-mediated release of, **2:**261, **2:**265
Arachidonic acid-regulated calcium channels
  activation of, **2:**37
  calcium signaling and, **2:**35, **2:**37–39
  carbachol effects, **2:**37–38
  characteristics of, **2:**35–37
  description of, **2:**35
  I_{ARC}, **2:**36–37
  I_{CRAC}, **2:**36–39
  identification of, **2:**35–37
  inhibition of, **2:**38
  I_{SOC}, **2:**36–39
  permeability of, **2:**36
  reciprocal regulation of, **2:**37–39
  summary of, **2:**38

ARAPs, **2:**205–206
ARC channels. *see* Arachidonic acid-regulated calcium channels
Arf6, **2:**211
Arf family
  activation of, **2:**728
  characteristics of, **2:**727
  coat complex binding to, **2:**729
  cofactors for, **2:**728
  co-translational modification of, **2:**727
  GTPase activating proteins, **2:**205–206
  guanine nucleotide exchange factors, **2:**205, **2:**728
  lipid metabolism modified by, **2:**728–729
  members of, **2:**727
  membrane traffic regulated by, **2:**727–729
  phosphatidylinositol 4-phosphate 5-kinase type I regulated by, **2:**125
  phosphatidylinositol 3,4,5-triphosphate regulation of, **2:**205
  phospholipase D activity regulated by, **2:**239, **2:**728
  similarities among, **2:**727
  transport processes, **2:**759
  vesicle biogenesis role of, **2:**729–730
Arfaptin, **2:**747–748
Arginine methylation
  description of, **3:**145
  function of, **3:**146
  histones, **3:**147
  signal transducers and activators of transcription, **3:**146
  signal transduction role of, **3:**147
Arginine methyltransferases
  description of, **3:**145
  S-adenosylmethionine use by, **3:**146
Argos, **1:**407
ark-1, **2:**807
ARNO, **2:**205
Arp2/3 complex, **2:**323–324, **2:**735, **3:**411–412
Arrestins, **1:**182, **2:**370–371, **2:**596, **3:**443
ASF/SF2, **3:**332
Asialoglycoprotein receptor, **1:**89
ASK1, **2:**371
Asp116, **1:**245
Aspartic acid residue, **1:**564
Aspergillus nidulans, **2:**84
ASV16, **2:**139
Ataxia-telangiectasia, **1:**558–559, **3:**225
Ataxia-telangiectasia-related protein, **1:**558–560
ATF4, **3:**360
ATF6, **3:**279
ATM
  amino acid motifs for, **3:**227
  cell cycle control, **3:**225
  checkpoint activation by
    description of, **3:**406
    G_2/M, **3:**230–231
    G_1/S-phase, **3:**229
    S-phase, **3:**229–230
  description of, **3:**225
  DNA double-strand breaks recognized by, **3:**227–229
  p53 and, **3:**228
  signaling role of, **3:**231–232
  structure of, **3:**225–226
  substrates for, **3:**228
ATP-binding cassette, **3:**369, **3:**397
ATP-sepharose, **2:**301–302
Atrial natriuretic peptide
  cGMP synthesis stimulated by, **2:**511
  description of, **2:**428
Atypical protein kinases
  ChaK
    catalytic domain of, **1:**568–569
    characteristics of, **1:**568
    description of, **1:**568
    hydrophobic ATP-binding pocket of, **1:**569
    kinase domains of, **1:**568–569
    protein kinase A vs., **1:**569
    regulation of, **1:**571
  description of, **1:**567
  domain organization of, **1:**568
  EF2 kinase, **1:**567–568
    calcium/calmodulin and, **1:**570
    cloning of, **1:**568
    description of, **1:**567
    p70 S6K effects, **1:**571
    regulation of, **1:**570–571
  functions of, **1:**571–572
  identification of, **1:**567–568
  regulation of, **1:**570–571
  structure of, **1:**568–570
  substrate specificity of, **1:**570
AUF1, **3:**284
AU-rich elements, **3:**319–320
Aurora kinases, **3:**95
Autocoid, **1:**1, **2:**266
Autocrine, **3:**54
Autophosphorylation
  calcium/calmodulin-dependent protein kinase II, **1:**544–545
  protein tyrosine kinase receptors, **1:**393
Axil, **2:**636
Axin, **2:**408, **2:**636, **3:**161
Axin dephosphorylation, **1:**623
Axin:APC complex, **3:**162
Axl, **1:**375
Axon
  modulatory signals for, **2:**873–874
  signaling, receptor protein tyrosine phosphatases in, **2:**869–870

B

B7, **1:**74
Bacillus subtilis
  PP2C-like phosphatases in, **1:**639
  redox activation of, **3:**194
  stress signaling in, **1:**639
Bacteria
  cAMP signaling in, **2:**531–534
  chemoreceptors
    clustering of, **1:**200
    description of, **1:**197
    sensitivity of, **1:**197
  chemotaxis
    adaptation of, **1:**199–200
    description of, **1:**197
  chemotaxis receptors
    cytoplasmic domain signaling, **1:**199
    periplasmic ligand binding domain signaling of, **1:**198–199
    transmembrane signaling, **1:**198–199
  ion channels, **1:**204–205

Bacteria *(continued)*
oxidative stress response in, **3:**191
transcription control, **2:**532
Bacteriorhodopsin, **1:**117–118
Bak, **3:**436
Basal transcription
definition of, **3:**11
description of, **3:**11
Basic fibroblast growth factor, **3:**537
Basic helix-loop-helix, **2:**816, **2:**819, **3:**42
Bax, **3:**436
BAY 43-9006, **1:**458
B-cell adaptor for phosphoinositide 3-kinase, **3:**560
B-cell antigen receptor
activation of, **1:**327, **1:**329
antigen binding, **3:**556
calcium response after engagement of, **1:**328
description of, **1:**327, **3:**555
functions of, **3:**555
IG-α/Ig-β heterodimer of, **1:**327
immunoreceptor tyrosine-based activation motif, **1:**327–328
ligation, **3:**561
mIg molecule, **1:**327
phospholipase Cγ activation, **3:**558–560
SHP-1 effects, **1:**329
signal transduction through
initiation of, **3:**555–557
propagation of, **3:**556–562
signaling initiation, **1:**328–329
structure of, **1:**327–328
summary of, **3:**562
bcl-2, **2:**409, **3:**435–437, **3:**596
Bcr-Abl
description of, **1:**444–445
kinase inhibitors, **1:**454
BCX-1812, **1:**110–111
Bem1, **2:**572
Benchmarking, **1:**18
Benign prostatic hyperplasia, **3:**597
β amyloid protein precursor, **2:**816
β_2-adrenergic receptor, **2:**408
BH-123 protein, **3:**436
BH3-only proteins, **3:**435
Bidentate inhibitors, of protein tyrosine phosphatases, **1:**679–681
Bile acid receptor, **3:**48–50
Binding affinity, **1:**27
Biogenic amine transporters, **2:**408
Bioinformatics
definition of, **1:**659
description of, **1:**12–13, **2:**890
protein tyrosine phosphatases, **1:**661–668
structural, **1:**665
websites for, **1:**661
Bioisosteres, **1:**455
Bioluminescence resonance energy transfer, **1:**187, **1:**189
Bip, **3:**315, **3:**360
Bistability, **1:**494
BLT_1 receptor, **2:**278
BLT_2 receptor, **2:**278
BM3.3 T-cell receptor, **1:**65–66
BMP-2–BR-IUA_{ec} complex, **1:**289–291
Bni1, **2:**735
Bnr1, **2:**735
Bombesin, **3:**478–479
Bone
development of
fibroblast growth factors in, **3:**503–504
growth factors associated with, **3:**501–503
hormones that affect, **3:**500–501
insulin-like growth factors in, **3:**503
low-density lipoprotein receptor-related protein 5 in, **3:**504
matrix metalloproteinases in, **3:**505
microfibrils in, **3:**505
transcription factors associated with, **3:**501–503
estrogen effects, **3:**500
extracellular matrix of, **3:**504–505
hormones that affect, **3:**500–501
mechanical strain on, **3:**499–500
remodeling of, **3:**499
Bone cells
estrogen effects on, **3:**500
osteoblasts, **3:**499
osteoclasts, **3:**498
osteocytes, **3:**499
Bone diseases
description of, **3:**497
genetic defects, **3:**498
malformations, **3:**498
osteoporosis, **3:**497–498
skeletal malignancies, **3:**498
Bone morphogenetic protein
BMP-2–BR-IUA_{ec} complex, **1:**289–291
description of, **1:**289, **3:**501–503
developmental role of
description of, **2:**833
extracellular modifiers, **2:**834
gradients, **2:**833
intracellular negative regulation of signaling, **2:**835–836
ligand gradients, **2:**833–834
loss-of-function studies in mammals, **2:**836
lung, **3:**511
ovary, **3:**536–537
receptors, **2:**834–835
signal transduction, **2:**835
summary of, **2:**836
lung development and, **3:**511
receptor–ligand complexes, **1:**290
signaling
description of, **1:**488
intracellular negative regulation of, **2:**835–836
Xenopus studies, **1:**489–490
Bone morphogenetic protein-4, **2:**800
Bone morphogenetic protein receptor 1a, **1:**24
53BP2, **1:**618
Bradykinin, **3:**582, **3:**586
Brain natriuretic peptide, **2:**428
Brain-derived growth factor, **2:**840–841, **3:**487
BRASSINOSTEROID-INSENSITIVE 1, **1:**581
BRCA1, **3:**230
Breast
description of, **3:**565
neoplastic
description of, **3:**565
epidermal growth factor receptor's role in, **3:**568

growth factors associated with, **3:**566–568
phosphoinositide 3-kinase/Akt pathway, **3:**568–569
signal transducers and activators of transcription in, **3:**569
signaling pathways in, **3:**566–568
transforming growth factor β, **3:**567–568
Bridging sheet, **1:**103
Brinker, **2:**835
Brody's disease, **2:**60
Bruton's tyrosine kinase, **2:**163
Brx, **2:**715
Btk, **1:**328, **2:**640–641
Bud2, **2:**733
BUD2, **2:**734
BUD5, **2:**734
Bystander effect, **3:**180
bZip proteins, **3:**99, **3:**313

C

C215, **1:**672
C1 domain, **2:**159
C2 domain
adenylyl cyclases, **2:**420–421
calcium binding mode of, **2:**97–98
classification of, **2:**95, **2:**97
function of, **2:**99
ligands of, **2:**99
phospholipase A_2, **1:**12
phospholipid binding mechanism of, **2:**98–99
protein kinase C, **2:**187
β-strands in, **2:**96–97
structure of, **2:**95–97
synaptotagmin 1, **2:**95, **2:**97–98
c-Abl, **3:**228, **3:**249
Cachectin, **1:**275
Cactus, **2:**779–780
Cadherins
adherens junctions, **1:**73
adhesive activity
regulatory modes for, **2:**893–895
schematic diagram of, **2:**892
apoptotic signals, **2:**895
β-catenin and, **1:**72, **2:**893–894
characteristics of, **2:**889
classical, **2:**889–890
Dcad 102F-like, **2:**891
description of, **1:**72, **2:**889
desmosomal, **2:**891
E-, **2:**408, **2:**895–896
extracellular domain of, **2:**895
fat-like, **2:**891
function of, **1:**72
intracellular proteins that regulate, **2:**893–894
MN-, **2:**896
molecular diversity of, **2:**890
N-, **2:**896
nonchordate classic cadherin domain, **2:**890
photocadherins, **2:**890–891
protein kinase, **2:**891
proteolytic cleavage for regulation of, **2:**895
seven-pass transmembrane, **2:**891
β-strand, **2:**892
structure of, **2:**889–890
structure-function relationships, **2:**891–892
synaptic clustering of, **2:**889
Caenorhabditis elegans
caspase in, **2:**352
description of, **1:**375–376
germline proliferation in, **2:**810–811
LET-23 signaling pathways, **2:**805–807
life span increases in, **3:**371
LIN-12/Notch pathway, **2:**810
meiotic differentiation in, **2:**810–811
microRNAs, **3:**329
mitogen-activated protein kinase phosphatase in, **1:**704
neurotransmission in, **3:**377
vulval development, epidermal growth factor-receptor signaling in, **2:**805–807
Cain/Cabin1, **1:**632
Cak, **3:**404
Calbindin D-28k, **2:**67
Calcineurin. *see also* PP2B
calcineurin B, **1:**632, **1:**634
calcium dependence, **1:**631
calmodulin effects, **1:**631–632
cellular trafficking role of, **1:**635
characteristics of, **1:**632
definition of, **1:**631
description of, **1:**591, **1:**603, **2:**399
distribution of, **1:**633–634
endogenous regulators of, **1:**632–633
enzymatic properties of, **1:**631–632
FK506 inhibition of, **1:**631
functions of, **1:**634
gene expression and, **1:**634
identification of, **1:**631
inhibition of, **1:**629
ion homeostasis and, **1:**634–635
isoforms, **1:**633–634
muscle differentiation role of, **1:**634–635
neuronal functions of, **1:**635
NFAT interactions with, **3:**120
NFATc and, **3:**126–127
NMDA receptor regulation, **2:**399–400
regulation of, **1:**632–633
structure of, **1:**632–633
substrates
description of, **1:**629
specificity of, **1:**631–632
suppression of, **1:**629
T-cell activation by, **1:**634
Calcium
calcineurin dependence on, **1:**631
calmodulin binding, **2:**53
capacitative entry of, **2:**31–32
cell proliferation and, **2:**84
cyclic adenosine diphosphate ribose regulation of, **2:**15
cytosolic, **3:**380
exocytotic secretion and, **3:**378–382, **3:**388
gastrointestinal hormone-stimulated signal transduction and, **3:**480–481
influx of, **3:**380
inositol 1,4,5-trisphosphate effects on, **2:**15
intracellular

Calcium *(continued)*
hydrogen peroxide regulation of, **2:**113–115
transients of, **2:**69
long-term potentiation and, **2:**400
mitochondrial
ambient calcium and, **2:**74–75
description of, **2:**55, **2:**73
disease and, **2:**76
function of, **2:**75–76
fundamentals of, **2:**73
mitochondrial metabolism effects, **2:**75–76
movement mechanisms, **2:**73–74
set point for uptake, **2:**74
uniporter movement, **2:**73–74
voltage-dependent anion channel, **2:**74
xNa^+/Ca^{2+} exchange, **2:**74
mitochondria-to-nucleus signaling mediated by, **3:**367
NMDA receptor, **2:**331
nucleus levels, **2:**55
presynaptic stores of, **2:**664–665
pulsatile increases in, **2:**53
in secretory cells, **3:**380
signal transduction mediated by, **1:**369
smooth muscle, **2:**54
sphingosine 1-phosphate-mediated release of
description of, **2:**19
intracellular target for, **2:**20–21
Calcium binding
C_2-domains, **2:**97–98
sites for, **2:**67
Calcium buffers
artificial, **2:**68
biological effects of, **2:**69–70
calcium homeostasis by, **2:**69
cytosolic concentration of, **2:**67, **2:**69
description of, **2:**67
metal-binding affinities of, **2:**68
metal-binding kinetics of, **2:**68
mobility of, **2:**68–69
parameters for, **2:**67–69
parvalbumin, **2:**67–70
Calcium channels
annexins as, **2:**101–102
arachidonic acid-regulated
activation of, **2:**37
calcium signaling and, **2:**35, **2:**37–39
carbachol effects, **2:**37–38
characteristics of, **2:**35–37
description of, **2:**35
I_{ARC}, **2:**36–37
I_{CRAC}, **2:**36–39
identification of, **2:**35–37
inhibition of, **2:**38
I_{SOC}, **2:**36–39
permeability of, **2:**36
reciprocal regulation of, **2:**37–39
summary of, **2:**38
calcium release-activated, **3:**558
cardiac cell activation of, **2:**23
description of, **2:**51–52
Gβγ subunit mediation of, **2:**667
high-voltage-activated
description of, **2:**668
G-protein βγ subunit interacting domain of, **2:**668–669
mechanically activated, **2:**52
in neurons, **3:**381
receptor-operated, **2:**52
regulators of, **2:**102
signal transduction and, **2:**24
smooth muscle cell activation of, **2:**23
store-operated, **2:**31–32, **2:**52
voltage-gated
calcium currents
L-type, **2:**23–26, **2:**583
N-type, **2:**25, **2:**640
pharmacological properties of, **2:**23–24
physiological properties of, **2:**23–24
P-type, **2:**24, **2:**26, **2:**640
Q-type, **2:**24, **2:**26, **2:**640
T-type, **2:**24, **2:**26
types of, **2:**23
voltage clamp studies of, **2:**24
cardiac, **2:**25
Ca_V 1, **2:**25
Ca_V 2, **2:**25, **2:**27
Ca_V 3, **2:**25
description of, **2:**23, **2:**51–52
diversity of, **2:**25–26
function of, **2:**26–27
molecular properties of, **2:**24–26
in neurons, **2:**23
physiological roles of, **2:**23
regulation of, **2:**27
second messenger signal transduction pathways effect on, **2:**27
subunits
α1, **2:**24–25
β-, **2:**24–25
γ-, **2:**25
functions of, **2:**25
S5 segments, **2:**26
S6 segments, **2:**26
structure of, **2:**24–25
summary of, **2:**27
transmembrane organization of, **2:**25–26
Calcium homeostasis
calcium buffers for. *see* Calcium buffers
calcium channels for. *see* Calcium channels
calcium pumps for. *see* Calcium pumps
description of, **2:**51
methods of, **2:**113
Na^+/Ca^{2+} exchanger for. *see* Na^+/Ca^{2+} exchanger
protein tyrosine phosphatase inactivation for, **2:**114–115
PTEN inactivation for, **2:**114–115
Calcium puffs, **2:**53–54
Calcium pumps
description of, **2:**57
enzyme cycle of, **2:**57
genetic diseases involving defects of, **2:**60
in lower eukaryotes, **2:**57
P type, **2:**57
PMCA
calmodulin regulation of, **2:**59
discovery of, **2:**59

genetic diseases involving defects of, **2:**60
inhibitors of, **2:**57–58
isoforms of, **2:**59
reaction cycle of, **2:**57–**2:**59
regulation of, **2:**59–60
reaction cycle of, **2:**57–58
SERCA
discovery of, **2:**58
genetic diseases involving defects of, **2:**60
inhibitors of, **2:**57–58
isoforms, **2:**58
N domain, **2:**58
P domain, **2:**58
phospholamban interactions with, **2:**58–59
reaction cycle of, **2:**57–58
regulation of, **2:**58–59
structure of, **2:**58–59
Calcium release
adenosine triphosphate, **2:**46–47
caffeine-induced, **2:**47
calcium-induced, **2:**664
channels involved in, **2:**52
inositol 1,4,5-trisphosphate 3-kinase, **2:**15, **2:**31
inositol 1,4,5-trisphosphate receptor role in, **2:**42
ryanodine receptors role in, **2:**52
sphingosine 1-phosphate
description of, **2:**19, **2:**249
intracellular target for, **2:**20–21
Calcium signaling
arachidonic acid-regulated calcium channels, **2:**35, **2:**37–39
calmodulin-mediated, **2:**83–85
C_2-domains
calcium binding mode of, **2:**97–98
classification of, **2:**95, **2:**97
function of, **2:**99
ligands of, **2:**99
phospholipid binding mechanism of, **2:**98–99
β-strands in, **2:**96–97
structure of, **2:**95–97
synaptotagmin 1, **2:**95, **2:**97–98
cellular, **2:**73
intracellular
calcium channels involved in. *see* Calcium channels
description of, **2:**51
messengers involved in, **2:**52
mechanisms of, **2:**51
molecules for, **2:**45
Na^+/Ca^{2+} exchanger modulation of, **2:**65
nicotinic acid adenine dinucleotide phosphate, **2:**16, **2:**52
S100 proteins in. *see* S100 proteins
temporal regulation of, **2:**53
Calcium signals
amplitude modulation, **2:**54
in electrically excitable cells, **2:**54
mitochondrial, **2:**55
neurons, **2:**54
in nonelectrically excitable cells, **2:**53–54
in organelles, **2:**55
spatial regulation of, **2:**53–54
Calcium-activated protein for secretion, **3:**384
Calcium/calmodulin-dependent effector proteins, **2:**83–84
Calcium/calmodulin-dependent phosphodiesterase, **2:**442
Calcium/calmodulin-dependent protein kinase II
activation of, **2:**331
autophosphorylation regulation of, **1:**544–545
calcium-independent activity, **1:**544–545
definition of, **1:**543
desensitization, **1:**545
EF2 kinase and, **1:**570
genes that encode, **1:**544
holoenzyme of, **1:**544, **2:**331
neuronal regulation by, **1:**545
NMDA receptor binding to, **2:**331
postsynaptic density-associated, **1:**545, **2:**400
structure of, **1:**543–544
subunits of, **1:**543–544
Calcium-dependent phospholipase A_2, **2:**262
Calcium-dependent protein kinases, **1:**377
Calcium-release activated calcium channels, **3:**558
Calmodulin
adenylyl cyclases activated by, **2:**422
in *Aspergillus nidulans,* **2:**84
calcineurin dependence on, **1:**631
calcium binding to, **2:**53
calcium signaling mediated by, **2:**83–85
cell cycle functions of, **2:**84–85
cyclic adenosine diphosphate ribose-induced calcium regulation and, **2:**15
description of, **1:**228–229, **2:**83
enzyme binding, **2:**84
immobility of, **2:**83
PMCA pump regulated by, **2:**59
structure of, **2:**83
Calmodulin binding domain, **1:**228–229
Calmodulin kinase I, **2:**84
Calmodulin kinase II, **2:**84, **3:**385
Calmodulin kinase IV, **2:**84, **2:**409
Calpain
-3, **2:**109
μ-, **2:**105, **2:**107
activators of, **2:**106–107
apoptotic role of, **2:**109
autoproteolysis of, **2:**107
calcium effects, **2:**106
calpastatin effects, **2:**107
cell adhesion modulation by, **2:**108–109
cell proliferation and, **2:**109
degradation of, **2:**107
domains of, **2:**105
family members, **2:**105
functions of, **2:**108
future studies of, **2:**109
inactivation of, **2:**107
inhibitors of, **2:**107–108
m-, **2:**105, **2:**107
muscular dystrophy and, **2:**109
phosphorylation of, **2:**106–107
platelet activation and, **2:**108
protein–protein interactions effect on, **2:**107
proteolysis of, **2:**107–108
regulation modes, **2:**106–107
signaling role of, **2:**107–108, **2:**352
structure of, **2:**105–106

Calpain *(continued)*
studies of, **2:**105
targets of, **2:**108
Calpastatin, **2:**107
Calyculin A, **1:**608–609
cAMP
adenylyl cyclase modulation of, **2:**535
bacterial functions, **2:**419
catabolite gene activator protein response to, **2:**532–534
cGMP-dependent protein kinase activated by, **2:**545–546
compartments of, **2:**461
exchange protein directly activated by. *see* Epacs
exocytotic secretion and, **3:**378–379, **3:**384, **3:**388
functions of, **2:**419
in heart cells, **2:**461
hyperpolarization-activated cyclic nucleotide-gated channel response, **2:**518
membrane-permeable analogs, **2:**539
phosphodiesterases
catalytic domains of, **2:**438
cGMP-inhibited, **2:**443–444
description of, **2:**437–438, **2:**441, **2:**461
expression of, **2:**439
PDE2, **2:**442–443
phosphorylation-related regulation of, **2:**439
regulation of, **2:**439
regulatory domains of, **2:**438
structure of, **2:**437–438
subcellular targeting of, **2:**438–439
production of, **1:**2
prostate gland and, **3:**598–599
protein kinase A
activation, **1:**614, **1:**627, **1:**631, **2:**326, **2:**330, **2:**377, **2:**383, **2:**419, **2:**471, **3:**598
catalytic domain of, **2:**497
catalytic subunits of, **2:**471–474
cGMP cross-activation of, **2:**546
D/D domain, **2:**475
description of, **2:**495, **2:**596
discovery of, **2:**471
inhibitors of
ATP binding site-targeted, **2:**488–491
cyclic nucleotide binding site-targeted, **2:**488
description of, **2:**487–488
peptide binding site-targeted, **2:**491
properties, **2:**489–490
peptide substrates of
acceptor loci, **2:**496
description of, **2:**495
optimum recognition sequences, **2:**496–498
phosphorylation sites, **2:**497–498
recognition of, **2:**495–496
specificity of, **2:**498
regulatory subunits, **2:**471, **2:**474–475
specificity of, **2:**498
T-cell proliferation and, **3:**551
recombinant, **2:**460
regulation of, **2:**431
ribose moiety of, **2:**552
selectivity of, **2:**546
sequestration of, **2:**466
signaling
in bacteria, **2:**531–534
in *Paramecium,* **2:**535–536
T-cell activation and, **3:**550–551
transcriptional response to, **3:**115
Trypanosoma brucei, **2:**539–540
in *Trypanosoma cruzi,* **2:**540
cAMP receptor protein, **2:**531
cAMP-dependent protein kinase isozyme I. *see* cAPK-I
cAMP-dependent protein kinase isozyme II. *see* cAPK-II
cAMP-response element, **3:**100
Cancer
eIF-4E and, **3:**346
ErbBs and, **1:**408
farnesyl transferase inhibitors for, **2:**739
FOXO transcription factors and, **3:**87–88
glycogen synthase kinase 3 and, **1:**549–550
G-protein signaling in, **2:**591–592
integrins' role in, **1:**466–467
mouse models of, **2:**672
oncogenic protein kinases
activation of
chromosomal translocations, **1:**444–445
gene amplification and overexpression, **1:**445
mutation, **1:**445–446
treatment for, **1:**446–447
upstream regulators, **1:**446
treatment for, **1:**446–447
PP2A's role in, **1:**622–625
prostate, **3:**598–600
Ras and, **2:**671–673
signal transduction therapy for, **1:**451
Cantharidin, **1:**608
CAP. *see* Catabolite gene activator protein
Capacitative calcium entry, **2:**31–32
cAPK-I, **2:**537, **2:**549
cAPK-II, **2:**537, **2:**549
CAR. *see* Constitutive androstane receptor
Carbachol, **2:**37–38
Carbohydrate masking, **1:**102
Carbohydrate recognition domain, **1:**88–89
Carbohydrate(s)
cross-linked arrays mediated by, **1:**91
description of, **1:**87
diversity of, **1:**87–88
glycolipids and, **1:**90
lectins and, **1:**88–89
protein interactions with, **1:**91
recognition of, **1:**87
signaling pathways regulated by, **1:**87
structure of, **1:**87–88
Carbon monoxide, **3:**578
Carboxypeptidase D, **2:**409
Cardiomyocytes
annexin effects on, **2:**102
calcium signals in, **2:**53–54
description of, **3:**463–464
CARM-1, **3:**27
Cas, **2:**408
Casein kinase I
ε, **1:**576
Cubitus interruptus synthesis by, **3:**169
δ, **1:**576

description of, **1:**575
double-time, **1:**575–576
in *Drosophila,* **1:**575–577
isoforms, **1:**576
in mammalian clock, **1:**576–577
in *Neurospora* clock, **1:**577
phosphatidylinositol 4-phosphate 5-kinase type I and, **2:**125
Casein kinase II, **2:**409, **3:**31, **3:**101
Caspase activator granzyme B, **2:**351
Caspase recruitment domain, **3:**434
Caspase-activated DNase, **3:**431
Caspase(s)
-3, **2:**353
-7, **2:**353
-8, **3:**432–434
-9, **3:**434–435
activation of, **2:**353
apoptotic role of, **2:**351–352
in *Caenorhabditis elegans,* **2:**352
classification of, **2:**351, **3:**432
cytokine responses and, **2:**351
executioner, **2:**353
induced proximity of, **2:**353
inhibitor regulation of, **2:**353–354, **3:**432
initiator, **2:**353
pro-caspase 9, **2:**353
recruitment domains, **1:**276
second mitochondrial activator of. *see* SMAC
Catabolite gene activator protein
background, **2:**531–532
cAMP interactions with, **2:**532–534
description of, **2:**531
differential gene regulation, **2:**532–533
history of, **2:**531–532
transcriptional regulation by, **2:**532
Catecholamines, **3:**386–387
β-Catenin
cadherins and, **2:**893–894
functions of, **1:**72–73
genetic changes that affect, **1:**623
glycogen synthase kinase 3 and, **1:**549, **2:**893
LEF-1/TCF protein binding, **3:**163
modulation of, **1:**73
presenilin-1 effects, **2:**895
protein phosphatase 2A interactions with, **2:**408
R5 subunit expression and, **2:**411
rapid proteolytic turnover of, **3:**161–163
receptor protein tyrosine phosphatase targeting, **2:**870
ubiquination of, **2:**483, **3:**130
Wnt signaling and, **2:**789
Cathepsin D, **2:**258–259
Cation channels, cyclic nucleotide-regulated
anatomic sites of, **2:**516
description of, **2:**515
expression of, **2:**516
features of, **2:**515–516
ion channel subunits, **2:**515–516
olfactory neuron expression of, **2:**516–517
subfamilies, **2:**516
Cation-dependent mannose 6-phosphate receptor, **1:**89
Cation(s), **1:**216
Caveolae, **1:**323, **1:**407
Caveolin, **1:**12, **1:**324–325
Caveolin-1, **1:**324
Cbl proteins
c-, **1:**407
description of, **1:**296
domains of, **1:**483
murine studies of, **1:**484–485
protein tyrosine kinase regulation by, **1:**483–484
regions of, **1:**483
CBP/P300, **3:**115, **3:**150
$CBTF^{98}$, **3:**339
$CBTF^{122}$, **3:**339
c-Cbl, **1:**407
CCR5, **1:**191–194
CD4, **3:**548
CD5, **3:**548
CD8
description of, **1:**59
T-cell signaling and, **3:**548
CD14, **2:**333
CD19, **3:**560
CD28
CD80 interactions with, **1:**81
description of, **1:**73, **1:**75
expression of, **1:**356
Itk effects on, **1:**478
phosphoinositide 3-kinase signaling pathway mediated by, **2:**343
T-cell activation by, **1:**355–356, **2:**343
T-cell signaling and, **3:**549
CD34, **1:**48
CD40
antigen-presenting cell maturation and, **1:**319
characteristics of, **1:**319
description of, **1:**315
ligand for, **1:**319
protein tyrosine kinases activated by, **1:**320
signaling
acid sphingomyelinase translocation triggered by, **1:**320
description of, **1:**319–320
mechanisms of, **1:**321
murine studies, **1:**321
tumor necrosis factor receptor-associated factor pathways involved in, **1:**320–321
CD45
cytoplasmic region of, **1:**689
description of, **1:**433, **1:**641, **1:**689
dimeric forms of, **1:**690
dimerization of, **1:**685
diseases and, **1:**647
function of, **1:**689–690
inhibitors, **1:**681
negative regulation role of, **1:**690
protein interactions with, **1:**691
regulation of, **1:**690–691
signaling effects, **1:**690
structure of, **1:**689–690
substrates for, **1:**689–690
T-cell signaling and, **3:**548
transmembrane domain of, **1:**691
CD80
CD28 interactions with, **1:**81
description of, **1:**319

CD86, **1:**319
CD95
  apoptosis mediated by, **1:**315
  description of, **1:**315
  Fas-associated death domain binding with, **1:**317
CD154, **1:**319
CD4 receptors, **1:**23
CD8 receptors, **1:**23
CD4 T cells, **2:**342
$CD8^+$ T cells, **2:**342
CD120a, **1:**315
CD120b, **1:**315
Cdc2, **1:**693
Cdc4, **1:**508
Cdc6, **2:**408, **3:**403
Cdc14
  A, **1:**700
  B, **1:**700
  budding yeast
    anaphase-promoting complex, **1:**697
    description of, **1:**697
    exit from mitosis and, **1:**697–698
    FEAR pathway, **1:**698–699
    MEN pathway, **1:**698–699
    oligomerization of, **1:**697
    regulation of, **1:**698–699
    signaling pathways, **1:**698
    structure of, **1:**697–698
    substrates, **1:**698
    Tyr residues, **1:**697
  description of, **1:**697
  fission yeast, **1:**699–700
  human, **1:**700
Cdc24, **2:**571–572
Cdc25
  activation of, **1:**694
  Cdc2 and, **1:**693
  description of, **1:**604, **1:**647, **1:**654, **1:**693
  localization of, **1:**695
  phosphatase activity of, **1:**693
  physiological functions of, **1:**693–694
  protein phosphatase 2A interactions with, **2:**408
  regulation of, **1:**694–695
  stability of, **1:**695
  suppression of, **1:**694–695
Cdc42
  actin cytoskeleton organization and, **2:**701, **2:**716
  activated kinase, **2:**745
  apoptosis and, **2:**716
  biological effects of
    description of, **2:**715–716
    molecular mechanisms underlying, **2:**716–717
  cell growth regulated by, **2:**715–716
  cellular differentiation and, **2:**716
  definition of, **2:**715
  description of, **1:**465, **2:**183, **2:**715
  downstream effectors of, **2:**703, **2:**715
  functions of, **2:**715
  guanine exchange activity of, **2:**715
  guanine nucleotide exchange factors, **2:**204
  mutations, **2:**715
  phosphoinositide interactions with, **2:**203
  Saccharomyces cerevisiae expression of, **2:**715
  sequence alignment of, **2:**746
  signaling pathways, **2:**702
Cdc42/ACK/Intersectin complex, **2:**717
Cdc25C, **3:**403, **3:**407
Cdc42Hs, **2:**715
Cdc28p, **1:**585
Cdc42/PAK/Cool(Pix), **2:**717
Cdc42/PAR6/PKCzeta, **2:**717
Cdc42/Rac interactive binding proteins. *see* CRIB proteins
cdh1, **1:**700
Cdk. *see* Cyclin-dependent kinase(s)
CD40L, **3:**549–550
Cell adhesion
  calpain regulation of, **2:**108–109
  fibronectin, **3:**252
Cell adhesion molecules, **1:**58
Cell cycle
  apoptosis vs., **3:**437
  arrest of, DNA-damage-related, **3:**204–205, **3:**213–215
  ATM effects on, **3:**225
  calmodulin's role in, **2:**84–85
  checkpoints for
    description of, **3:**197–201, **3:**204, **3:**406–407
    genes involved in control of
      description of, **3:**209–210
      mutations in, **3:**210
    G_2/M, **3:**230–231, **3:**407
    G_1/S-phase, **3:**229
    identification of, **3:**206, **3:**208
    screening for defects in, **3:**208–209
    S-phase, **3:**229–230
  c-Jun regulation of, **3:**103
  cyclin-dependent kinase regulation of, **3:**332, **3:**401–403
  description of, **2:**711
  gene expression silencing, **3:**332
  inhibitor proteins, **3:**407
  phase of, **3:**401–403
  progression of
    cyclin D/Cdk4 in, **3:**402
    description of, **3:**197
    G1, **2:**711–712
    platelet-derived growth factor receptor signaling and, **1:**401
    Rac's role in, **2:**712–713
    Rho's role in, **2:**712–713
  regulation of
    FOXO transcription factor's role in, **3:**87
    PPARγ's role in, **3:**43
  splicing regulation and, **3:**332–333
Cell growth
  cdc42 and, **2:**715–716
  eIF-4E and, **3:**346
  guanylyl cyclase and, **2:**429–430
  heptahelical receptors and, **2:**595
  mTOR and, **1:**558
  TOR and, **1:**525
Cell membrane, **1:**117
Cell migration
  integrin signaling and, **1:**464–466
  Rac's role in, **2:**701–702, **2:**851
  Rho GTPases and, **2:**701–702, **2:**851
  steps involved in, **1:**465

Cell polarity
description of, **2:**716
importance of, **2:**733
molecular basis of, **2:**733–735
planar, **2:**785
Cell signaling
definition of, **1:**1
extracellular initiation of, **1:**21
future of, **1:**3
initiation of, **1:**21
membrane-bound receptors in, **1:**117–118
molecular level, **1:**2–3
molecules involved in, **1:**21
origins of, **1:**1–2
paradigms of, **1:**21–23
Cell–cell interactions
kidney, **3:**573–575
in ovary development, **3:**535–538
overview of, **3:**453–454
in pancreatic development, **3:**472–473
in testis development, **3:**531–535
Cell–matrix interactions
overview of, **3:**453–454
in pancreatic development, **3:**472–473
Cell-surface adhesion receptors
description of, **1:**71
focal adhesions, **1:**71
interferon-γ, **1:**271
mechanosensory mechanisms, **1:**71–72
T-cell costimulation, **1:**73–75
cellular, **2:**73
Central core disease, **2:**47
Central nervous system
cGMP-dependent protein kinase and, **2:**512–513
G protein effects, **2:**581–582
Na^+/Ca^{2+} exchanger distribution in, **2:**65
semaphorins' role in development of, **2:**877
Central supramolecular activation cluster, **1:**80
Centromere binding protein-E, **2:**740
Ceramide
apoptosis induced by, **2:**257
cell regulation mediated by, **2:**257
de novo biosynthesis of, **2:**257–258
generation of, **2:**257–258
physical properties of, **2:**259
protein interactions with, **2:**259
sphingomyelin cycle-derived, **2:**258
targets of, **2:**258–259
Ceramide-activated protein kinases, **2:**258
Ceramide-activated protein phosphatases, **2:**258
c-Erb-B2, **3:**566
c-Erb-B3, **3:**566
c-Erb-B4, **3:**566
Cerebrosterol, **3:**54
cGKI, **2:**511
cGKII, **2:**511
cGMP
cAMP-dependent protein kinase A activated by, **2:**546
description of, **2:**423
effectors, **2:**429
GAF domain-induced release of, **2:**455
growth factor activation inhibited by, **2:**429
guanylyl cyclase's role in formation of, **2:**427
Paramecium, **2:**536–537
phosphodiesterase-5. *see* Phosphodiesterase-5
protein kinase
activation of, **2:**480
biochemistry of, **2:**479–481
cAMP cross-activation of, **2:**545–546
β-catenin phosphorylation by, **2:**483
cell function roles of, **2:**481–482
description of, **2:**479, **2:**511
domain structure of, **2:**487–488
G protein phosphorylation by, **2:**611
gastrointestinal function and, **2:**512
genes, **2:**511
hypertension and, **2:**511–512
inhibitors of
ATP binding site-targeted, **2:**488–491
cyclic nucleotide binding site-targeted, **2:**488
description of, **2:**487–488
peptide binding site-targeted, **2:**491
properties, **2:**489–490
isoforms of, **2:**479–481
knockout mice studies of, **2:**481, **2:**511–513
mitogen-activated protein kinase regulation by, **2:**482
myosin light chain phosphatase binding subunit, **2:**481–482
nervous system effects, **2:**512–513
in *Paramecium,* **2:**537
peptide substrates of
acceptor loci, **2:**496
description of, **2:**495
optimum recognition sequences, **2:**496–498
phosphorylation sites, **2:**497–498
recognition of, **2:**495–496
specificity of, **2:**498
physiologic roles of, **2:**481–483
protein tyrosine phosphatase regulation by, **2:**482
Rp-cyclic nucleotide phosphorothioates, **2:**488
smooth muscle cell functions of, **2:**480
specificity of, **2:**498
substrates
peptide, **2:**495–498
physiological, **2:**502, **2:**507–508
type I, **2:**479–480
type Iα, **2:**480–481
type Iβ, **2:**481
type II, **2:**479, **2:**481
urogenital function and, **2:**512
vasorelaxation effects, **2:**511–512
receptors of, **2:**549
regulation of, **2:**431
ribose moiety of, **2:**552
selectivity of, **2:**546
sequestration of
description of, **2:**466
phosphodiesterase 5-induced, **2:**467–469
phosphodiesterase 6-induced, **2:**466–467
signaling pathways regulated by, **2:**468
signal compartmentalization, **2:**438–439
CG-NAP, **2:**385, **2:**389, **2:**409–411
ChaK
catalytic domain of, **1:**568–569
characteristics of, **1:**568

ChaK *(continued)*
description of, **1:**568
hydrophobic ATP-binding pocket of, **1:**569
kinase domains of, **1:**568–569
protein kinase A vs., **1:**569
regulation of, **1:**571
Channel kinase. *see* ChaK
Chaperone protein, **3:**127
Chaperones, **3:**271–273, **3:**359
Charge–charge interactions, **1:**11
C_H2/C_H3 hinge of Fc fragment, **1:**51, **1:**54
Checkpoint-dependent phosphorylation, **3:**209
Checkpoints, cell cycle
ATM regulation of
G_2/M, **3:**230–231, **3:**407
G_1/S-phase, **3:**229
S-phase, **3:**229–230
description of, **3:**197–201, **3:**204
genes involved in control of
description of, **3:**209–210
mutations in, **3:**210
identification of, **3:**206, **3:**208
screening for defects in, **3:**208–209
C-helix, **1:**235
Chemoattractant receptor–homologous molecule, **2:**268
Chemokine receptors
constitutive signaling of, **1:**174
CXCR2, **2:**408
description of, **1:**151–152
endogenous, **1:**173–174
herpesvirus-encoded, **1:**175
human immunodeficiency virus use of, for host cell entry
binding of, **1:**192
CCR5, **1:**191–194
CXCR4, **1:**191–194
description of, **1:**151, **1:**191
domains, **1:**193
Env domains involved in, **1:**192–193
in vivo, **1:**192
mechanisms of, **1:**191–192
presentation and processing, **1:**193–194
signaling, **1:**194
poxvirus-encoded, **1:**175
Chemokine(s)
α, **1:**150
β, **1:**150
CC, **1:**151, **1:**173
CCR5, **1:**193
classification of, **1:**150–151
CXC, **1:**150–151, **1:**173, **1:**193
definition of, **1:**149
description of, **1:**173
function of, **1:**149–151
induced, **1:**151
mechanism of action, **1:**173
molecular inhibitors of, **1:**151
overexpression of, **1:**151
redundant, **1:**173–174
structure of, **1:**149–151
types of, **1:**150
Chemomechanical gating model, **1:**230
Chemoreceptors
clustering of, **1:**200
description of, **1:**197
sensitivity of, **1:**197
Chemosensing proteins, **1:**18
Chemotaxis
adaptation of, **1:**199–200
bacterial uses of, **1:**197
definition of, **1:**197, **2:**217, **2:**645
description of, **1:**197
Dictyostelium
description of, **2:**217–218, **2:**645
phosphoinositide 3-kinase
activation of, **2:**218–220
localization of, **2:**218
pi3k1/s null cells, **2:**219–220
signaling pathways that control, **2:**218–219
G-protein βγ subunit signaling in, **2:**645–647
lipid phosphatases in, **2:**647
methyl-accepting proteins, **1:**197
phosphatase and tensin homology deleted on chromosome 10 in, **2:**647
phosphatidylinositol 3-kinase in, **2:**645–647
phosphoinositide 3-kinase's role in, **2:**218–220
receptors
cytoplasmic domain signaling, **1:**199
periplasmic ligand binding domain signaling of, **1:**198–199
transmembrane signaling, **1:**198–199
regulation of, **2:**217
SHIP in, **2:**647
CheW, **1:**198
Chico, **1:**413
Chimaerins, **2:**243
Chk1, **1:**694, **3:**200–201, **3:**407
Cholecystokinin, **3:**381, **3:**472
Cholecystokinin-8 receptor, **1:**118
Cholesterol
derivatives of, **2:**289
functions of, **3:**353
HMG-CoA reductase, **2:**287, **3:**353
intracellular levels of, **3:**353
liver X receptor regulation of, **3:**48, **3:**53–54
nuclear receptor ligands, **2:**289
oxysterols synthesized from, **2:**287
signaling molecules generated by synthesis of, **2:**287–288
sources of, **3:**353
sterol regulatory element binding protein and, **2:**288–289
synthesis of, **2:**288–289
CHOP, **3:**361
Chromosomal translocations, **1:**444–445
Cilostazol, **2:**434
CISK, **2:**172, **2:**174
11-*cis*-retinol chromophore, **1:**140–141
CIT2, **3:**367
c-Jun
cell cycle regulation by, **3:**103
DNA-binding domain of, **3:**101
JunB, **3:**101–102
loss of, **3:**101
NH2-terminal kinase. *see* JNK
N-terminal kinase, **1:**414–415, **1:**493–495, **1:**634, **2:**594

CK1ε, **3:**162
CL100, **1:**705
Cla4p, **1:**585–586
CLAVATA 1, **1:**580
Cleft lip/palate, **3:**498
Clg, **2:**715
clp1, **1:**699
c-Myc, **3:**437, **3:**567
Coat protein, **2:**729
γ-Coatomer, **2:**715
Collagen, **3:**504
Collapsin-reponse-mediator protein, **2:**878–879
Colon, gut peptides in, **3:**479
Colony stimulating factor-1, **3:**536, **3:**619–620
Colony stimulating factor-1 receptor, **3:**619–620
Complementarity-determining region loops
  CDR1β, **1:**64–65
  CDR2β, **1:**64–65
  description of, **1:**34, **1:**39
  H3, **1:**36
  inhibitors of, **1:**457
  olfactory receptors, **1:**145
  rearrangement of, **1:**36
  T-cell receptor, **1:**64–65
Compound 24, **1:**680
Conformational flexibility, **1:**25
Conformational freedom, **1:**28
Constant fragment. *see* Fc
Constitutive androstane receptor
  activation of, **3:**54–55
  description of, **3:**48
  ligands for, **3:**54–55
  sequestration of, **3:**54
COP9, **3:**131
Coprecipitation, for studying protein–protein interactions
  antibodies, **2:**297
  considerations for, **2:**295–296
  epitope tags for, **2:**297–298
  glutathione S-transferase-tagged proteins, **2:**296–297
Core binding factor 1, **3:**501
Co-Smads, **3:**171
COUP-TF
  description of, **3:**58
  vascular development role of, **3:**58–59
Covalent modification
  G proteins, **2:**585–587
  RNA polymerase II general transcription factors, **3:**16
COX-1. *see* Cyclooxygenase-1
COX-2. *see* Cyclooxygenase-2
CPEB
  description of, **3:**324
  vertebrate development mediated by, **3:**323–325
CPI-17, **1:**628
CRAC, **2:**218
cRaf1 protein kinase, **2:**390
Craniosynostosis, **2:**862
CREB
  basal, **3:**115
  description of, **3:**115
  mixed lineage leukemia protein and, **3:**117
  repressive complexes that affect, **3:**116
  secondary phosphorylation of, **3:**116
  signal discrimination via, **3:**116
  transactivation domain of, **3:**115
  transcriptional activation mechanisms via, **3:**115–116
CRIB proteins, **2:**745–747, **2:**759
Crm1, **3:**423
Cryptochrome, **1:**577
C124S, **1:**654
Csk
  description of, **1:**477
  T-cell antigen receptor function and, **1:**477
Csk-binding protein/PAG, **1:**477
C-SMAC, **2:**340, **2:**342
c-SRC, **3:**36
c-Src, **1:**407, **1:**446
Csw, **1:**711–712
C-terminal helix, **1:**124
CTLA-4
  degradation of, **1:**356
  description of, **1:**73–75
  expression of, **1:**356
  lysosome localization of, **1:**356
  negative signaling mediated by, **1:**357–358
  protein trafficking, **1:**356–357
  regulation of, **1:**356
  T-cell activation by, **1:**355–356
C-type leptin-like natural killer cell surface receptors, **1:**84–85
C-type leptin(s), **1:**88–89
C-type natriuretic peptide, **2:**423, **2:**428
Cubitus interruptus
  description of, **3:**167
  expression patterns of, **3:**167
  Hedgehog regulation of, **3:**167–169
  N-terminus of, **3:**167
  protein kinase A regulation of, **3:**169
  protein structure of, **3:**167
  transcriptional regulation of, **3:**169–170
CUP/AP-2α, **3:**42
CV1 cells, **2:**211
CXCR1, **3:**444
CXCR4, **1:**191–194
CXCR2 chemokine receptor, **2:**408
Cyclic adenosine diphosphate ribose
  antagonists, **2:**16
  calcium regulation by, **2:**15–16
  description of, **2:**15
  structure of, **2:**16
Cyclic adenosine monophosphate. *see* cAMP
Cyclic cGMP-specific and -regulated cyclic nucleotide phosphodiesterases, Anabaena adenylyl cyclase, and E.coli transcription factor FhlA. *see* GAF domains
Cyclic guanosine-3,5-monophosphate. *see* cGMP
Cyclic nucleotide
  cAMP. *see* cAMP
  cation channels regulated by
    anatomic sites of, **2:**516
    description of, **2:**515
    expression of, **2:**516
    features of, **2:**515–516
    ion channel subunits, **2:**515–516
    olfactory neuron expression of, **2:**516–517
    subfamilies, **2:**516

Cyclic nucleotide *(continued)*
cGMP. *see* cGMP
fluorescence resonance energy transfer monitoring of, **2:**460
formation of, **2:**466
hyperpolarization-activated cyclic nucleotide-gated channels
activation of, **2:**517
anatomic sites of, **2:**517–518
cAMP response, **2:**518
description of, **2:**515
family of, **2:**517
features of, **2:**515–516
intracellular levels of, **2:**525
loss of, **2:**465–466
monitoring of, **2:**460
in *Paramecium,* **2:**537
receptor binding of, **2:**515
sequestration of, **2:**465–466
signaling
cyclic nucleotide analogs for studying, **2:**549–553
description of, **2:**459–460
in neurons, **2:**462
in *Paramecium,* **2:**535–537
spatio-temporal aspects of, **2:**462
in trypanosomatids, **2:**539–542
Cyclic nucleotide monophosphates
analogs
activating, **2:**549–550
axial, **2:**550
chemical structure of, **2:**551–553
equatorial, **2:**550
future of, **2:**553
inhibitory, **2:**550–551
properties of, **2:**551–552
purine ring structure, **2:**552
cAMP. *see* cAMP
cGMP. *see* cGMP
discovery of, **2:**549
Cyclic nucleotide receptors, **2:**546–547
Cyclic nucleotide-binding domain
cyclic nucleotide-gated ion channel, **2:**546–547
description of, **2:**515–516
specificity of, **2:**546
Cyclic nucleotide-binding protein, **2:**466
Cyclic nucleotide-dependent protein kinases
cGMP-dependent protein kinase. *see* cGMP, protein kinase
inhibitors of
ATP binding site-targeted, **2:**488–491
cyclic nucleotide binding site-targeted, **2:**488
description of, **2:**487–488
peptide binding site-targeted, **2:**491
properties, **2:**489–490
peptide substrates of
acceptor loci, **2:**496
description of, **2:**495
optimum recognition sequences, **2:**496–498
phosphorylation sites, **2:**497–498
recognition of, **2:**495–496
specificity of, **2:**498
protein kinase A
activation, **1:**614, **1:**627, **1:**631, **2:**326, **2:**330, **2:**377, **2:**383, **2:**419, **2:**471
catalytic subunits of, **2:**471–474
D/D domain, **2:**475
discovery of, **2:**471
regulatory subunits, **2:**471, **2:**474–475
Rp-cyclic nucleotide phosphorothioates, **2:**488
Cyclic nucleotide-gated channels
allosteric regulation studies, **1:**235
CNGB1, **1:**235
CNGB3, **1:**235
description of, **1:**233
family of, **1:**234
genes, **1:**234
isoforms, **1:**235
ligand gating in, **1:**235
olfactory neurons and
molecular basis for, **1:**234–235
signal transduction of, **1:**233–234
photoreceptors and
molecular basis for, **1:**234–235
signal transduction, **1:**233–234
subunits, **1:**234–235
Cyclic nucleotide-mediated signaling, **1:**370
Cyclin A, **3:**403
Cyclin B/Cdc2, **3:**403
Cyclin D1
description of, **1:**623, **2:**672
expression of, **3:**401
phosphorylation of, **3:**402
Rho's role in expression of, **2:**713
transcription of, **3:**62
Cyclin D/cdk4/6, **2:**713
Cyclin E/Cdk2, **3:**402–403
Cyclin G1, **2:**409, **2:**411
Cyclin G2, **2:**408, **2:**411
Cyclin H/Cdk7, **3:**404
Cyclin I, **2:**411
CYCLIN L, **3:**333
Cyclin-dependent kinase 5, **1:**614
Cyclin-dependent kinase(s)
in alternative splicing, **3:**333
Cdk4, **1:**446, **3:**401
Cdk6, **3:**401
cell cycle regulation by, **3:**332, **3:**401–403
degradation, **3:**405
description of, **1:**443, **1:**446, **1:**693, **1:**697, **2:**711
inhibitors of, **1:**457–458, **1:**508
inhibitory phosphates on, **3:**404
inhibitory protein regulation of, **3:**404
phosphorylation of, **3:**404–405
substrates, **3:**405–406
Cyclooxygenase
definition of, **2:**265
metabolites, **3:**577–578
prostanoid formation, **2:**265
Cyclooxygenase-1
acetylation of, **2:**283
amino acid sequence of, **2:**265
expression of, **2:**265
thromboxane A_2 production by, **2:**266–267
Cyclooxygenase-2
acetylated recombinant, **2:**283
acetylation of, **2:**283
amino acid sequence of, **2:**265
deletion of, **2:**265
expression of, **2:**265

patent ductus arteriosus and, **2:**265
prostacyclin production by, **2:**267
protective role for, **2:**283–284
vascular, **2:**283–284
CYP7A1, **3:**50, **3:**54
cyp2b10, **3:**50
Cystatin A, **1:**29
Cysteine, **1:**329
Cysteine-dependent aspartate specific protease. *see* Caspases
Cystic fibrosis transmembrane conductance, **1:**638
Cytochrome c, **3:**434
Cytochrome P-450 system, **3:**50, **3:**578
Cytohesins, **2:**205
Cytokine homology domain, **1:**251
Cytokine receptors
chains of, **1:**427–428
classification of, **1:**343, **3:**617
conformational reorganizations of, **1:**256–257
description of, **1:**2, **1:**343, **1:**362, **3:**617
epidermal growth factor receptors. *see* Epidermal growth factor receptors
erythropoietin receptor. *see* Erythropoietin receptor
granulocyte cell signaling factor. *see* Granulocyte cell signaling factor
Janus tyrosine kinases associated with, **1:**344–345, **1:**362–363, **1:**431
overview of, **1:**239–240
signal transduction pathways activated by, **1:**429
signaling by
description of, **1:**343, **1:**427–429
gp 130 subunit, **3:**622–623
homodimerization of non-tyrosine kinase receptor polypeptide chain, **3:**620–622
mediation of, **1:**432
negative feedback regulation of, **3:**523
suppressors of. *see* Suppressors of cytokine signaling
tyrosine kinase-containing receptors, **3:**618–620
tyrosine phosphorylation events involved in, **1:**431–433
tumor necrosis factor receptors. *see* Tumor necrosis factor receptors
type I
characteristics of, **1:**362–363
description of, **1:**343
erythropoietin receptors. *see* Erythropoietin receptors
growth hormone receptors. *see* Growth hormone receptors
Janus kinases associated with, **1:**428
type II
characteristics of, **1:**343, **1:**362–364
Janus kinases associated with, **1:**428
Cytokine-binding homology regions, **1:**259
Cytokine(s)
caspases effect, **2:**351
characteristics of, **1:**431
definition of, **1:**343, **1:**431
function of, **1:**429
gene expression regulation, **3:**521–522
gp130–cytokine complex
description of, **1:**259–260
granulocyte colony-stimulating factor–granulocyte colony-stimulating factor receptor extracellular signaling complex, **1:**261–262
mutagenesis of, **1:**260
α-receptor interactions with, **1:**260–261
receptor/ligand interactions, **1:**259
site 2 interface, **1:**260–261
site 3 interface, **1:**261
structure of, **1:**260
viral, **1:**260
hematopoietic, **3:**615–617
hetero-oligomerization of, **1:**259
insulin receptor substrate-protein tyrosine phosphorylation promoted by, **1:**414
janus tyrosine kinases activated by, **1:**343
NFκB regulation of, **3:**111
Shp2's role in signaling by, **1:**718
Cytoplasmic polyadenylation elements, **3:**324
Cytoplasmic signaling
description of, **1:**369–370, **3:**257–258
Eph receptors, **1:**421–422
future of, **1:**370
radiation-induced, **3:**258–261
receptor tyrosine kinase activation, **3:**441–442
Cytoskeleton, actin
cdc42's role in, **2:**701, **2:**716
disruptions of, **3:**411
dynamin and, **3:**413–414
in growth cones, **2:**878
polarity of, **2:**735
rho GTPases and
cell migration, **2:**701–702, **2:**851
description of, **2:**701, **3:**294
remodeling, **3:**414–416
signaling, **2:**702–703
in yeast, **3:**411–413
Cytosolic calcium-independent phospholipase A_2, **2:**262–263
Cytotoxic T cells, **1:**319
Cytotoxic T-lymphocyte antigen 4. *see* CTLA-**1:**4
CytR-regulated promoters, **2:**532

D

D2 domains, **1:**664, **1:**687–688, **1:**744–745
DAF-16, **3:**88
Darier's disease, **2:**60
DARPP-32
description of, **1:**616, **1:**628
knockout mice studies, **2:**399
$PP1_c$ interaction with, **2:**398, **2:**400
Db family
Dbl domains
description of, **2:**751–752
regulation of, **2:**754
description of, **2:**751
domains of, **2:**751–752
pleckstrin homology domains
characteristics of, **2:**752–753
description of, **2:**751
regulation of, **2:**754
structure of, **2:**751–752
Dcad 102F-like cadherins, **2:**891
DC-SIGN, **1:**89, **1:**99
DC-SIGNR, **1:**89
Dead-phosphatases
description of, **1:**741
types of, **1:**742–743
Death effector domain, **3:**432–433

Death receptors
caspase-8 activation by, **3:**432–434
characteristics of, **3:**432–433
death domains, **3:**432
description of, **1:**305
DR5
Apo2L/TRAIL bound to, **1:**305–307
description of, **1:**24
ligand binding, **1:**307
loops of, **1:**306
Shp1 effects, **1:**713
signaling requirements, **2:**353
Death-inducing signaling complex, **1:**279, **3:**254
Degrons, **2:**349
7-Dehydrocholesterol, **2:**288
Deltex proteins, **3:**151
Dendritic cells, **2:**336
Dendritic protein phosphatases
calcineurin. *see* Calcineurin
description of, **2:**397
protein phosphatase 1. *see* Protein phosphatase 1
substrates, **2:**399–400
DEP1, **1:**647
Dephosphorylation
axin, **1:**623
Ins (1,3,4,5,6)P_5, **2:**233
PPP family, **1:**602
protein, **1:**591
protein kinase C, **1:**552–553, **2:**188–189, **2:**393
Depolarization, **1:**209
Desensitization of G-protein coupled receptors
definition of, **1:**181
description of, **1:**220
heterologous, **1:**183–184
homologous, **1:**182
mechanisms of, **1:**182–184
Desmosomal cadherins, **2:**891
Deubiquinating enzymes, **2:**347–348
Development
bone
fibroblast growth factors in, **3:**503–504
growth factors associated with, **3:**501–503
hormones that affect, **3:**500–501
insulin-like growth factors in, **3:**503
low-density lipoprotein receptor-related protein 5 in, **3:**504
matrix metalloproteinases in, **3:**505
microfibrils in, **3:**505
transcription factors associated with, **3:**501–503
bone morphogenetic proteins in
description of, **2:**833
extracellular modifiers of, **2:**834
gradients of, **2:**833
intracellular negative regulation of signaling, **2:**835–836
ligand gradients for, **2:**833–834
loss-of-function studies in mammals, **2:**836
receptors, **2:**834–835
signal transduction, **2:**835
summary of, **2:**836
endocytic signaling in, **3:**444–445
eye, in *Drosophila melanogaster,* **2:**827–830
fibroblast growth factor receptor in, **2:**861–862
hedgehog proteins in, **2:**795–796
hedgehog signaling in, **2:**793–796
lung
bombesin-like peptides in, **3:**511
bone morphogenic proteins in, **3:**511
description of, **3:**509
epidermal growth factor in, **3:**510
fibroblast growth factor in, **3:**510
fibroblast-pneumonocyte factor in, **3:**511
growth factors involved in, **3:**509–511
hepatocyte growth factor in, **3:**511
insulin-like growth factors in, **3:**510–511
platelet-derived growth factor in, **3:**510
transforming growth factor α, **3:**510
transforming growth factor β in, **3:**511
vascular endothelial growth factor in, **3:**511
neurotrophins in
cellular effects of, **2:**839
description of, **2:**839
interacting proteins, **2:**842
ligands, **2:**840
receptors, **2:**840–841
retrograde transport, **2:**841–842
signaling specificity, **2:**840–841
notch signaling pathways in
basic helix-loop-helix factors, **2:**819
description of, **2:**817
lateral inhibition, **2:**819
limbs, **2:**820–821
lymphoid development, **2:**821
neurogenesis, **2:**817–819
notch ligands, **2:**814–817
notch receptors, **2:**813–814
organ systems, **2:**821–822
segmentation, **2:**819–820
signal transduction, **2:**815–816
vascular development, **2:**821
nuclear receptor corepressors in, **3:**31
platelet-derived growth factor receptor signaling in, **2:**845–847
testis, **3:**532–533
vulval, in *Caenorhabditis elegans,* **2:**805–807
wnt signaling in, **2:**790
DFCP1, **2:**181
dHAND, **3:**466
DHR39, **3:**69
DHR83, **3:**69
Diabetes mellitus
AMP-activated protein kinase applications, **1:**536
autosomal forms of, **1:**416
IA-2 in, **1:**745
insulin resistance in, **1:**415–416
Diacylglycerol
description of, **1:**369, **1:**501, **1:**553
diacylglycerol kinase regulation of, **2:**245
fatty acid specificity and, **2:**245
phosphatidic acid as precursor to, **2:**237
phospholipase Cγ production of, **3:**560
phospholipase D production of, **2:**240, **2:**243
protein kinase C activation by, **1:**369, **2:**120, **2:**187, **2:**390–391, **3:**260–261, **3:**550
proteins activated by, **2:**243
signaling of, **2:**245

Diacylglycerol kinases
α, **2:**244
ε, **2:**245
compartmentalized function of, **2:**245
description of, **2:**243
diacylglycerol regulation by, **2:**245
family of, **2:**243
myristoylated alanine-rich C kinase substrate, **2:**244
nuclear, **2:**245
paradigms of, **2:**244–245
regulation of, **2:**244
structure of, **2:**243–244
type I, **2:**243
type II, **2:**243
type III, **2:**243
type IV, **2:**243–244
visual signal transduction and, **2:**245
Diacylglycerol receptors, **2:**243
Dictyostelium chemotaxis
description of, **2:**217–218, **2:**645
phosphoinositide 3-kinase
activation of, **2:**218–220
localization of, **2:**218
pi3k1/s null cells, **2:**219–220
signaling pathways that control, **2:**218–219
Diethyl pyrocarbonate, **1:**736
Dihydrofolate reductase, **1:**254
Dihydropyridine-modulated receptors, **2:**46
Dihydropyridines, **1:**217
1α, 25-Dihydroxyvitamin D_3, **3:**500
Dimerization
CD45, **1:**685
early endosome antigen 1, **2:**179
ErbBs, **1:**408
erythropoietin receptors, **1:**255
G-protein coupled receptors, **1:**188–189
ligand binding-induced, **1:**392–393
nuclear receptors, **3:**23
phosphodiesterase-5, **2:**450
platelet-derived growth factor, **1:**399
protein tyrosine kinase receptors, **1:**392–393
receptor protein tyrosine phosphatases regulated by, **1:**685–686
tumor necrosis factor receptors, **1:**364–365
tyrosine kinase-containing receptors, **1:**361–362
dinI, **3:**185
Diphosphoryl inositol synthase, **2:**231
Diseases
CD45 and, **1:**647
hedgehog proteins and, **2:**795–796
mitochondrial calcium and, **2:**76
myotubularins and, **2:**145–146
nuclear receptor corepressors and, **3:**31
omega-3 polyunsaturated fatty acids for, **2:**283
protein tyrosine phosphatases and, **1:**646–647, **1:**667–668
Rab proteins and, **2:**692
Shp1 and, **1:**647
Shp2 and, **1:**647, **1:**719–720
Disheveled protein, **3:**162
Dissociation
definition of, **1:**30–31
protein complex, **1:**30–31
DNA damage
cell cycle arrest secondary to, **3:**204–205, **3:**213–215
checkpoints for
description of, **3:**197–201
G_1/S-phase, **3:**229
protein kinases associated with, **3:**406
S-phase, **3:**229–230
cytoplasmic signaling, **3:**259–260
double-strand breaks
adaptation responses to, **3:**209
ATM recognition of, **3:**227–229
cellular response in, **3:**220
checkpoint activation for
description of, **3:**205–206
G_2/M checkpoint, **3:**230–231
G_1/S-phase checkpoint, **3:**229
screening for alterations in, **3:**209
S-phase checkpoint, **3:**229–230
description of, **3:**203, **3:**219
detection of, **3:**226
genes involved in, **3:**206–207
genetic effects of, **3:**205
homologous recombination of, **3:**219
induction of, **3:**203–204
Ku, **3:**221–222
nature of, **3:**205
nondamage-related occurrence of, **3:**228
nonhomologous end joining for, **3:**219–223
recognition of, **3:**221
repair of, **3:**205, **3:**219–222, **3:**226
sensor proteins for detecting, **3:**226–227
signaling networks for, **3:**206
signaling responses to, **3:**205–206
genotoxic agents
activating protein 1 and, **3:**102
stress signal caused by, **3:**179
overview of, **3:**203–204
radiation-induced
Drosophila melanogaster studies
apoptosis, **3:**214, **3:**216
cell cycle arrest secondary to, **3:**213–215
description of, **3:**213
DNA damage, **3:**213
DNA repair, **3:**214
effectors, **3:**213–214
sensors, **3:**213
transmitters, **3:**213
sensing of, **3:**226–227
responses to, **3:**203–204
signaling pathways, **3:**203
DNA damage regulon, **3:**201
DNA polymerase α-primase, **2:**408
DNA replication, **3:**402–403
DNA-dependent protein kinase catalytic subunits, **1:**557, **1:**559
DNA-PK, **3:**179, **3:**242
Docking, **1:**12, **2:**663
Dok-1, **1:**584
Dopamine, **3:**584
Dorsal root ganglion cells, **3:**607
Dorsal root reflexes, **3:**611–612

Double-strand breaks
adaptation responses to, **3:**209
ATM recognition of, **3:**227–229
cellular response in, **3:**220
checkpoint activation for
description of, **3:**205–206
G_2/M checkpoint, **3:**230–231
G_1/S-phase checkpoint, **3:**229
screening for alterations in, **3:**209
S-phase checkpoint, **3:**229–230
description of, **3:**203, **3:**219
detection of, **3:**226
genes involved in, **3:**206–207
genetic effects of, **3:**205
homologous recombination of, **3:**219
induction of, **3:**203–204
Ku, **3:**221–222
nature of, **3:**205
nondamage-related occurrence of, **3:**228
nonhomologous end joining for, **3:**219–223
recognition of, **3:**221
repair of, **3:**205, **3:**219–222, **3:**226
sensor proteins for detecting, **3:**226–227
signaling networks for, **3:**206
signaling responses to, **3:**205–206
Downregulation, **1:**182
Downstream promoter element, **3:**13
Downstream signaling pathways
description of, **1:**471
insulin, **3:**329
modular interactions, **1:**471–473
Ras/Ras proteins, **2:**671–672
receptor protein tyrosine phosphatases, **2:**869–870
sphingosine 1-phosphate receptors, **2:**248–249
Dpo4, **3:**188
dpp, **3:**463
DPTP10D, **2:**869
DPTP69D, **2:**867, **2:**869
Drosophila corkscrew gene, **1:**707–708
Drosophila melanogaster
bone morphogenetic proteins in, **2:**833
CPEB in development of, **3:**324
dorsal-ventral patterning in, **2:**779–781, **2:**828
embryogenesis of, **3:**69
eye development in, **2:**827–830
FYVE domain in, **2:**178
G-protein function in, **2:**572–573
immune response, **2:**780–781
JNK pathway in morphogenesis of
cellular stress response, **2:**785–786
description of, **2:**783
follicle cell morphogenesis, **2:**785
planar cell polarity, **2:**785
signaling in dorsal closure, **2:**783–785, **2:**815
thorax closure, **2:**785
wound healing, **2:**785–786
KSR cloning, **2:**595
LIN-12/Notch pathways, **2:**810
m4, **3:**154
mitogen-activated protein kinase phosphatases in, **1:**704
nuclear receptors
description of, **3:**69
ecdysone regulatory hierarchies, **3:**69, **3:**71
embryonic pattern formation and, **3:**69
neuronal development role of, **3:**71
subfamilies, **3:**69–70
Pax2, **3:**154
phosphoinositide 3-kinase studies, **2:**139
photoreceptors, **1:**349–351
phototransduction, **1:**349
protein kinases of, **1:**375
R2 subunits in, **2:**407
radiation response in
apoptosis, **3:**214, **3:**216
cell cycle arrest secondary to, **3:**213–215
description of, **3:**213
DNA damage, **3:**213
DNA repair, **3:**214
effectors, **3:**213–214
sensors, **3:**213
transmitters, **3:**213
receptor protein tyrosine phosphatases in
neuromuscular system, **2:**868
visual system, **2:**867
sim, **3:**154–155
Toll-Dorsal signaling in, **2:**779–781
translational control in, **3:**327–329
DRP1, **2:**768
dsRNA-binding motif, **3:**335
DTIR, **3:**208
Dual-specificity phosphatases
active site cleft of, **1:**655
Cdc25. *see* Cdc**1:**25
crystal structures of, **1:**654
description of, **1:**643, **1:**653
STYX and, **1:**745
VHR, **1:**654
DUN1, **3:**208
Dynamin
-2, **2:**768
actin regulatory protein interactions with, **2:**768, **3:**413–414
description of, **2:**763, **3:**411
domain structure of, **2:**763
dyn (K44A), **2:**767–768
dyn (K694A), **2:**767
dyn (R725A), **2:**767
endocytic vesicle formation, **2:**765–767
features of, **2:**763–765
GAP domain, **2:**765
in vivo function, **2:**766–767
proline-rich domain of, **2:**764
proteins interacting with, **3:**414
in *Saccharomyces cerevisiae,* **2:**767
signaling molecule, **2:**767–768
signaling pathways affected by, **2:**768
subfamilies, **2:**767
summary of, **2:**768
topology of, **2:**764
unconventional GTPase activity, **2:**765

E

E1, **2:**347
E2, **2:**347
Early endosome antigen 1
dimerization of, **2:**179

FYVE domain of, **2:**177
properties of, **2:**181
E-cadherin, **2:**408, **2:**895–896
Ecdysone pulse, **3:**69, **3:**71
EEA1. *see* Early endosome antigen 1
EF2 kinase
calcium/calmodulin and, **1:**570
cloning of, **1:**568
description of, **1:**567
p70 S6K effects, **1:**571
regulation of, **1:**570–571
EF-hand proteins
description of, **2:**79
neuronal calcium sensors. *see* Neuronal calcium sensors
egl-17, **2:**807
Eicosanoids
definition of, **2:**265
endothelial cells, **3:**577–578
inflammation mediation by, **2:**261
renal tubules affected by, **3:**585–586
vasoconstriction by, **3:**579
eIF2, **3:**339
eIF2α, **3:**314–316
eIF4B, **3:**346
eIF-4E
cancer and, **3:**346
cap-dependent translation repressed by, **3:**344
cell growth and proliferation controlled by, **3:**346
4E-BP regulation of, **3:**301–302
mTOR phosphorylation of, **3:**344–345
phosphorylation of, **3:**344
eIF-4E binding proteins
description of, **1:**524
mTOR phosphorylation of, **1:**526–527, **1:**530
overexpression of, **1:**530
eIF4F, **3:**343–344
eIF4G, **3:**346
Electrosomes
definition of, **1:**207
ion channels as, **1:**207
Electrostatic interactions, in molecular binding, **1:**11
EMP1, **1:**252
EMP33, **1:**252
Endocrinology, **1:**1–2
Endocytosis
actin dynamics in, **3:**411–413
description of, **2:**677–678, **3:**411
in developmental systems, **3:**444–445
G-protein coupled receptor signaling and, **3:**444
microtubule cytoskeleton in, **3:**413
receptor tyrosine kinase signaling, **3:**441–443
Endofin, **2:**181
Endoplasmic reticulum
calcium release, **2:**75
degradation apparatus, **3:**263
description of, **3:**311
functions of, **3:**311
inositol 1,4,5-trisphosphate receptors in, **2:**42
mitochondria proximity to, **2:**75
perturbations that affect, **3:**311
signaling pathways, **3:**263–265
SREBP cleavage-activating protein and, **3:**356
sterol regulatory element binding proteins and, **3:**356
stress responses, **3:**263–266, **3:**279, **3:**359–362
unfolded protein response. *see* Unfolded protein response
Endoplasmic reticulum degradation apparatus, **3:**359
Endothelial cells
activation of, **3:**459
Ang-1 effects on survival of, **2:**852
Ang-2 expression in, **2:**851
angiogenesis survival of, **2:**851–852, **3:**459
characteristics of, **3:**457
connections for, **3:**578
description of, **2:**850
endothelin release by, **3:**577
kidney, **3:**575–579
migration of, **2:**851
number of, **3:**459
vascular endothelial growth factor-induced proliferation of, **2:**850
Endothelial differentiation gene, **2:**19, **2:**247
Endothelial nitric oxide synthase, **2:**851, **3:**584
Endothelial-derived hyperpolarizing factor, **3:**578
Endothelin, **3:**576–577, **3:**585
Endothelin receptors, **3:**585
ENTH domain, **2:**156–157
Enthalpy, **1:**28
Entropy, **1:**28
Env proteins, **1:**191–193
Epacs
-1, **2:**521
-2, **2:**521–522
cAMP effects mediated by, **2:**549–550
cAMP-binding domain of, **2:**521
cellular function of, **2:**522, **2:**524
description of, **2:**475, **2:**521
domain organization of, **2:**521
evolutionary conservation of, **2:**522
expression of, **2:**522
family of, **2:**521
properties of, **2:**522
sequence alignment of, **2:**523
subcellular localization of, **2:**522
Eph receptors
activation of, **1:**421
cell adhesion and, **1:**423
cell proliferation effects, **1:**423
cytoplasmic interactions, **1:**422
description of, **1:**392, **1:**421
effectors of, **1:**422
ligand binding of, **1:**421
NMDA receptor interactions with, **1:**422
PDZ binding motif in, **1:**423
signaling of
cytoplasmic protein tyrosine kinases, **1:**421–422
PDZ-domain-containing proteins for, **1:**423
Rho family GTPases, **1:**422–423
Src kinases effect on, **1:**422
structure of, **1:**422
summary of, **1:**424–425
EphB2 receptor, **1:**399
Ephrins
definition of, **1:**421
Eph receptor activation by, **1:**421
EphrinA, **1:**421
Ephrin-B2, **1:**75

Ephrins *(continued)*
EphrinB reverse signaling, **1:**424
Ephrin-B2/EphB2 receptor complex, **1:**75–76
reverse signaling by, **1:**423–424
signaling by, **1:**423–424, **2:**873
summary of, **1:**424–425
Epidermal growth factor
breast tissue signaling and, **3:**566–567
cellular effects of, **1:**323
cGMP effects on, **2:**481
description of, **1:**2, **1:**323
functions of, **1:**323
lung development and, **3:**510
wild-type, **3:**441
Epidermal growth factor receptors
amplification of, **1:**445
angiogenesis and, **1:**455
cancer-related overexpression of, **1:**323, **1:**445, **1:**453
caveolae localization of, **1:**407
caveolin and, **1:**324–325
characteristics of, **1:**323
description of, **1:**239, **1:**323, **1:**391, **1:**405
domain structure of, **1:**405
in *Drosophila* eye development, **2:**827–831
G-protein coupled receptors and, **1:**407
insulin receptor and, similarities between, **1:**293
internalization, **3:**442
kinase inhibitors, **1:**453–454, **1:**456
ligands
binding, **3:**441
types of, **3:**566
lipid raft localization of, **1:**323–325
mitogen-activated protein kinase activation and, **1:**324
negative regulatory pathways, **1:**407–408
Notch signaling pathways and, in *Drosophila* eye development, **2:**827–831
role of, **1:**256
signaling
in *Caenorhabditis elegans* vulval development, **2:**805–807
pathways for, **1:**407
Epitope tags, **2:**297–298
Epoxysterols, **2:**287
Epsin, **2:**156
ErbBs
cancer and, **1:**408
dimerization of, **1:**408
domain structure of, **1:**405
ErbB2, **1:**323
ErbB3, **1:**323
ErbB4, **1:**323, **1:**394
negative regulatory pathways, **1:**407–408
proteins
pathological conditions associated with, **1:**408
subcellular localization of, **1:**405, **1:**407
signaling
in breast, **3:**567
pathways for, **1:**407
specificity of, **1:**408
ERECTA, **1:**580
Ergocalciferol, **3:**500
ERK. *see* Extracellular signal-regulating protein kinases
Erkl, **2:**365
ERM proteins, **2:**211
Erythropoietin, **1:**432, **3:**620
Erythropoietin receptors
activation of, **1:**255
binding mechanisms, **1:**251
characteristics of, **1:**363
crystal structures of, **1:**253
cytokine signaling through, **3:**620
description of, **1:**24, **1:**239, **1:**251, **3:**620
dimerization of, **1:**255
EMP1 binding to, **1:**252
EMP33 binding to, **1:**252
erythropoietin binding to, **1:**252
function of, **1:**245, **1:**429
homodimerization of, **1:**245
hot spot in, for ligand binding, **1:**252, **1:**254
juxtamembrane domain of, **1:**254–255
plasticity of, **1:**254
preformed dimers in, **1:**254–256
structural studies of, **1:**251–252
transmembrane domain of, **1:**254
unliganded, **1:**252
WSXWS sequence, **1:**251
Escherichia coli
catabolite gene activator protein
background, **2:**531–532
cAMP interactions with, **2:**532–534
description of, **2:**531
differential gene regulation, **2:**532–533
history of, **2:**531–532
transcriptional regulation by, **2:**532
cell signaling pathways, **1:**16, **1:**18
chemotaxis receptor dimer, **1:**197–198
flagellar proteins in, **1:**16, **1:**18
glucose metabolism by, **2:**531
Q mutant, **1:**199
SOS response
description of, **3:**185
dinI gene, **3:**185
ending of, **3:**185–186
genes induced by, **3:**187
LexA repressor, **3:**185–187
posttranscriptional control in, **3:**185
regulation of, **3:**186–187
self-cleavage responses, **3:**186
SoxR regulatory system of, **3:**191
E-selectin, **1:**88
Estrogen
bone effects, **3:**500
prostate gland development and, **3:**594
Estrogen receptors
α, **3:**35, **3:**597
β, **3:**35, **3:**597
description of, **3:**23
Estrogen related receptors
characteristics of, **3:**36
description of, **3:**36
ligands for, **3:**55
Eukaryotic ion channels, **1:**206–207
Eukaryotic protein kinases
Caenorhabditis elegans, **1:**375–376
catalytic domain of, **1:**373–374

comparative kinomics, **1:**376–377
discovery of, **1:**373
Drosophila melanogaster, **1:**375
Homo sapiens, **1:**375–376
nematodes, **1:**376
Saccharomyces cerevisiae, **1:**374–375
Schizosacharomyces pombe, **1:**374–375
structure of, **1:**373
Eukaryotic termination factor-1, **2:**409
Exchange protein directly activated by cAMP. *see* Epacs
Excitation-contraction coupling, **2:**53
Exocytosis
calcium sensor in, **3:**383–385
cAMP sensors in, **3:**383–385
cell variations in, **3:**375–376
description of, **2:**664, **3:**375–376
functional aspects of, **3:**376
G proteins' role in, **3:**379
morphological aspects of, **3:**376
protein kinase C's role in, **3:**385–386
rate of, **3:**375
regulation of, **3:**380–382
secretagogues
calcium influx regulation by, **3:**380
properties of, **3:**377
target cell receptor functions of, **3:**377–378
types of, **3:**376
secretion
calcium's role in, **3:**378–382, **3:**388
cAMP's role in, **3:**378–379, **3:**388
description of, **3:**375
far upstream regulation of, **3:**387
guanosine triphosphate's role in, **3:**379
negative regulation of, **3:**386
upstream regulation of, **3:**386–387
secretory vesicles, **3:**386–387
sensors for, **3:**383–385
signal propagation mediated by, **3:**378
signal summation in, **3:**385
SNAREs and, **3:**382–383
summary of, **3:**387–388
Extracellular matrix
bone, **3:**504–505
cancer cell growth and behavior, **1:**466
integrins and, **1:**123
Extracellular signal-regulating protein kinases
cassette, **2:**592–594
classification of, **2:**365
description of, **1:**493–494, **1:**673, **3:**259
dual phosphorylation motif, **2:**365
ERK1, **2:**365, **2:**367, **3:**101
ERK2, **2:**365, **3:**101
ERK3, **2:**366
ERK5, **2:**366
ERK7, **2:**366
ERK8, **2:**366
G-protein coupled receptor signaling and, **2:**593
mRNA turnover and, **3:**286
tyrosine kinases that mediate, **2:**592
Ezrin/radixin/moesin proteins. *see* ERM proteins

F

Fab1, **2:**181
Fab fragment
complementarity-determining region loops
antigen binding, **1:**51
description of, **1:**34, **1:**36
rearrangement of, **1:**36
conformational changes of
description of, **1:**34
main-chain rearrangements, **1:**34–35
side-chain rearrangements, **1:**34–35
V_L-V_H rearrangements, **1:**36
definition of, **1:**34
discovery of, **1:**33
F-actin
Abl regulation of, **3:**252
characteristics of, **1:**465, **1:**499, **2:**217–218, **2:**398, **2:**716, **2:**878
Factor XIII, **1:**120
FADD. *see* Fas-associated death domain
Falvopiridol, **1:**458
Familial advanced sleep phase syndrome, **1:**577
FAP-1, **1:**647
FAPP1, **2:**166
Farnesoid X receptor
bile acids that bind to, **3:**54
description of, **2:**289, **3:**48
ligands for, **3:**54
Farnesyl diphosphate, **2:**287
Farnesyl transferase, **2:**675
Farnesyl transferase inhibitors
Akt and, **2:**742
animal models of, **2:**739
CAAX-containing proteins as targets of, **2:**741–742
cancer uses of, **2:**739
cell culture activity of, **2:**739
description of, **2:**683, **2:**737
development of, **2:**738–739
identification of, **2:**738–739
inhibition of signaling by, **2:**741–742
pharmacological uses of, **2:**740
signaling studied using, **2:**740
targets of, **2:**740–741
Farnesylation
description of, **1:**333
protein function and, **2:**737
Fas ligand, **3:**536
Fas-associated death domain
CD95 binding with, **1:**317
description of, **1:**317, **3:**432, **3:**596
Fat-like cadherins, **2:**891
Fatty acid synthase, **3:**48
Fatty acid transport protein 1, **3:**39
F-box proteins, **3:**131, **3:**153
Fc
binding domains, **1:**52
binding peptide, **1:**53–54
binding site on, **1:**52–53
characteristics of, **1:**53–54
plasticity of, **1:**51–54
Fc fragment, C_H2/C_H3 hinge of, **1:**51, **1:**54

Fc receptors
FCαRI, **1:**45
FCγRI, **1:**45
FCεRII, **1:**45
immunoglobulin E interactions with
description of, **1:**45
FCεRI, **1:**47–48
FCεRII/CD23, **1:**48
immunoglobulin G interactions with
description of, **1:**45
FCγR, **1:**46
FCRn, **1:**46
types of, **1:**45
FCγR, **1:**46
FCεRI, **1:**47–48
FCγRI, **1:**45
FCεRII, **1:**45, **1:**48
FCRn
binding sites of, **1:**51–52
immunoglobulin G interaction with, **1:**46, **1:**52
FEAR pathway, **1:**698–699
Fem-2, **1:**638–639
FERM domain, **2:**157
Fgd1, **2:**715
Fibrin
fibrinogen conversion to, **1:**119–120
signaling events involving, **1:**119–120
Fibrinogen
carboxyl-terminal segments of, **1:**120
cell surface proteins involved in, **1:**120
characteristics of, **1:**119
composition of, **1:**119
function of, **1:**119
signaling events involving, **1:**119–120
Fibrinopeptides, **1:**119
Fibroblast growth factor
bone development role of, **3:**503–504
breast neoplasms and, **3:**568
export pathways for, **3:**393–394
FGF-1
bone development role of, **3:**503–504
description of, **3:**393–394
multiprotein complex export of, **3:**394–396
Syt1's role in stress-induced release of, **3:**396
FGF-2, **3:**393–394
FGF-7, **3:**592
FGF-10, **3:**592
homologs, **1:**265
lung development and, **3:**510
lung injury and, **3:**512
polypeptides, **1:**265
prostate gland development and, **3:**592
signaling system, **1:**265
testis expression of, **3:**533
Fibroblast growth factor receptor
activation of, **2:**861
adapter proteins and, **2:**863
antagonists, **2:**887
craniosynostosis and, **2:**862
definition of, **2:**861
description of, **1:**362, **1:**391
in development, **2:**861–862
expression of, **1:**391, **2:**861
FGFR1, **1:**24, **2:**861, **2:**863
FGFR2, **1:**24
FGFR3, **1:**24
FGFR4, **2:**861
heparan sulfate and, **1:**267
immunoglobulin-like modules, **1:**266
intracellular signal transduction, **1:**268–269
isotypes, **1:**268
MAP kinase signaling pathway activation, **1:**268
monomer, **1:**265
phospholipase C-γ binding to, **2:**862
signaling complex
description of, **1:**266
oligomeric, **1:**267–268
signaling pathways mediated by, **2:**862–863
substrate 2, **2:**862
summary of, **2:**863–864
syndromes associated with, **2:**862
Tyr766 and, **1:**268
tyrosine kinases, **1:**265–268
Fibroblast-pneumonocyte factor, **3:**511
Fibronectin, **3:**505
Fibronectin type III, **1:**51, **1:**260, **1:**271
Fimbrim, **3:**411
Finger-helix-groove, **1:**290
Fish oils, **2:**283
Five-lipoxygenase, **2:**275–277
Five-lipoxygenase activating flap, **2:**275–277
FK506
calcineurin inhibition by, **1:**631
description of, **1:**523
FK506 binding protein, **2:**45
FKBP12.6, **2:**15–16
Flagellar proteins, **1:**16, **1:**18
FLAGELLIN-SENSING 2, **1:**581
Flavocytochrome b_{558}, **2:**705, **2:**709
flgE, **1:**16, **1:**18
Flt 3 ligand, **1:**287, **3:**618–619
Fluorescence lifetime imaging microscopy, **2:**308
Fluorescence resonance energy transfer
C/EBP detection, **3:**136
cyclic nucleotide monitoring using, **2:**460
description of, **1:**187–188, **1:**212, **3:**135–136
fluorescent probes for, **2:**306
green fluorescent protein variants, **2:**305–306
mechanisms of, **2:**305
photobleaching-based techniques, **2:**307–308, **3:**136–137
properties of, **3:**135–136
ratio imaging, **2:**306
sensitized emission measurements, **2:**306–307
sensors for, **2:**305
signal detection, **3:**136–138
summary of, **2:**308
techniques for, **2:**306–308
Fluorescent proteins
discovery of, **3:**135
green, **2:**305, **3:**135
4-Fluoromethylphenylphosphate, **1:**678
Focal adhesion kinase, **1:**423, **1:**465–466, **2:**851, **3:**481

Focal adhesions
conformational switches, **2:**318
connectivity-based ordering of, **2:**318–319
cytoskeletal domain of, **2:**317
description of, **1:**71, **2:**317
domains of, **2:**317
dynamic turnover of, **2:**318
molecular switches in, **2:**318–320
molecular-interference switches, **2:**320
plaque constituents, **2:**317–318
protein stability switches, **2:**318
protein–protein interactions with, **2:**320
Rho and, **2:**701
SH2 domains of, **2:**317
tension switches, **2:**320
transcriptional switches, **2:**318
transmembrane receptors, **2:**317
tyrosine phosphorylation switches, **2:**318
Focal-adhesion complexes, **1:**499
Follicle growth and differentiation, **3:**535–536
Follicle-stimulating hormone, **3:**535
Follistatin, **3:**593
Forkhead box, **3:**86
Forkhead transcription factors
description of, **3:**83
DNA-binding domain of, **3:**83
FOXO subfamily. *see* FOXO transcription factors
Forkhead-associated domains, **1:**506–507
Forskolin, **2:**422, **3:**379
Fostriecin, **1:**607–609
14-3-3 proteins
description of, **1:**410, **1:**505–506, **2:**390
FOXO transcription factor binding, **3:**85
FOXO transcription factors
14-3-3 binding to, **3:**85
aging and, **3:**88
apoptosis regulated by, **3:**86–87
cancer development and, **3:**87–88
cell cycle regulation by, **3:**87
DAF-16, **3:**88
description of, **3:**83
family of, **3:**84
FOXO1, **3:**84
FOXO2, **3:**84
FOXO4, **3:**84–85
FOXO3a, **3:**84, **3:**87
growth factor regulation of, **3:**85
identification of, **3:**83–84
metabolism regulation and, **3:**88
nuclear translocation of, in response to growth factor stimulation, **3:**85
phosphatidylinositol 3-kinase-Akt pathway regulation of, **3:**84
phosphorylation sites in, **3:**84–85
regulation of, **3:**84
stress responses and, **3:**88
target genes, **3:**86
transcriptional activator properties of, **3:**86
FP, **2:**269
Fra-1, **3:**101–102
Frabin, **2:**181
Fractalkine, **1:**150
FRAP/mTOR, **3:**345–346
Free energy profile, **1:**28
Free-radical signaling, **3:**194
Frequency protein, **1:**577
Frequentin, **2:**79, **2:**81, **3:**384
Frizzleds
description of, **1:**177
G-protein coupled receptors, **1:**178–179
Wnt signaling, **1:**177–179, **2:**789
ft-arrestin, **2:**370–371
Fucosyl transferase-1, **3:**593
Fumonisin B_1, **2:**257
Functional genomics, **3:**182
FUS3, **1:**493
Fus3, **1:**704, **2:**571
Fyn, **1:**475–476, **1:**714
FYVE domains
conservation of, **2:**179
description of, **2:**177
in *Drosophila,* **2:**178
early endosome antigen 1, **2:**177
identification of, **2:**177–178
membrane trafficking by, **2:**179–182
phosphoinositide 3-kinases, **2:**136–137, **2:**158
proteins
description of, **2:**179, **2:**181
in mammals, **2:**181
phosphatidylinositol 3-phosphate metabolism, **2:**182
properties of, **2:**181
SARA, **2:**182–183
in yeast, **2:**181
signaling by, **2:**182–183
structural basis for, **2:**178–179
summary of, **2:**183
in yeast, **2:**181
FYVE-like domains, **2:**183

G

G domain, **2:**757–758
Gab2, **1:**720
Gab proteins, **2:**149
$GABA_B$ receptors, **1:**187
GADD34, **1:**618, **3:**265, **3:**361
Gads, **3:**546
GAF domains
A, **2:**526–528
atomic structure of, **2:**526–528
B, **2:**526–528
cGMP release induced by, **2:**455
description of, **2:**525
PDE2A, **2:**526–528
phosphodiesterases, **2:**432, **2:**449–450
sequences, **2:**528
GAL4, **1:**673
Galectins, **1:**90
γ-phosphate linked ATP-sepharose, **2:**301–302
Gap-1, **2:**805
GAP domain, **2:**765
GAP junctions, **3:**586–587
Gap-1m, **2:**805
GARPs, **1:**352

Gastrin
  description of, **3:**477–478
  mitogenic pathway of, **3:**481
Gastrin releasing peptide, **3:**478, **3:**511
Gastrointestinal tract hormones
  characteristics of, **3:**478
  in colon, **3:**479
  description of, **3:**477
  gastrin, **3:**477–478
  receptors for, **3:**479–480
  signal transduction pathways for
    calcium mobilization, **3:**480–481
    description of, **3:**479–480
    downstream transcription factors, **3:**482
    mitogen-activated protein kinase, **3:**481–482
    phosphatidylinositol activation, **3:**480–481
    phospholipase C, **3:**480–481
    protein kinase C, **3:**480–481
    tyrosine kinases, **3:**481
  in small intestine, **3:**478–479
  in stomach, **3:**477–478
Gastrulation, **3:**463
GATA4, **1:**634
GATA proteins, **3:**464
GCAP 1, **2:**80–81
GCAP 2, **2:**80–81
GCAP 3, **2:**80–81
GCN2, **3:**299–300
Gcn4, **3:**130
GCN system, **3:**299–300
Gdf11, **2:**887
GDI, **2:**692
GDP-GTP exchange factor, **2:**733
Gene cluster, **1:**16
Genomics, **3:**182
Genotoxic agents
  activating protein 1 and, **3:**102
  stress signal caused by, **3:**179
Geranylgeranyl diphosphate, **2:**287
G-γ-like domains, **1:**132
Gic1, **2:**735
Gic2, **2:**735
Gi/Go coupled receptors, **2:**668
gip2, **2:**592
Gleevec, **1:**446–447, **1:**454
Gli2, **2:**795
Gli3, **2:**795
Glial-derived neurotrophic factor, **2:**79
Gln, **2:**760
Gln-61, **2:**558
Glomerular filtration rate, **3:**573, **3:**575
glp-1, **2:**811
Glu276, **1:**107
Glu277, **1:**107
Glu278, **1:**107
Gluamic-acid-rich region, **1:**352
Glucagon-like peptide-1, **3:**379
Glucagon-like peptide-2, **3:**478–479
Glucocorticoid receptor, **3:**35
Glucocorticoids, **3:**61
Glucose homeostasis, **3:**305, **3:**316
Glucose transport, insulin signaling for stimulation of, **1:**295–296
Glucose-independent insulinotropic polypeptide, **3:**379
Glucose-regulated proteins, **3:**279–280, **3:**312
GLUT4
  cell surface transport of, **1:**296
  description of, **1:**295, **1:**536
  Gαq/11 subunit tyrosine phosphorylation effects, **2:**611
Glutamate, **2:**329
Glutamate receptors
  agonist binding domains of, **1:**219–221
  AMPA type, **2:**385, **2:**397
  dendritic localization of, **2:**397
  domain organization of, **1:**219–220
  eukaryotic, **1:**219
Glutamic acid-rich protein, **1:**235
Glutamine, **3:**301
Glutathione S-transferase-Rac2, **2:**707
Glutathione S-transferase-tagged proteins, for coprecipitating interacting proteins, **2:**296–297
Glycogen synthase, **1:**614, **3:**193
Glycogen synthase kinase 3
  cancer and, **1:**549–550
  β-catenin and, **1:**549, **2:**893
  CK2 phosphorylation of, **1:**547
  Cubitus interruptus synthesis by, **3:**169
  definition of, **1:**547
  drug targeting of, **1:**549
  embryonic development role of, **1:**549
  growth factor regulation of, **1:**548–549
  inhibition of, **1:**548
  insulin regulation of, **1:**548
  isoforms of, **1:**547
  proteins phosphorylated by, **1:**547
  substrates of, **1:**547–548
Glycogen synthase kinase 3β, **2:**411, **3:**130, **3:**161
Glycolipid enriched microdomains, **1:**476
Glycolipid(s), **1:**90
Glycoprotein hormone receptors
  description of, **1:**161
  ectodomain of
    serpentine portion and, intramolecular signal transduction between, **1:**163–165
    structure and function, **1:**162–163
  molecular pathophysiology of, **1:**161
  serpentine portion
    activation of, **1:**163
    ectodomain and, intramolecular signal transduction between, **1:**163–165
  structure of, **1:**161
  structure–function relationships, **1:**162–165
Glycoprotein(s)
  description of, **1:**89–90
  gp120, **1:**99–100, **1:**103
  gp160, **1:**99
  human immunodeficiency virus-1, **1:**99–100
Glycosaminoglycans, **1:**90
Glycosphingolipid-enriched microdomains, **3:**556
Glycosylphosphatidylinositols, **1:**333, **1:**421
Glycosyltransferases, **1:**87–88
Goldman–Hodgkin–Katz voltage equation, **1:**216
GoLoco motifs, **1:**132, **2:**567, **2:**573
Gonadotropin-releasing hormone, **3:**385

Goodpasture antigen binding protein, **2:**166
gp120 glycoprotein, **1:**99–100, **1:**103, **1:**192–193
gp160 glycoprotein, **1:**99
gp130–cytokine complex
  description of, **1:**259–260
  granulocyte colony-stimulating factor–granulocyte colony-stimulating factor receptor extracellular signaling complex, **1:**261–262
  mutagenesis of, **1:**260
  α-receptor interactions with, **1:**260–261
  receptor/ligand interactions, **1:**259
  site 2 interface, **1:**260–261
  site 3 interface, **1:**261
  structure of, **1:**260
  viral, **1:**260
GPR motifs, **1:**132
G-protein coupled receptor kinases
  description of, **2:**205
  G-protein coupled receptor phosphorylation by, **2:**596
G-protein coupled receptor(s)
  adenylyl cyclase regulation by, **2:**422
  agonist regulation of, **1:**182, **2:**631
  agonist-exposed, **1:**188
  aminergic, **1:**156
  amino acids, **2:**565
  angiogenesis by, **2:**590–591
  Arg, **1:**156
  Arg131, **1:**156
  binding pocket of, **1:**155–158
  biological outcomes and, **2:**592–594
  calcium-sensing, **2:**590
  characteristics of, **1:**117, **1:**365, **2:**589
  chemokines. *see* Chemokine(s)
  chimeras
    constitutive activity, **2:**620
    construction of, **2:**619
    defining of signal for, **2:**619–620
    description of, **2:**619
    guanine nucleotide exchange assays, **2:**620
    inverse agonism, **2:**620–621
    mutations, **2:**620
    sensitivity of, **2:**620–621
  constitutive dimerization of, **1:**188–189
  control of, **3:**443–444
  coupling of, **2:**586
  definition of, **1:**117, **2:**565
  description of, **1:**2, **1:**127, **1:**155, **1:**187, **1:**365
  desensitization
    description of, **1:**181
    heterologous, **1:**183–184
    homologous, **1:**182
    mechanisms of, **1:**182–184
    rapid, **1:**184
  dimeric complexes formed from, **1:**187–189
  downregulation of
    description of, **1:**182
    proteolytic, **1:**184
  drug targeting of, **1:**117
  effectors of, **2:**595–596
  endocytosis of
    agonist-induced, **1:**184
    functional consequences of, **1:**184–185
    rapid desensitization role, **1:**184
    signal transduction specificity controlled by, **1:**184–185
  endothelial differentiation gene of, **2:**247
  epidermal growth factor receptors and, **1:**407
  family members, **2:**589
  frizzleds as, **1:**178–179
  functional coupling of, from heterotrimeric G proteins, **1:**182–183
  Gα interactions with, **1:**132–133
  gastrointestinal hormone-stimulated signal transduction and, **3:**479
  gene expression pathways, **2:**593
  growth factor binding to, **2:**590
  heterodimerization of, **1:**189
  leukotrienes, **2:**276
  ligands, **2:**590
  mechanism of action, **1:**365
  mitogen-activated protein kinases and, **2:**592, **3:**444, **3:**480
  monomeric nature of, **1:**365
  mutations, **2:**620
  NH_2-terminal domains, **1:**118
  oligomers, **1:**188
  phosphoinositide 3-kinase class I and, **2:**135
  phospholipase C-β and, **2:**5
  phospholipase D and, **2:**240
  physiological functions of, **2:**597
  prostaglandin effects on
    description of, **3:**577
    $PGF_2\alpha$, **2:**269
  prostanoids, **2:**266
  proteolyic downregulation of, **1:**184
  rapid desensitization of, **1:**184
  regulation of, **1:**181–182
  resensitization of, **1:**184
  rhodopsin
    activation of, **1:**142
    amino-terminal tail of, **1:**140
    characteristics of, **1:**139–140
    crystal structure of, **1:**139
    cytoplasmic domain of, **1:**141
    description of, **1:**178
    extracellular loops, **1:**158–159
    interhelical loops of, **1:**140
    ligand-binding pocket of, **1:**139
    membrane-embedded domain of, **1:**140
    molecular structure of, **1:**140–141
    R* transition, **1:**141–142
    solvent-accessible surface area analysis, **1:**156
    transmembrane helices, **1:**142
    transmembrane segments of, **1:**155
  signal transducers and activators of transcription activated by, **3:**77
  signaling
    description of, **2:**589
    endocytosis and, **3:**444
    extracellular signal-regulating protein kinases in, **2:**593
    independent, **2:**594–595
    paradigms of, **3:**443
    scaffolding proteins involved in, **2:**595–596, **3:**444
  sphingosine 1-phosphate and, **2:**21
  subcellular localization of, **1:**181–182
  taste transduction by, **2:**658–660

G-protein coupled receptor(s) *(continued)*
tumorigenesis role of, **2:**590–591
types of, **1:**174–175
upregulation of, **1:**182
viral, **2:**590
G-protein-gated inwardly rectifying potassium channels
activation of
coupling to specific receptors, **2:**668
description of, **2:**667
kinetics of, **2:**668
basal activity of, **2:**668
description of, **2:**639–640, **2:**664, **2:**667
Gβγ interacting domain, **2:**667–668
transmembrane domains of, **2:**667
G-proteins, heterotrimeric
activation of
description of, **1:**337
Gβγ involvement in, **1:**338
g-protein coupled receptors role in, **2:**565–566, **2:**631, **3:**443
receptor-induced, **2:**565
requirements for, **2:**560
calcium channel mediation by
description of, **2:**667
voltage-independent, **2:**669
central nervous system role of, **2:**582–583
chemotaxis involvement by, **2:**645–647
classification of, **1:**335
covalent modification regulation of, **2:**585–587
description of, **1:**127, **1:**335, **2:**557–558
in developmental processes, **2:**583
in *Drosophila,* **2:**572–573
exocytosis and, **2:**664, **3:**379
family of, **1:**335
feedback loops, **2:**572
frizzleds, **1:**179
Gα subunit
α3–β5 loop, **1:**129
α4–β6 loop, **1:**129
adenylyl cyclase co-crystallization with, **1:**337
ADP-ribosylation of, **2:**585
architecture of, **2:**575–576
cellular functions modulated by, **2:**563
C-terminal residues of, **1:**337
description of, **1:**127–129, **1:**333, **1:**335–336
domains of, **1:**336–337
effector complexes, **1:**129, **2:**563
effector molecule interactions with, **1:**337–338, **2:**586, **2:**589
enzymes modulated by, **2:**562
Gα12, **2:**610
Gβγ subunit and, **2:**576
G-protein coupled receptor interactions with, **1:**132–133
GTP and, **2:**575
GTPase domain, **2:**605
GTPase-activating proteins
description of, **2:**566, **2:**631–633
steady-state measurements, **2:**633
guanosine diphosphate state, **1:**127–128, **2:**576
guanosine triphosphate hydrolysis, **1:**127, **1:**130–131
α-gustducin, **2:**657–658
helical domain of, **2:**723
hemostasis and, **2:**583
lipid modifications, **2:**560–561, **2:**585
mutations, **1:**130, **2:**566, **2:**591
myocardial growth and, **2:**583
N-terminal acylation of, **2:**585–586
palmitoylation in
activation-regulated, **2:**651–652
depalmitoylation and, **2:**653
description of, **2:**586, **2:**651
plasma membrane localization by, **2:**653
reversible, **2:**652–654
sites of, **2:**651
phosphorylation of, **2:**609
posttranslational modifications of, **2:**585
RGS-box and, **2:**631–633
sensory systems and, **2:**583
serine phosphorylation of, **2:**609–610
structure of, **1:**128
switching mechanism of, **2:**575–576
taste transduction by, **2:**657–658
transition states of, **2:**576
tumorigenesis by, **2:**591
tyrosine phosphorylation of, **2:**611
variants of, **1:**127, **2:**563–564, **2:**591
Gβ subunit
characteristics of, **1:**127, **1:**131–132, **1:**333, **1:**337
functions of, **2:**627
genes that encode, **2:**623–625
modification of, **2:**616
N-terminal helix of, **2:**576
RNA suppression, **2:**627
Gβγ subunit
architecture of, **2:**576–577
assembly of, **2:**625–626
calcium channel mediation by, **2:**667
cellular functions modulated by, **2:**563
composition of, **2:**639
conformation of, **1:**339
C-terminus of, **2:**577
description of, **1:**337, **2:**623, **2:**639
dimer assembly, **2:**625–626
diversity of, **2:**623–625
effectors of
adenylyl cyclases, **2:**640
description of, **1:**338–339, **2:**564, **2:**587, **2:**589, **2:**626–627
G-protein-gated inwardly rectifying potassium channels, **2:**639–640
GRK, **2:**641
interaction mechanisms, **2:**639–641
mitogen-activated protein kinase pathway, **2:**640
N-type calcium channels, **2:**640
phosducin, **2:**641
phospholipase Cβ, **2:**640
phospholipase Cε, **2:**641
P/Q-type calcium channels, **2:**640
functions of, **2:**627
Gα subunit and, **2:**576
inhibition of, **2:**669
lipid modifications, **2:**560–561
N-terminal domain of, **2:**667
phosducin effects, **2:**577
proteins that interact with, **1:**338

receptor, **2:**626
signaling specificity, **2:**626–627
structure of, **2:**560, **2:**639
taste transduction by, **2:**658
voltage-gated calcium channel gating affected by, **2:**664
Gγ subunits
βγ dimer assembly, **2:**625–627
carboxymethylation of, **2:**587
characteristics of, **1:**127, **1:**131–132, **1:**333, **1:**337
C-terminal modification of, **2:**586–587
functions of, **2:**627
gene targeting, **2:**627
genes that encode, **2:**624
prenylation of, **2:**586–587
RNA suppression, **2:**627
structure of, **2:**635
taste transduction by, **2:**658
GDP-form of, **2:**573
$G\alpha_o$
characteristics of, **2:**605–606
description of, **2:**582, **2:**605
Rap1GAP regulation by, **2:**606
signaling effectors, **2:**605–607
GoLoco motifs, **1:**132
GPR motifs, **1:**132
G_q, **2:**582
GSα, **2:**578
guanine nucleotide release from, **2:**758
$G\alpha_z$
biochemical properties of, **2:**601
covalent modifications of, **2:**601–602
description of, **2:**582
knockout mice studies, **2:**603
plasma membrane localization of, **2:**602
properties of, **2:**601–602
Rap1GAP interactions with, **2:**602–603
receptors that couple, **2:**602
signaling by
effectors of, **2:**602–603
regulators of, **2:**602
summary of, **2:**603–604
tissue distribution of, **2:**601
immune system role of, **2:**582–583
in vivo functions of, **2:**581–584
intracellular targets of, **1:**127
isoforms, **2:**561
kinase interactions with, **1:**499–502
knockout mice studies of, **2:**581–584
lipid modifications, **2:**560–561, **2:**587
molecular organization of, **1:**335–337, **2:**561–565
mono-ADP-ribosylation of
bacterial toxin-induced, **2:**614–615
cellular, **2:**616
cycle of, **2:**614
description of, **2:**613–614
endogenous, **2:**615–616
substrates, **2:**616
name origin of, **2:**562
neurotransmitter release modulated by, **2:**663
phosphorylation of
cGMP-dependent protein kinase in, **2:**611
description of, **2:**609, **2:**665
epidermal growth factor receptor, **2:**611
insulin receptor in, **2:**611
p21-activated protein kinase, **2:**610–611
protein kinase C, **2:**609–610
serine, **2:**609–611
Src tyrosine kinases in, **2:**611
tyrosine, **2:**611
physiological responses mediated by, **2:**575
posttranslational modifications of, **2:**609
presynaptic inhibition by, **2:**664
receptor interactions with, **1:**337
receptor signal propagation by, **1:**370
regulation of, **2:**585–587
semaphorin signaling, **2:**879
signal transduction by, **2:**557–558
signaling by
activators of, **2:**567
A-kinase anchoring proteins signaling complexes for, **2:**384–385
cancer and, **2:**591–592
description of, **1:**130, **1:**370
regulators of, **1:**335, **2:**566–567, **2:**601–602
in *Saccharomyces cerevisiae,* **2:**571–572
in yeast, **2:**571–572
small molecular weight, **3:**561–562
stimulatory, **2:**422
structure of, **2:**577
subunits. *see also specific subunit*
adenylyl cyclase regulation by, **2:**563, **2:**586, **2:**640
description of, **2:**559–560, **2:**605
synaptic fusion regulated by, **2:**663–665
taste transduction by
description of, **2:**657
Gβγ subunits, **2:**658
α-gustducin, **2:**657–658
α-transducin, **2:**658
transforming, **2:**591–592
vesicle fusion inhibition by, **2:**664
Granulocyte cell signaling factor
crystal structure of, **1:**256
description of, **1:**251
Granulocyte colony-stimulating factor, **1:**259–262, **3:**620
Granulocyte colony-stimulating factor receptor, **1:**24, **1:**259–260, **3:**620
Granulocyte-macrophage colony-stimulating factor
cytokine signaling through, **3:**621
description of, **1:**343
ovary expression of, **3:**537
Granulosa cells, **3:**535
Gravin, **2:**390
Grb2, **1:**381–382, **2:**193, **2:**863
GRB2/SOS complex, **3:**305, **3:**481
GRE, **3:**64
Green fluorescent protein
discovery of, **3:**135
fusions, **2:**305
Growth differentiation factor 9, **3:**537
Growth factors. *see also specific growth factor*
angiogenic properties of, **3:**599
antegrade transport of, **3:**488
bone development and, **3:**501–503
breast signaling pathways, **3:**566–568

Growth factors *(continued)*
cyclic guanosine-3,5-monophosphate inhibition of, **2:**429
FOXO transcription factor regulation by, **3:**85
glycogen synthase kinase 3 regulated by, **1:**548–549
G-protein coupled receptor binding, **2:**590
growth factor receptor phosphorylation in response to binding of, **3:**260
lung development, **3:**509–511
mRNA localization induced by, **3:**293–294
transforming growth factor β. *see* Transforming growth factor β
Growth hormone
cross-reactivity of, **1:**243
description of, **1:**241
endotoxin-induced postreceptor resistance, **3:**524
functions of, **1:**242
high-affinity variant of, **1:**245
interleukin-4 and, comparisons between, **1:**247–248
specificity of, **1:**243
suppressors of cytokine signaling protein downregulation of signaling, **3:**524–525
Growth hormone receptors
binding energetics, **1:**244–245
binding sites for, **1:**243–246
characteristics of, **1:**363
description of, **1:**24, **1:**241
extracellular domains, **1:**242–244
homodimerization
altered mode for, **1:**245
structural basis for, **1:**242–243
prolactin hormone receptor interactions, **1:**244
Grp1, **2:**163
GRP58, **3:**279
GRP78, **1:**618, **3:**279–280
GRP94, **3:**279
GRP170, **3:**279
GS4071, **1:**110
GSK-3, **1:**616
gsp, **2:**592
GTPase activating proteins
Arf, **2:**205–206, **2:**727
description of, **1:**130, **2:**203, **2:**631, **2:**759
GTPase reaction affected by, **2:**759
RGS-box as, **2:**631–634
single-turnover assays of, **2:**632–633
steady-state measurements of, **2:**633–634
GTPase effector domain, **2:**764
GTPase(s)
cdc42p Rho-like module, **2:**734–735
description of, **2:**558, **2:**733
G domain, **2:**757–758
high molecular weight, **2:**772–774
Rab. *see* Rab proteins
Ran. *see* Ran
Ras. *see* Ras
R-Ras proteins, **2:**684
structure of, **2:**559, **2:**757
yeast studies, **2:**733–735
GTP-binding proteins
description of, **2:**757
effectors for, **2:**758–759
G domain, **2:**757–758
GBP1, **2:**764
Guanine nucleotide, **2:**758
Guanine nucleotide dissociation inhibitor, **2:**565
Guanine nucleotide exchange assays, **2:**620
Guanine nucleotide exchange factors
Arf
description of, **2:**728
phosphatidylinositol 3,4,5-triphosphate regulation of, **2:**205
cAMP-binding. *see* Epacs
cdc42, **2:**204, **2:**715
description of, **1:**423, **2:**203, **2:**758
Epacs. *see* Epacs
hormone receptor complexes as, **2:**558
Rab protein activation and, **2:**691
Rac, **2:**203
Ran, **2:**695–696
Ras protein interaction with, **2:**677, **2:**758
Rho GTPases
allosteric regulation of, **2:**753
DB family. *see* DB family
description of, **2:**751
external regulation of, **2:**754
modulation of, **2:**754
nucleotide exchange mechanisms, **2:**753
pleckstrin homology domains, **2:**753
structure of, **2:**758
Guanine-nucleotide binding pockets, **2:**723
Guanine-nucleotide dissociation inhibitor, **3:**416
Guanosine triphosphate
exocytotic secretion and, **3:**379
Gα hydrolysis of, **1:**127, **1:**130–131, **1:**335
receptor-mediated release of, **1:**337
Guanosine triphosphate exchange factor, **1:**382
Guanosine triphosphate-activating proteins, **1:**75
Guanylate cyclase, **2:**879
Guanylate kinase associated protein, **2:**330
Guanylate-binding protein 1, **2:**772
Guanylyl cyclase
bone expression of, **2:**513
cell growth regulation and, **2:**429–430
description of, **2:**419, **2:**427
domain structure of, **2:**420, **2:**427–428
historic perspectives of, **2:**427
ligands, **2:**428–429
nitric oxide effects, **2:**428, **2:**443
Paramecium, **2:**536–537
particulate, **2:**427
soluble, **2:**427–428
structure of, **2:**427–428
subunits, **2:**427–428
in *Trypanosoma brucei,* **2:**540
Guanylyl cyclase activating proteins
description of, **2:**428
GCAP 1, **2:**80–81
GCAP 2, **2:**80–81
GCAP 3, **2:**80–81
properties of, **2:**80–81
Guanylyl-cyclase-containing receptors, **1:**362, **1:**364
Gustducin, **2:**583, **2:**657–658
Gut hormones
characteristics of, **3:**478
in colon, **3:**479
description of, **3:**477

gastrin, **3:**477–478
receptors for, **3:**479–480
signal transduction pathways for
calcium mobilization, **3:**480–481
description of, **3:**479–480
downstream transcription factors, **3:**482
mitogen-activated protein kinase, **3:**481–482
phosphatidylinositol activation, **3:**480–481
phospholipase C, **3:**480–481
protein kinase C, **3:**480–481
tyrosine kinases, **3:**481
in small intestine, **3:**478–479
in stomach, **3:**477–478
GW4064, **3:**54

H

H8, **1:**141
HAC1, **3:**264, **3:**360
HAESA, **1:**581
hairy2, **3:**155
α-Haloacetophenone, **1:**678
α-Halobenzylphosphonate, **1:**678
Haptens, **1:**34
H2AX, **3:**227
Hck, **1:**389
hCl9, **3:**164
Heart
aortic arch development, **3:**467–468
cardiac looping, **3:**464–465
description of, **3:**463
growth of, **3:**467
left-right asymmetry, **3:**464–466
outflow tract, **3:**467–468
valve formation, **3:**467
Heart tube
formation of, **3:**463–464
neural crest, **3:**467
patterning of, **3:**465–467
transcription factors involved in, **3:**465
Heat shock transcription factor 2, **2:**408
Heat-shock proteins
characteristics of, **3:**272–273
HuR effects, **3:**287
mRNA turnover and, **3:**286–287
neurodegenerative diseases and, **3:**273
Heat-shock response
causes of, **3:**269
definition of, **3:**269
molecular chaperones, **3:**271–273
neurodegenerative diseases and, **3:**273
sequelae of, **3:**269
transcriptional regulation of, **3:**269–271
Heat-shock transcription factors
description of, **3:**269
genes, **3:**269
HSF2, **3:**271
stress-induced activation of, **3:**269–270
Heavy chains, **1:**39
Hedgehog proteins
Cubitus interruptus and, **3:**167–169
description of, **1:**333
developmental role of, **2:**795–796
diseases and, **2:**795–796
distribution of, **2:**793
generation of, **2:**793
N-terminal fragment of, **2:**793
patterning functions of, **2:**796
Hedgehog signaling
description of, **2:**793
embryonic development and, **2:**793–796
left-right determination of vertebrates by
description of, **2:**799
genes involved in, **2:**799
mice, **2:**800–802
transmitting of, **2:**793–795
HEK 293 cells, **2:**35, **2:**248, **2:**399, **2:**659
Helix C, **1:**541
Hemangioblasts, **2:**856
Hematopoiesis
cytokine receptor signaling, **3:**617–623
cytokines involved in, **3:**615–617
description of, **3:**615
Hematopoietic cells, **3:**615–617
Hematopoietins
description of, **3:**77
Janus kinase signaling, **3:**78
Heme oxygenase, **3:**578
Hemostasis, **2:**583
Hensen's node, **2:**887, **3:**464–465
Heparan sulfate, **1:**267
Heparin-binding domain, of vascular endothelial growth factor, **1:**285–286
Hepatocellular carcinoma, **3:**526
Hepatocyte growth factor
lung development and, **3:**511–512
ovary development and, **3:**536–537
receptors, **1:**392
testis production of, **3:**534
Hepatocyte nuclear receptor 4
description of, **3:**53
ligands for, **3:**55
Hepatocytes, **2:**53
Heptahelical receptors. *see also* G-protein coupled receptor(s)
cell growth stimulated by, **2:**595
characteristics of, **2:**589–590
genes that encode, **2:**590
HER-2, **3:**599
HER-3, **3:**599
HER-4, **3:**599
HER2, **1:**446
Heregulins, **3:**566
Herpesvirus
β-, **1:**175
γ-, **1:**175
HES1, **3:**155
15-HETE, **2:**283
20-HETE, **3:**578, **3:**586
Heterochromatin protein 1, **3:**30
Heterodimerization
G-protein coupled receptors, **1:**189
protein tyrosine kinase receptors, **1:**393
Heterologous desensitization, **1:**183–184
HH signaling. *see* Hedgehog signaling

High molecular weight GTPases, **2:**772–774
High-copy suppressors of synthetic lethality, **3:**209
High-molecular-weight protein tyrosine phosphatases, **1:**733
Hill equation, **1:**384
HIPK2, **3:**242
Hippocalcin, **2:**80–81
Histidine kinases, **1:**563–565
Histidine-containing phosphotransfer, **1:**563
Histone
  amino-terminal tails of, **3:**92
  arginine methylation of, **3:**147
  description of, **3:**91
  epigenetic modification of, **3:**127
  H3
    description of, **3:**92–93
    phosphorylation of, **3:**95
  H2AX, **3:**227
  phosphorylation
    acetylation and, **3:**93–94
    apoptosis and, **3:**94–95
    cellular processes associated with, **3:**95–96
    chromatin structure alterations and, **3:**91
    DNA repair and, **3:**94
    gene activation and, **3:**91–94
    mitosis and, **3:**95
Histone acetyltransferase complexes
  description of, **1:**560
  Su(H) effects mediated by, **3:**150
Histone code, **3:**91
Histone deacetylase
  description of, **2:**711, **3:**116
  HDAC4, **3:**127
  HDAC5, **3:**127
  repressed transcription and, **3:**127
HL60 cells, **3:**340
HMG-CoA reductase
  cholesterol from, **2:**287, **3:**353
  description of, **2:**287
HNF4. *see* Hepatocyte nuclear receptor 4
hnRNP-k, **1:**584
Hog1, **1:**703, **2:**357
Hog1 mitogen-activated protein kinase, **1:**703, **2:**357, **2:**360
Homer proteins
  definition of, **2:**43
  N-terminal of, **2:**43
Homo sapiens, **1:**375–376
Homodimerization
  erythropoietin receptors, **1:**245
  growth hormone receptors
    altered mode for, **1:**245
    structural basis for, **1:**242–243
  platelet-derived growth factor receptors, **1:**393
  protein tyrosine kinase receptors, **1:**393
Homologous desensitization, **1:**182
Homologous recombination, for DNA double-strand breaks, **3:**219
Homology approach, to protein functions, **1:**16, **1:**18
Horizontal receptor signaling, **1:**239
Hormone. *see also specific hormone*
  discoveries regarding, **1:**2
  history of term, **1:**1
Hormone response elements, **3:**25, **3:**35
Hot spots, **1:**31, **1:**40
HOX11, **2:**408
Hox genes, **2:**886–887
hPEM, **2:**715
H-Ras
  amino acid sequence of, **2:**682
  description of, **2:**675–676
  farnesyl transferase inhibitors effect on, **2:**739
  prenylation of, **2:**739
Hrs, **2:**180–182
HRX, **2:**408
HSP40, **3:**272
HSP60, **3:**272
HSP70, **3:**272, **3:**368
HSP90, **3:**272
HSP 100, **3:**272
HSPC300, **2:**326
HSPDE71A1, **2:**431
Human factor C1, **1:**617
Human genome
  phosphatase and tensin homology deleted on chromosome 10 genes, **2:**144
  protein tyrosine phosphatase genes, **1:**665–666
Human immunodeficiency virus
  characteristics of, **1:**191
  chemokine receptor coreceptor use by, for host cell entry
    binding of, **1:**192
    CCR5, **1:**191–194
    CXCR4, **1:**191–194
    description of, **1:**151, **1:**191
    domains, **1:**193
    Env domains involved in, **1:**192–193
    in vivo, **1:**192
    mechanisms of, **1:**191–192
    presentation and processing, **1:**193–194
    signaling, **1:**194
  Env proteins, **1:**191–193
  signaling in, **1:**194
  tropism, **1:**191
  types of, **1:**191
  Vpr protein, **2:**410
Human immunodeficiency virus-1
  atomic details of, **1:**100–102
  carbohydrate masking, **1:**102
  CD4 receptor binding, **1:**100
  genome of, **1:**102
  glycoproteins, **1:**99–100
  gp120 glycoprotein, **1:**99
  gp160 glycoprotein, **1:**99
  humoral immune evasion mechanisms, **1:**102–103
  molecular interactions of, **1:**99–100
  receptor recognition, **1:**103
  structure of, **1:**99–100
  V3 loop, **1:**103
  variable loops, **1:**102–103
Human rhinoviruses
  classification of, **1:**95
  description of, **1:**95
  intercellular adhesion molecule-1 binding to, **1:**95–97
  low-density lipoprotein receptor binding to, **1:**95
  receptor sites of, **1:**95–96

very-low-density lipoprotein receptor binding to, **1:**95–97
viral coat proteins, **1:**95, **1:**97
Humoral immunity, **1:**319
HuR, **3:**284
Hydrogen peroxide
antagonism of, **2:**114
chemical properties of, **2:**113–114
Inositol 1,4,5-trisphosphate 3-kinase activation by, **2:**114
OxyR activation by, **3:**193–194
production of, **2:**113
protein tyrosine phosphatase inactivation by, **2:**114–115
PTEN inactivation by, **2:**114–115
ryanodine receptor activation by, **2:**114
sources of, **2:**113
Hydrophobic effect, **1:**11, **1:**27
Hydroxyl radicals, **2:**113
(4-Hydroxyphenyl) retinamide, **2:**724
Hyperplastic alveolar nodules, **3:**567
Hyperpolarization-activated cation channels, **1:**235–236
Hyperpolarization-activated cyclic nucleotide-gated channels
activation of, **2:**517
anatomic sites of, **2:**517–518
cAMP response, **2:**518
description of, **2:**515
family of, **2:**517
features of, **2:**515–516
Hypoxia
endoplasmic reticulum stress and, **3:**280
GRP78 antisense inhibition during, **3:**280
mitochondria effects, **3:**370
mRNA turnover and, **3:**287
p53 activation induced by, **3:**243
Hypoxia-inducible factor 1
description of, **1:**435, **3:**277
diseases associated with, **3:**277–278
HIF-1α, ubiquitination stimulated by, **3:**131
phosphorylation of, **3:**278
regulation of, **3:**278
signaling by, **3:**277–279
stability of, **3:**277
structure of, **3:**277
vascular endothelial growth factor targeting, **3:**278–279

I

I-1, **1:**628
I-2, **1:**628–629
IA-2, **1:**647, **1:**745
I_{ARC}, **2:**36–37
ICK, **2:**368–369
I_{CRAC}, **2:**36–39
I_h channels, **1:**236
IκB
description of, **3:**108, **3:**491–492
IκBα, **3:**130
phosphorylation of, **3:**109
IκB kinases
activation of, **3:**132
description of, **3:**109
IKK1, **3:**109–110
IKK2, **3:**109–110
NFκB activity regulated by, **3:**112
sequence analysis of, **3:**109–110
ubiquination activation of, **3:**132
Ileal bile acid binding protein, **3:**50
Immediate early genes, **3:**92
Immune system
G protein's role in, **2:**582–583
immunoglobulins, **1:**51
semaphorins signaling in, **2:**879
Immunoglobulin E–Fc receptor interactions
description of, **1:**45
FCεRI, **1:**47–48
FCεRII/CD23, **1:**48
Immunoglobulin fold, **1:**39
Immunoglobulin G
antigen binding sites, **1:**51–52
description of, **1:**33–34
Fc receptor interactions with
description of, **1:**45
FCγR, **1:**46
FCRn, **1:**46
Immunoglobulin G-superfold
characteristics of, **1:**57
functional context, **1:**60
heterotypic interactions, **1:**59
homotypic interactions, **1:**59–60
interaction modes, **1:**58–59
molecular architecture of, **1:**60
protein–protein interactions, **1:**59
β-strands, **1:**57
structure of, **1:**57–58
Immunoglobulin(s)
immune system functions of, **1:**51
protein A effects, **1:**53
protein G effects, **1:**53
superfamily
cell adhesion molecules, **1:**58
description of, **1:**57
proteins, **1:**58
recognition, **1:**58–60
Immunological synapse
adaptive responses, **1:**81
C-SMAC, **2:**340, **2:**343
cytoskeleton and, **1:**79–80
definition of, **1:**79, **2:**340
formation of, **1:**80, **2:**343
function of, **2:**342–343
innate responses, **1:**81
T-cell activation and, **1:**79–80, **2:**340–341
T-cell receptor downregulation and, **2:**343–344
Immunoreceptor tyrosine-based activation motif, **1:**327–329, **3:**546, **3:**556
Immunoreceptor tyrosine-based inhibitory motif, **2:**138, **2:**150
Immunoreceptors
affinities, **1:**84
description of, **1:**83
Importin α
description of, **3:**420
isoforms, **3:**422
Importin β
description of, **3:**421–422
export receptors, **3:**423–424

Importin β *(continued)*
import receptors, **3:**422
transport adaptors, **3:**422–423
Inactivation/noafterpotential D protein
description of, **1:**349
signaling complexes
anchoring of, **1:**351–352
assembly of, **1:**351–352
photoreceptor, **1:**349–351
phototransduction, **1:**351
targeting of, **1:**351–352
vertebrates, **1:**352
INAD. *see* Inactivation/noafterpotential D protein
Indian Hedgehog, **2:**802
Inflammation
characteristics of, **3:**608
lipoxins for, **2:**282, **2:**284
neurogenic, **3:**608–609
skin, **3:**611–613
Influenza A virus, **2:**771–772
Influenza virus neuraminidase
active binding site, **1:**107, **1:**109
inhibitors of
Abbott compounds, **1:**111
BCX-1812, **1:**110–111
development of, **1:**105
observations regarding, **1:**111
Relenza, **1:**109
Tamiflu, **1:**109–110
ligands, **1:**106
role of, **1:**105
sialic-acid-binding active site, **1:**107
structure of, **1:**107
Inhibin, **3:**536, **3:**538
Inhibitors of apoptosis
antagonists, **2:**354
baculovirus domains, **2:**354
caspase regulation by, **2:**353
description of, **2:**353
proteins, **3:**432
X-linked, **2:**353
Inhibitory postsynaptic current, **2:**70
Ink, **3:**404
Inositol (1,4,5) P_3-receptor-associated cGKI substrate, **2:**481
Inositol phospholipids, **2:**245
Inositol polyphosphate
description of, **2:**229
diphosphoryl, **2:**229, **2:**234
DNA metabolism role of, **2:**231
Ins(1,3,4,5,6)P_5, **2:**233–234
IP_4, **2:**230
IP_5, **2:**230
lipid, **2:**230
nuclear processes regulated by, **2:**230
signaling by
description of, **2:**229–230
nuclear function and, **2:**230–231
synthesis pathways for, **2:**230
Inositol polyphosphate kinases, **2:**231
Inositol polyphosphate 5-phosphatase
description of, **2:**156
signaling role of, **2:**156
Inositol triphosphate, **1:**3
Inositol 1,4,5-trisphosphate 3-kinase
calcium release by, **2:**15, **2:**31
characteristics of, **2:**12–13
description of, **2:**11
enzymes that metabolize, **2:**12
hydrogen peroxide activation of, **2:**114
isoforms, **2:**12
Inositol 1,4,5-trisphosphate 5-phosphatase
description of, **2:**11
isoforms, **2:**12
type 1, **2:**11
Inositol 1,4,5-trisphosphate receptors
adenosine triphosphate modulation of, **2:**42
binding sites of, **2:**41
cytosolic calcium regulation of, **2:**42
description of, **2:**41, **2:**52
in endoplasmic reticulum, **2:**42
eukaryotic expression of, **2:**41
luminal calcium regulation of, **2:**42
modulators of, **2:**42
regulation of, **2:**42
size of, **2:**41
structure of, **2:**41–42
subunits, **2:**41
Ins(1,3,4,5,6)P_5, **2:**233–234
Ins(1,4,5,6)P_4, **2:**234
Ins(1,3,4,5,6)P_5 1-phosphatase, **2:**233
Ins(1,3,4,5,6)P_5 3-phosphatase, **2:**234
Insulin
β cells that release, **3:**379
amino acid uptake regulated by, **3:**307
deficiency of, **1:**293
description of, **1:**293
downstream targets of, **3:**329
eIF4F complex control, **3:**306
function of, **3:**305
glucose homeostasis by, **3:**305
glucose transporter regulated by, **1:**295
glycogen synthase kinase 3 regulated by, **1:**548
high-affinity binding of, **1:**295
nutrient uptake stimulated by, **3:**307
phosphoinositide 3-kinase signaling and, **2:**139
PP1G/R_{GL} effects, **1:**614
protein synthesis and, **3:**307
release of, **3:**378
signal transduction pathway, **3:**305–306
T-loop of, **2:**195
Insulin receptor
α subunits, **1:**294, **1:**299, **1:**362, **1:**391, **1:**409
β subunits, **1:**299, **1:**362, **1:**391, **1:**409
activation loop
autoinhibition of, **1:**300–302
location of, **1:**398
protein tyrosine phosphatase-1B and, **1:**647
activation mechanism, **1:**300
architecture of, **1:**300
binding determinants of, **1:**294–295
characteristics of, **1:**409
description of, **3:**441
domain structure of, **1:**293–294
ectodomain of, **1:**294

epidermal growth factor receptor and, similarities between, **1:**293
Gα subunit phosphorylation by, **2:**611
kinase, **1:**295
ligand binding, **1:**294
oligomerization of, **1:**311
structure of, **1:**299, **1:**409
studies of, **1:**299–302
tyrosine kinases, **1:**299–302, **1:**541
Insulin receptor kinase domain
definition of, **1:**299
IRK-3P, **1:**301
Insulin receptor substrate-proteins
14-3-3 binding sites, **1:**410
composition of, **1:**409–410
COOH-terminal end of, **1:**410
degradation of, **1:**415
description of, **3:**305
discovery of, **1:**409
function of, **1:**410
growth and, **1:**413
heterologous regulation of, **1:**414–415
insulin signaling and, **1:**409–410
IRS1, **1:**409–410, **1:**413, **1:**415
IRS2, **1:**409–410, **1:**413–415
IRS3, **1:**409–410
IRS4, **1:**409–410
longevity and, **1:**413
murine studies, **1:**409, **1:**413
nutrition and, **1:**413
pancreatic β-cells and, **1:**415–416
PH domain, **1:**410
phosphatidylinositol 3-kinase and, **1:**412–413, **3:**306
sequence alignment of, **1:**410
serine phosphorylation of, **1:**414
signaling
pathways, **1:**412
tumor necrosis factor α-induced inhibition of, **1:**415
structure of, **1:**410–411
summary of, **1:**416
tyrosine phosphorylation
cytokines that promote, **1:**414
sites of, **1:**410
YMXM motifs in, **1:**412
Insulin resistance
description of, **1:**415
obesity and, **3:**39
PPARγ and, **3:**42–43
Insulin signaling
ATM's role in, **3:**231
description of, **1:**295–296
glucose transport stimulated by, **1:**295–296
insulin receptor substrate proteins and, **1:**409–410
pathways activated by, **1:**299
PTP1B's role in, **1:**729
suppressors of cytokine signaling protein-related downregulation of, **3:**523–524
translational control and, **3:**305–307
Insulin-like growth factor I
bone development and, **3:**503
description of, **1:**300
lung development and, **3:**510–511
nuclear metabolism stimulated by, **2:**230
ovary development and, **3:**537–538
prostate gland development and, **3:**592, **3:**595
PTP1B and, **1:**730
receptors, **1:**409
Insulin-like growth factor II/cation-independent mannose 6-phosphate receptor, **1:**89
Insulin-like growth factor-binding proteins, **3:**510
Insulin-receptor-related receptor, **1:**293
Int-1, **2:**789
Integrated stress response, **3:**263, **3:**265
Integrins
cancer and, **1:**466–467
cell migration as model for studying, **1:**464–466
C-terminal helix, **1:**124–125
definition of, **1:**123
description of, **1:**463
domain organization of, **1:**123–124
in fibrinogen binding, **1:**120
β-I domain, **1:**125
integrin-PKCα-ezrin association, **2:**392
mechanosensory signaling mechanism of, **1:**71–72
multi-protein complex formation, **1:**463–464
platelet-derived growth factor receptors and, **1:**402
protein recruitment by, **1:**463–464
quaternary changes of, **1:**124
signaling
description of, **1:**123–125
Eph receptor effects on, **1:**423
Shp2's role in, **1:**718
signaling by, **1:**464–466
structure of, **1:**123–124
tail interactions, **1:**125
tertiary changes of, **1:**124–125
Interaction domains, **1:**380–381
Intercellular adhesion molecule, **1:**75
Intercellular adhesion molecule-1, **1:**95–97
Interferon-γ
cell surface receptors, **1:**271
composition of, **1:**271
definition of, **1:**271
domains of, **1:**271
IFN-γR1, **1:**271–272
IFN-γR2, **1:**271–272
structure of, **1:**272
suppressors of cytokine signaling effects on, **1:**436, **3:**525
Interferon-γ receptor α, **1:**24, **1:**363
Interferon regulatory factor 9, **3:**77
Interleukin-1
export pathways for, **3:**396–397
family members, **3:**396
IL-1α, **3:**396
IL-1β, **3:**396
ovary development and, **3:**537
receptor, **1:**24, **2:**333, **2:**335–336
testis production of, **3:**533
Interleukin-3, **3:**621
Interleukin-4
α-chain receptor, **1:**246–247
γ-chain receptor, **1:**246–247
cytokine signaling through, **3:**622
description of, **1:**241, **1:**246, **1:**414

Interleukin-4 *(continued)*
growth hormone and, comparisons between, **1:**247–248
insulin receptor substrate 2 signaling and, **1:**414
receptor activation induced by, **1:**246–247
receptors, **1:**241
signaling cascade of, **1:**246
Interleukin-5, **3:**621–622
Interleukin-6
characteristics of, **1:**343
crystal structure of, **1:**259, **1:**261
cytokine signaling through, **3:**623
viral, **1:**260–261
Interleukin-7, **3:**622
Interleukin-9, **3:**622
Interleukin-11, **3:**623
Interleukin-15, **3:**622
Interleukin-4 receptor, **1:**24, **3:**622
Interleukin-5 receptor, **3:**621–622
Interleukin-9 receptor, **3:**622
Interleukin-10 receptor, **1:**24, **1:**363
Interleukin-11 receptor, **3:**623
Interleukin-2 receptors
α-chains of, **1:**427
cytokine signaling through, **3:**622
description of, **1:**343
subunits, **1:**363
Interleukin-6 receptors
description of, **1:**363, **3:**623
gp130 subunits, **1:**24, **1:**363
Interleukin-8 receptors, **1:**151
Interneurons, **2:**883–884
Interstitial cells, **3:**587
Intracrine signals, **3:**54
Invertebrates
development of
translational control in, **3:**327–329
wnt signaling in, **2:**789–790
Shp2 deficiency in, **1:**711–712
Ion channels
accessory subunits, **1:**207
aqueous pore, **1:**215
architecture of, **1:**203–204
bacterial, **1:**204–205
barrel-stave architecture of, **1:**203–204
blocking of, **1:**217
C-terminal ends, **1:**206
cyclic nucleotide regulation of. *see* Cyclic nucleotide-gated channels
description of, **1:**115, **1:**203, **1:**215
electron microscopy studies of, **1:**204
as electrosomes, **1:**207
eukaryotic, **1:**206–207, **1:**219–220
full-length, **1:**204
functions of, **1:**203–204, **1:**215
gating of, **1:**220
KcsA, **1:**115, **1:**204
mechanosensation, **1:**115–116
N-terminal ends, **1:**206
opening of, **1:**206
pore helices of, **1:**205–206
pore-forming domains of, **1:**203–204
protein kinase C interactions with, **2:**391
proteins, **1:**203
regulation of, **1:**233–236
selectivity of. *see* Ion selectivity
soluble subunits, **1:**207
Ion selectivity
examples of, **1:**216
mechanisms of, **1:**216–217
theory of, **1:**216
Ionizing radiation. *see* Radiation
Ionophoric receptors, **1:**224
Ionotropic receptors
activation of, **3:**381
description of, **3:**377
IP, **2:**267
IP_3 receptors. *see* Inositol **2:**1,**2:**4,**2:**5-trisphosphate receptors
ipk1, **2:**230
ipk2, **2:**230
IQGAP1, **2:**893
IRE1, **3:**264, **3:**279, **3:**313, **3:**315, **3:**360
IRE1α, **3:**313
IRE1β, **3:**313
Ire1p, **3:**264
IRSp53, **2:**326
I-Smads, **3:**171
I_{SOC}, **2:**36–39
Itk, **1:**478

J

JAK–STAT signaling
definition of, **1:**343, **3:**77
inhibition of, **1:**346–347
paradigm of, **3:**77
proteins that deactivate, **1:**346–347
schematic diagram of, **1:**344
summary of, **1:**348
suppressor of cytokine signaling proteins effect, **1:**347
Janus tyrosine kinases. *see also* JAK–STAT signaling
activation of, **1:**343, **1:**428, **3:**77
carboxyl domain of, **1:**427–428
cytokine effects, **1:**343, **1:**427
cytokine receptors and, **1:**344–345, **1:**362–363, **1:**428–429
description of, **1:**246, **1:**271, **1:**343, **1:**427
family of, **1:**343, **3:**78
FERM domain of, **1:**345
hematopoietin signaling by, **3:**77
homology domains of, **1:**345
interferonγR1 dimer association with, **1:**363
Jak1, **3:**78
Jak2, **1:**452, **1:**730–731, **3:**526
JAK3, **1:**345
kinase-like domains of, **1:**343
localization of, **1:**344–345
receptor tyrosine kinases phosphorylated by, **3:**77
structure of, **1:**344–345, **1:**428
suppressors of cytokine signaling 1 effect on, **3:**526
JNK pathways
activation of, **3:**239, **3:**260
description of, **1:**494–495, **2:**368
Drosophila melanogaster morphogenesis role of
cellular stress response, **2:**785–786
description of, **2:**783

follicle cell morphogenesis, **2:**785
planar cell polarity, **2:**785
signaling in dorsal closure, **2:**783–785, **2:**815
thorax closure, **2:**785
wound healing, **2:**785–786
IRE1 activation of, **3:**316
JSP-1, **2:**371
Jun, **3:**99–100
Juxtacrine signals, **3:**54
Juxtaglomerular apparatus, **3:**580–582

K

K201, **2:**101
K^+ channel interacting proteins
KChIP 1, **2:**80–81
KChIP 2, **2:**80–81
KChIP 3, **2:**80–81
KChIP 4, **2:**80–81
properties of, **2:**80–81
Kallikrein, **3:**586
Kaposi's sarcoma herpesvirus, **1:**260
KAR2, **3:**264, **3:**360
Karyorrhexis, **3:**486
KChIP 1, **2:**80–81
KChIP 2, **2:**80–81
KChIP 3, **2:**80–81
KChIP 4, **2:**80–81
KcsA ion channel, **1:**115
Keratinocyte growth factor, **3:**510
Kidney
adenosine production, **3:**586
adenosine triphosphate, **3:**586
anatomy of, **3:**573–574
blood flow, **3:**575, **3:**581
cell–cell interactions in, **3:**573–575
dopamine, **3:**584
eicosanoids, **3:**577–578
electrolyte transport, **3:**583
endothelial cells, **3:**575–579
endothelial-derived hyperpolarizing factor, **3:**578
endothelin levels, **3:**576–577, **3:**585
functions of, **3:**573–575
GAP junctions, **3:**586–587
glomerular filtration rate, **3:**573, **3:**575, **3:**581
heme oxygenase expression, **3:**578
interstitial cell–tubule communication, **3:**587
juxtaglomerular apparatus, **3:**580–582
nitric oxide effects, **3:**577
pressure natriuresis, **3:**583
receptors in, **3:**576
regions of, **3:**573–574
renin, **3:**580
signaling pathways, **3:**576
solute carrier proteins, **3:**583
structural–functional relationships in, **3:**575
structure of, **3:**573–574
summary of, **3:**587
tubule–tubule communication in, **3:**583–587
tubuloglomerular feedback, **3:**581–582
vascular resistance of, **3:**574
vascular smooth muscle cells, **3:**579–580
vasculotubular communication, **3:**582–583
vasopressin effects, **3:**575
Killer inhibitory receptors, **1:**714
Kinase inducible activators, **3:**115
Kinase inhibitory domain, **1:**501
Kinase suppressor of Ras. *see* KSR
Kinase-associated phosphatase, **1:**656
KIR2DL1, **1:**84
KIR2DL2, **1:**84
KIX domain
description of, **3:**115
methylation of, **3:**117
kni, **3:**69
Knirps, **3:**69
Knockout mice studies
cGMP-dependent protein kinase, **2:**481
CPEB, **3:**325
G-proteins, **2:**581–584
phospholipase C, **2:**8
protein kinase C, **2:**392
protein tyrosine phosphatases, **1:**645–646
SHIP1, **2:**149
suppressors of cytokine signaling, **1:**436
Knuckle epitope, **1:**290
KRas4B protein, **2:**675
KSR, **2:**369, **2:**595
Ku70, **3:**219, **3:**339
Ku80, **3:**339

L

Lactotrophs, **3:**379
Laforin, **1:**647
Lag-3, **3:**150
lag-2, **2:**810
Lamellipodia, **1:**465
Lanthanum, **2:**57–58
Large dense-core vesicles, **3:**375, **3:**387
LAR-interacting protein 1, **1:**687
Latent transforming growth factor-β binding proteins, **3:**504–505
Lck, **1:**388, **1:**475–476, **1:**714, **2:**343
Lectins
carbohydrates and, **1:**88–89
glycolipids and, **1:**90
glycoproteins and, **1:**89–90
LEF-1/TCF proteins, **3:**163–164
Left-right determination in vertebrates by hedgehog signaling
description of, **2:**799
genes involved in, **2:**799
mice, **2:**800–802
Legless, **3:**164
Leishmania spp., **2:**539–541
Leptomycin B, **3:**85
LET-23, **1:**405, **1:**484
LET-23 signaling pathways, **2:**805–807
Leucine/isoleucine/valine binding protein, **1:**220
Leucine-rich repeat receptor protein kinases
in *Arabidopsis thaliana,* **1:**579–581
description of, **1:**579
functional view of, **1:**580–581
Leucine-rich repeats, **1:**162–163, **1:**508

Leukemia inhibitory factor, **3:**533–534, **3:**536, **3:**623, **3:**630
Leukemia-associated protein, **3:**164
Leukocytes, **3:**457
Leukotrienes
  biosynthesis of, **2:**275–277
  cascade
    enzymes in, **2:**276, **2:**278
    intermediates in, **2:**276
  cys-, **2:**278
  description of, **2:**275
  five-lipoxygenase, **2:**275–277
  G-protein coupled receptors, **2:**276
  leukotriene A_4 hydrolase, **2:**277–278
  leukotriene C_4 synthase, **2:**278
  receptors, **2:**278
LexA repressor, **3:**185–187
Leydig cells, **3:**532–533
L-Glutamate, **1:**219–220
Ligand binding
  death receptor 5, **1:**307
  dimerization induced by, **1:**392–393
  Eph receptors, **1:**421
  epidermal growth factor receptors, **3:**441
  insulin receptor, **1:**294
  lysophosphatidylcholine/sphingosylphosphorylcholine, **2:**254
  NFAT family, **3:**120
  receptor tyrosine kinases, **1:**389
  T-cell receptor, **3:**546
Ligand gating, **1:**235
Ligand-binding domain
  nuclear receptors
    antagonists, **3:**23
    ligand-binding pockets, **3:**21–22
    repression by, **3:**23
    structure of, **3:**21–22
  steroid hormone receptors, **3:**35
Ligand-binding pockets, **3:**21–22
Ligand(s)
  G-protein coupled receptor, **2:**590
  guanylyl cyclase, **2:**428–429
  neurotrophin, **2:**840
  orphan nuclear receptors, **3:**47, **3:**53–55
  transforming growth factor β, **1:**289–290
Light chains, **1:**39
LIM kinases, **1:**499
Limiting ligand, **1:**74
LIN-1, **2:**805
LIN-12, **2:**809–811
LIN-31, **2:**805
lin-12, **2:**810
Linear peptide ligands, **1:**472
lip-1, **2:**807
Lipid A molecules, **1:**117
Lipid mediators, **2:**120
Lipid rafts
  actin assembly and, **2:**212
  description of, **1:**331
  epidermal growth factor receptor localization to, **1:**323–325
  protein lipidation and, **1:**333–334
  proteins, **1:**324
  signaling role of, **1:**331–332
Lipid second messengers
  C1 domains and, **2:**159
  characteristics of, **2:**153
  description of, **2:**153
  enzyme recognition of, **2:**153–156
  inositol polyphosphate 5-phosphatase production of, **2:**156
  non-phosphoinositide recognition of, **2:**159
  phosphatase and tensin homology deleted on chromosome 10 production of, **2:**156
  phosphatidylinositol phosphate 4-kinase production of, **2:**156
  phosphatidylinositol phosphate 5-kinase production of, **2:**156
  phosphoinositide 3-kinases, **2:**154–156
  phosphoinositide-binding domains
    ENTH, **2:**156–157
    FERM, **2:**157
    pleckstrin homology domains, **2:**157
    Tubby C-terminal DNA-binding domain, **2:**157
  phospholipase A_2 production of, **2:**156
Lipid(s)
  covalent attachment of, **1:**331
  G protein regulation by modifications of, **2:**585
  metabolism, ARF modification of, **2:**728–729
  signaling protein localization mediated by, **1:**331–334
Lipopolysaccharide-inducible kinase, **1:**495
Lipopolysaccharides, **3:**62, **3:**321
Lipoprotein lipase, **3:**39
Lipoxins
  anti-inflammatory actions of, **2:**282, **2:**284
  aspirin-triggered 15-epi-LX
    anti-inflammatory actions of, **2:**282
    biosynthesis of, **2:**282
  bioactions of, **2:**281–282
  biosynthesis of, **2:**281
  diseases and, **2:**281–282
Liver, suppressors of cytokine signaling proteins' role in regeneration of, **3:**525
Liver X receptor
  cholesterol regulation by, **3:**53–54
  description of, **2:**289, **3:**48–49
  ligands for, **3:**53–54
  LXRα
    characteristics of, **3:**39, **3:**48–49
    fatty acid synthase targeting, **3:**48
  LXRβ, **3:**48
LMP1, **1:**311
Long-term potentiation, **2:**400, **2:**512
Long-term synaptic depression, **2:**385, **2:**512
Low-density lipoprotein receptor
  description of, **3:**353
  human rhinovirus binding to, **1:**95
Low-density lipoprotein receptor-related protein 5, **3:**504
Low-molecular-weight protein tyrosine phosphatases
  activators of, **1:**736–737
  amino acid sequence alignments of, **1:**734
  biological role of, **1:**737
  bovine, **1:**733, **1:**735–736
  catalytic mechanisms, **1:**735–736
  characteristics of, **1:**733
  cysteine residues, **1:**736
  description of, **1:**604, **1:**654
  diethyl pyrocarbonate inactivation of, **1:**736
  discovery of, **1:**733
  inhibitors of, **1:**736–737

isoenzymes, **1:**737
phosphate binding loop of, **1:**734
pyridoxal phosphate binding to, **1:**736
regulation of, **1:**737
structure of, **1:**735
substrate specificity of, **1:**737
vascular endothelial growth factor and, **1:**737
in yeast, **1:**737
LPC. *see* Lysophosphatidylcholine
L-selectin, **1:**88
Lst8p, **3:**368
LT-1, **2:**614
LT-2, **2:**614
L-type calcium currents, **2:**23–26, **2:**583
Lumenal cells, **3:**457
Lung
cell types, **3:**509
description of, **3:**509
development of
bombesin-like peptides in, **3:**511
bone morphogenic proteins in, **3:**511
description of, **3:**509
epidermal growth factor in, **3:**510
fibroblast growth factor in, **3:**510
fibroblast-pneumonocyte factor in, **3:**511
growth factors involved in, **3:**509–511
hepatocyte growth factor in, **3:**511
insulin-like growth factors in, **3:**510–511
platelet-derived growth factor in, **3:**510
transforming growth factor α, **3:**510
transforming growth factor β in, **3:**511
vascular endothelial growth factor in, **3:**511
injury of, **3:**512
LY294002, **1:**513
Ly49A, **1:**84–85
Lymphangiogenesis
overview of, **2:**855–856
regulation of, **2:**857
VEGFR-3 in, **2:**857
VEGFR-C in, **2:**857
VEGFR-D in, **2:**857
Lymphocyte enhancer factor-1. *see* LEF-1
Lymphocyte function-associated antigen, **2:**340
Lymphocyte phosphatase-associated phosphoprotein, **1:**691
Lymphoid enhancer binding factor, **2:**893
Lymphotactin, **1:**149
Lyn, **1:**328
Lys47, **1:**586
Lysophosphatidylcholine
description of, **2:**253
physiological and pathological functions of, **2:**253
receptors for, **2:**254
Lysophospholipase-D, **2:**253
LYVE-1, **3:**458

M

Macula densa, **3:**580
Main-chain rearrangements, in Fab fragment, **1:**34–35
Major histocompatibility complex
CD4 receptors in, **1:**23
CD8 receptors in, **1:**23
class I, **1:**63
class II, **1:**63
peptide binding to, **1:**63
Major histocompatibility complex peptide
self, **1:**80–81, **2:**341
T-cell receptor and
alloreactivity, **1:**66–67
altered peptide ligands effect, **1:**65, **1:**67, **2:**341
antagonism, **1:**65
complexes
bound water molecules in, **1:**67
description of, **1:**64–65
interactions
description of, **1:**63–64
signaling complex, **1:**64
superantagonism, **1:**65
Major urinary proteins, **3:**522
mAKAP protein, **2:**379, **2:**386
Malignant hyperthermia, **2:**47
Mammalian circadian timing system
central pacemaker, **3:**140
clock genes, **3:**139–140
description of, **3:**139
oscillators
description of, **3:**139–140
phase entrainment of, **3:**142
suprachiasmatic nuclei
description of, **3:**139–140
outputs of, **3:**140–141
subsidiary clocks, **3:**141
synchronization, **3:**141
Mammary-derived growth factor 1, **3:**568
Mannose binding protein, **1:**89
Mannose-6-phosphate receptor, **2:**409
MAPKKKKs, **1:**496, **1:**637, **1:**703
MAPKKKs, **1:**495–496, **1:**637, **1:**703, **2:**592, **3:**444, **3:**516
MAPKKs, **1:**495, **1:**637, **1:**703, **2:**366, **2:**592, **3:**516
MARCKS. *see* Myristoylated alanine-rich C kinase substrate
Maskin, **3:**324
Masking, **3:**125
Mass spectrometry, for protein–protein interactions, **2:**298–299
Mast cells
description of, **3:**458
prostaglandin D_2 by, **2:**268
Matrix metalloproteinases, **3:**458, **3:**498, **3:**505
Maturation promoting factor, **1:**693
mDia 1, **2:**703
mDia 2, **2:**703
Mdm2, **3:**239, **3:**406
MdmX, **3:**239
Mec1
description of, **3:**198–199
substrate of, **3:**200
MEC2, **3:**208
MEC3, **3:**208
Mechanically activated calcium channels, **2:**52
Mechanosensation, **1:**115–116
Mec1p, **1:**560
Mediator, **3:**14–16
Medullipins, **3:**587
MEF2D, **1:**634
Mek-1, **2:**295, **3:**31, **3:**441
Mek-1 kinase, **3:**31
Membrane immunoglobulin, **3:**555

Membrane rafts, **1:**319
MEN pathway, **1:**698–699
Messenger ribonucleic acid. *see* mRNA
Metabolism
  FOXO transcription factor regulation of, **3:**88
  lipid, ARF modification of, **2:**728–729
  mitochondrial, **2:**75–76
  phosphatidylinositol 3-phosphate, **2:**182
  phospholipid, **2:**237
  sphingolipids, **2:**19
Metabotropic receptors, **3:**378
Metarhodopsin II, **1:**141
Methyl-accepting chemotaxis proteins
  description of, **1:**197
  subfamilies of, **1:**197–198
Mevinolin, **2:**289
Mga2, **3:**132
mGluR1, **1:**221
m^7GpppX, **3:**343
MICAL, **2:**872
Michaelis–Menten enzymes, **1:**384
Microcystin-LR
  affinity tagging of, **1:**609–610
  structure of, **1:**608
Microfibrils, **3:**505
MicroRNAs, **3:**329
Microtubules, **2:**697, **3:**413
Mid-1, **2:**409
mIg molecule, **1:**327
Mineralocorticoid receptor, **3:**35
MIPP. *see* Multiple inositol polyphosphate phosphatase
Mitochondria
  apoptosis and, **3:**434–435
  biogenesis, **3:**366
  calcium
    ambient calcium and, **2:**74–75
    description of, **2:**55, **2:**73
    disease and, **2:**76
    function of, **2:**75–76
    fundamentals of, **2:**73
    mitochondrial metabolism effects, **2:**75–76
    movement mechanisms, **2:**73–74
    set point for uptake, **2:**74
    uniporter movement, **2:**73–74
    voltage-dependent anion channel, **2:**74
    xNa^+/Ca^{2+} exchange, **2:**74
  calcium signals, **3:**366–367
  calcium stores, **3:**381
  cytochrome c release from, **3:**434–435
  description of, **3:**365
  DNA, **3:**365
  dysfunctional
    description of, **3:**366
    genome-wide transcriptional responses to, **3:**370
    oxidative stress caused by, **3:**520
    p38 mitogen-activated protein kinase signaling pathway and, **3:**520–521
  hypoxia effects, **3:**370
  outer membrane permeabilization, **3:**435
  research milestones for, **3:**365–366
  retrograde regulation, **3:**365
  signaling
    in animals, **3:**366–367
    NAD^+ in, **3:**367
    in yeast, **3:**367–371
Mitogen-activated protein kinase phosphates
  in *Caenorhabditis elegans,* **1:**704
  cytosolic, **3:**519
  mitogen-activated protein kinase inactivation by, **3:**519
Mitogen-activated protein kinases
  activating protein 1 regulation by, **3:**101
  activation of, **2:**365, **2:**872, **3:**116, **3:**257, **3:**259
  canonical, **3:**109
  characteristics of, **1:**493
  description of, **1:**673, **2:**365, **2:**783
  discovery of, **1:**493
  DNA damage-related activation of, **3:**259
  docking interactions, **2:**369
  enzyme that encode, **2:**365
  epidermal growth factor stimulation effects on activation of, **1:**324
  extracellular signal-regulating protein kinases
    classification of, **2:**365
    description of, **1:**493–494, **1:**673
    dual phosphorylation motif, **2:**365
    ERK1, **2:**365, **2:**367
    ERK2, **2:**365
    ERK3, **2:**366
    ERK5, **2:**366
    ERK7, **2:**366
    ERK8, **2:**366
  Fus3, **2:**361
  gastrointestinal hormone-stimulated signal transduction and, **3:**481–482
  G-protein $\beta\gamma$ subunit and, **2:**640
  G-protein coupled receptor activation of, **2:**592, **3:**444, **3:**480
  H3 kinase and, **3:**92
  hierarchical organization of, **1:**495
  Hog1, **1:**703, **2:**357, **2:**360
  JNK, **2:**366, **2:**368
  Kss1, **2:**357, **2:**359
  MAPKKKKs, **1:**496, **1:**637, **1:**703
  MAPKKKs, **1:**495–496, **1:**637, **1:**703, **2:**592, **3:**444, **3:**516
  MAPKKs, **1:**495, **1:**637, **1:**703, **2:**366, **2:**592, **3:**516
  mechanism of activation, **2:**365
  molecular cloning of, **1:**493
  Mpk1, **2:**357
  mRNA turnover and, **3:**286
  nomenclature of, **2:**366
  nuclear receptor corepressors and, **3:**31
  p38, **1:**495, **2:**366–367, **2:**594
  phosphatases
    description of, **1:**643
    in *Drosophila melanogaster,* **1:**704
    in mammals, **1:**705
    MAPK phosphatase-1, **1:**705
    MAPK phosphatase-3, **1:**656
    in yeast, **1:**703–704
  platelet-derived growth factor effects, **1:**423
  PP2A and, **1:**624
  protein kinases, **2:**368–369
  protein tyrosine phosphatase effects, **1:**653
  Ras triggering of, **2:**862
  in *Saccharomyces cerevisiae,* **1:**703–704, **2:**358–359

scaffold proteins
description of, **2:**369
ft-arrestin, **2:**370–371
JIP, **2:**370
JSP-1, **2:**371
KSR, **2:**369
MPI, **2:**370
nomenclature of, **2:**370
SKRP1, **2:**371
signaling pathway
fibroblast growth factor receptors and, **1:**268
p38, **1:**320–321
Smk1, **2:**357
steroid hormone receptor targeting by, **3:**36
stress signal transduction by, **3:**182
stress-activated
description of, **1:**494–495
PP2C regulation of, **1:**637–638
substrate
docking site on, **2:**369
specificity of, **2:**365
tristetraprolin and, **3:**321
yeast cells
activation of, **2:**357–358
cascades
cell architecture in, **2:**361–362
downregulation, **2:**361–362
dynamic localization of, **2:**360–361
inactivation of, **2:**361
kinase interactions, **2:**359–360
cascades in, **2:**357–358
regulatory mechanisms, **2:**359–362
scaffold proteins, **2:**360
crossregulation of, **2:**362
description of, **2:**357
Saccharomyces cerevisiae, **1:**703–704, **2:**358–359
Mitosis
description of, **2:**697
function of, **3:**401
histone phosphorylation and, **3:**95
Mixed lineage leukemia protein, **3:**117
MKK4, **2:**369
MKP3, **1:**744
Mks1p, **3:**368–369
MN cadherins, **2:**896
Mnk1, **3:**344
Mnk2, **3:**344
Modular interaction domains, **1:**471–473
Molecular chaperones, **3:**271–273
Molecular recognition
binding, **1:**11–12
description of, **1:**11
nonspecific association with membrane surfaces, **1:**12
protein–protein interactions, **1:**12, **1:**23
Molten globule, **3:**397–398
Mono-ADP-ribosylation, of G proteins
bacterial toxin-induced, **2:**614–615
cellular, **2:**616
cycle of, **2:**614
description of, **2:**613–614
endogenous, **2:**615–616
substrates, **2:**616
Monoclonal antibody, antibody–antigen interface studies in, **1:**40–42
Monomeric G proteins, phosphatidylinositol 4-phosphate 5-kinase type I stimulated by, **2:**125
MPI, **2:**370
MPM2, **3:**340
MPP4, **3:**340
M-Ras, **2:**686–687
M-RdgB1, **2:**227
Mre11/Rad50/Nbs1 complex, **3:**220–221
Mre11/Rad50/Xrs2, **3:**227
mRNA
β-actin, **3:**293–294
description of, **3:**283
instability elements, **3:**319–320
insulin effects on translation of, **3:**307
localization of
cytoskeleton, **3:**294
extracellular matrix signaling, **3:**294
growth factors that induce, **3:**293–294
GTPase signals regulating actomyosin interactions involved in, **3:**294–295
regulation of proteins associated with, **3:**294
neuronal movement of, **3:**294–295
stability of, **3:**283, **3:**319
suppressors of cytokine signaling, **3:**521
tumor necrosis factor, **3:**321
turnover of
AMP-activated protein kinase and, **3:**287
AU-rich elements, **3:**319–320
cis elements that regulate, **3:**283–284
heat-shock proteins and, **3:**286–287
hypoxia and, **3:**287
mitogen-activated protein kinase regulation of, **3:**286
models of, **3:**285
phosphatidylinositol 3-kinase and, **3:**287
protein kinase C regulation, **3:**286
regulation of, **3:**285, **3:**319–321
S6 kinase and, **3:**287
schematic diagram of, **3:**284
stress-activated signaling molecules associated with, **3:**285–287
trans factors, **3:**284–285
tristetraprolin effects, **3:**320–321
mRpd3, **3:**29
MsbA, **1:**117
MSG5, **1:**703
mSiah2, **3:**30
MTM1, **1:**647
MTMR2, **1:**647
MTMR4, **2:**181
MTMR2, **2:**145
mTOR
amino acid regulation of, **1:**529–530, **3:**300–301
cell growth regulated by, **1:**558
description of, **1:**524–525
eIF-4E binding protein phosphorylation by, **1:**526–527, **3:**344–345
energy sufficiency regulation of, **1:**530–531
FRAP/mTOR, **3:**345–346
glutamine response, **3:**301
insulin translation and, **3:**307

mTOR *(continued)*
p70 S6K regulation by, **1:**527–528
rapamycin effects, **3:**300
regulation of, **1:**528–531
RTK-PI-3K effects on, **1:**528–529
signaling pathways, **1:**528, **1:**558, **3:**300–302
S6K control by, **3:**302
mT2r5, **2:**658
Multidrug resistance ABC transporter group, **1:**117
Multiple endocrine neoplasia type 2B, **1:**445
Multiple inositol polyphosphate phosphatase, **2:**234
Munc18c, **1:**296
Mural cells, **3:**457
Murine mammary epithelial cells, **3:**569
murine studies of, **3:**101–103
Musashi protein, **3:**329
Muscular dystrophy, calpain 3's role in, **2:**109
Mx proteins
antiviral activity of, **2:**771–772
cellular interaction partners of, **2:**774–775
central interactive domain of, **2:**773
cytoplasmic, **2:**771
description of, **2:**771
functional domains of, **2:**773
high molecular weight GTPases, **2:**772–774
human, **2:**772
molecular interactions of, **2:**773
mouse, **2:**772
Mx1, **2:**774–775
MxA, **2:**771, **2:**773–774
MxB, **2:**771
oligomerization of, **2:**774
Mycobacterium tuberculosis mechanosensation ion channel, **1:**116
MyD88, **1:**311
MyD88 factor, **2:**335–336
Myelin basic protein, **3:**147
Myelin-associated glycoprotein signaling, **2:**873
Myeloma, **3:**498
Myocyte enhancer factors, **1:**527
myo-inositol, **2:**231
Myosin, **2:**409
Myosin heavy chain kinase
description of, **1:**567–568
phosphorylation of, **1:**570
substrate binding, **1:**570
Myosin light chain phosphatase binding subunit, **2:**481–482
Myotonic dystrophy kinase-related Cdc42-binding kinase, **1:**501–502
Myotonic dystrophy protein kinase, **1:**501
Myotubularins
characteristics of, **1:**643, **1:**745–746
diseases and, **2:**145–146
family members, **2:**145
FYVE domain of, **2:**146
MTMR2, **2:**145
PH domain of, **2:**146
phosphatase activity of, **2:**145
phosphatidylinositol 3-phosphate utilization by, **2:**145
structural features of, **2:**146
subgroups of, **2:**145
Mypt2, **1:**617
Myristic acid, **1:**632, **2:**585
Myristoylated alanine-rich C kinase substrate, **1:**12, **2:**212, **2:**243
Myristoylation, **1:**128
Myt1, **3:**404
Myxovirus resistance proteins. *see* Mx proteins

N

Na^+/Ca^{2+} exchanger
calcium signaling and, **2:**65
cardiac, **2:**64
cellular expression of, **2:**65
cytoplasmic loop of, **2:**64
description of, **2:**63
epithelial expression of, **2:**65
families of, **2:**63–64
inhibition of, **2:**64, **2:**66
localization of, **2:**64–65
membrane typology of, **2:**63–64
NCKX, **2:**63–64
NCX1, **2:**63–64
NCX2, **2:**63–64
NCX3, **2:**63–64
nervous system distribution of, **2:**65
operation modes of, **2:**64
physiological roles of, **2:**65–66
regulation of, **2:**64
transport reactions mediated by, **2:**65
x, **2:**74
Na^+/Ca^{2+} exchanger regulatory factor, **3:**443
N-acetylglucosamine, **1:**90
NADPH oxidase
components of, **2:**705–706
description of, **2:**174
electron transfer steps of, **2:**706
formation of, **2:**705–706
Rac and
interactions between, **2:**705
regulatory role, **2:**706–708
Nanos, **3:**328
1-Naphthylmethyl-PP1, **1:**585–586
Natural killer cell surface receptors
classification of, **1:**83–84
C-type leptin-like, **1:**84–85
description of, **1:**83–84
immunoglobulin G-type, **1:**84
KIR2DL1, **1:**84
KIR2DL2, **1:**84
Ly49A, **1:**84–85
NKG2D, **1:**84
Natural killer cell(s)
C-SMAC, **2:**342
description of, **1:**83
effector functions of, **1:**84
functions of, **1:**83
NKG2D, **1:**83, **1:**85
P-SMAC, **2:**342
Shp1 effects on signaling of, **1:**714
synapses, **2:**342
N-cadherin, **2:**896
NCK, **2:**863

NCKX, **2:**63–64
NCKX2, **2:**63–64
Necrosis, **3:**485
NEDD8, **3:**131
Negative glucocorticoid response elements, **3:**63
Negative signaling
  CTLA-4-mediated, **1:**357–358
  definition of, **1:**355
  protein localization in, **1:**355–358
Nej1p, **3:**222
Nerve growth factor
  description of, **1:**2, **1:**281
  discovery of, **1:**2
  nerve growth factor–TrkA-D5 complex
    crystal structure of, **1:**282–283
    description of, **1:**282
    N-terminal residues in, **1:**283
  N-terminal residues of, **1:**283
  ovary development and, **3:**537
  testis production of, **3:**534
Nerve growth factor protein, **3:**486
Nerve growth factor-TrkA, **2:**841–842
Nervous system
  anterograde signaling in, **3:**607
  cell-to-cell signaling in, **3:**607–608
  central
    cGMP-dependent protein kinase and, **2:**512–513
    G protein effects, **2:**581–582
    Na^+/Ca^{2+} exchanger distribution in, **2:**65
    semaphorins' role in development of, **2:**877
  neurogenic inflammation, **3:**608–611
  retrograde signaling in
    description of, **3:**607–608
    dorsal root reflexes, **3:**611–612
N-ethylmaleimide sensitive factor, **1:**296, **3:**382
Netrins, **2:**871–872
Neu/ErbB2, **1:**445
Neurabin, **2:**398–399
Neural cell adhesion molecules, **1:**60
Neuraminidase
  active binding site, **1:**107, **1:**109
  inhibitors of
    Abbott compounds, **1:**111
    BCX-1812, **1:**110–111
    development of, **1:**105
    observations regarding, **1:**111
    Relenza, **1:**109
    Tamiflu, **1:**109–110
  ligands, **1:**106
  role of, **1:**105
  sialic-acid-binding active site, **1:**107
  structure of, **1:**107
Neuregulin, **3:**566
Neurocalcins, **2:**80–81
Neurodegenerative diseases, **3:**273
Neurofilament proteins, **2:**409
Neurogenesis
  description of, **3:**625
  neural stem cells
    isolation and culture of, **3:**626–628
    regulation of differentiation into neurons, **3:**628–631
Neuroligins, **2:**330
Neuronal calcium sensors
  description of, **2:**79, **3:**384
  guanylate cyclase activating proteins, **2:**80–81
  hippocalcin, **2:**80–81
  K^+ channel interacting proteins, **2:**80–81
  NCS-1, **2:**79, **2:**81
  neurocalcins, **2:**80–81
  properties of, **2:**79–80
  recoverins, **2:**80–81
Neuron(s)
  apoptosis, **3:**485–486, **3:**489
  calcineurin effects on, **1:**635
  calcium channels in, **3:**381
  calcium signaling in, **2:**54
  cyclic nucleotide signaling in, **2:**462
  neurotrophins and, **3:**488
  signaling, **3:**445
  voltage-gated calcium channel activation in, **2:**23
Neuropilins, **2:**872, **2:**877–878
Neurotensin, **3:**478
Neurotransmitter release
  G-protein's role in, **2:**663–665
  modulation of, **2:**664–665
Neurotrophic growth factors
  description of, **3:**485
  neural cell death, **3:**485–486
Neurotrophic hypothesis, **3:**486–488
Neurotrophin receptors
  description of, **1:**392, **3:**488–489
  $p75^{NTR}$, **1:**281–283, **2:**839, **3:**488–489, **3:**596–597
Neurotrophin(s)
  apoptosis regulation by, **3:**488–489
  cellular effects of, **2:**839
  definition of, **1:**281
  description of, **2:**839, **3:**487–488
  developmental role of, **2:**839–842
  fold of, **1:**282
  interacting proteins, **2:**842
  ligands, **2:**840
  nerve growth factor and, **1:**281
  neurological disorders and, **1:**281
  neuronal activity and, **3:**488
  receptors, **2:**840–841
  retrograde transport, **2:**841–842
  signaling pathways, **3:**489–490
  signaling specificity, **2:**840–841
  transcriptional regulation, **3:**490
  types of, **3:**487
NF45
  cellular regulation of, **3:**340
  description of, **3:**335
  properties of, **3:**337
NF110, **3:**335, **3:**337–338
NF90 family
  cDNA, **3:**337
  cellular regulation of, **3:**340
  description of, **3:**335
  domain structure of, **3:**337–338
  homologues
    characteristics of, **3:**336–337
    functions of, **3:**340

NF90 family *(continued)*
members of, **3:**337
nucleic acid binding properties of, **3:**339–340
protein kinase PKR interactions with, **3:**335
proteins that interact with, **3:**338–339
in *Saccharomyces cerevisiae,* **3:**340
TCP80 isoform of, **3:**337, **3:**340
NFAT family
activation of, **3:**550
activator protein-1 interactions with, **3:**121
biological functions regulated by, **3:**121–122
calcineurin interactions with, **3:**120
description of, **3:**119
DNA-binding domain of, **3:**120
gene transcription, **3:**120
isoforms, **3:**121
ligand binding of, **3:**120
mass spectrometry analysis of, **3:**121
NFAT1, **3:**119–122
NFAT2, **3:**119–122
NFAT3, **1:**634, **3:**119–122
NFAT4, **3:**120–122
NFAT5, **1:**634, **3:**119, **3:**121–122
NFATc, **3:**126–127
regulation of, **3:**120–121
rephosphorylation of, **3:**120
structure of, **3:**120
transcriptional functions of, **3:**121
NFAT-Fos-Jun, **3:**121
NF-κB
activation of, **3:**108–109, **3:**492
apoptosis and, **3:**433
cellular transformation and, **3:**112
characteristics of, **3:**107–112, **3:**550
cytoplasmic localization of, **3:**108, **3:**491–492
description of, **3:**62–63, **3:**491
family members, **3:**107–108
functions of, **3:**491
gene regulation by, **3:**108
identification of, **3:**107
IκBs, **3:**108
inhibitors of, **3:**112
intracellular localization of, **3:**491–492
NF-κB2/p100, **3:**111
signal transduction pathway, **3:**109
NF-L, **2:**399
Nibrin, **3:**227
Nicotinic acetylcholine receptors
α subunit, **1:**224
binding sites, **1:**224–225
channel, **1:**225
cytoplasmic domain, **1:**226
description of, **1:**204–205
discovery of, **1:**223
extracellular domain of, **1:**224
function of, **1:**223
homopentameric, **1:**226
neuronal-type, **1:**223
quaternary structures, **1:**224
secondary structures, **1:**224
structure of, **1:**223–226
subunit composition, **1:**223
tertiary structures, **1:**224
Nicotinic acid adenine dinucleotide phosphate
calcium signaling by, **2:**16, **2:**52
description of, **2:**15, **3:**380–381
structure of, **2:**16
NIH3T3 cells, **2:**709
NIPP-1. *see* Nuclear inhibitor of protein phosphatase **1:**1
NIPP1, **1:**617
Nitric oxide
cGMP synthesis stimulated by, **2:**511
granular cell effects, **3:**580
guanylyl cyclase and, **2:**428, **2:**443
platelet homeostasis and, **2:**512
renal tubule cells affected by, **3:**584–585
renal vasculature effects, **3:**577
vasoconstriction by, **3:**579
Nitric oxide synthase
description of, **2:**330
endothelial, **2:**851
isoforms, **3:**584
targeted inactivation of, **2:**511
Nitrosothiols, **3:**193–194
NKG2D, **1:**83, **1:**85
NMDA receptors
calcineurin's role in, **2:**399–400
calcium/calmodulin-dependent protein kinase II binding to, **2:**331
calcium-related response, **2:**331
description of, **2:**329, **2:**399–400
Eph receptor interactions with, **1:**422
NR2A-D units, **2:**329
protein phosphatase 2A interactions with, **2:**408
scaffold proteins, **2:**330–331
signaling complex, **2:**329–331
nm23H1, **3:**414
N-Myristoylation, **1:**332
Nodal, **2:**800
Nogo, **2:**873
Nonhomologous end joining, for DNA double-strand breaks, **3:**219–223
Non-homologous end-joining, **2:**231
Non-obligate complexes, **1:**23
Non-phosphoinositides ligands
pleckstrin homology domain binding to, **2:**165
second messenger recognition, **2:**159
Nonsense-mediated RNA decay, **1:**559
Noonan syndrome, **1:**668, **1:**719
Nos-response element, **3:**328
Notch ligands
description of, **2:**814–815
regulation of, **2:**816–817
Notch proteins
description of, **2:**809–811, **3:**149
intracellular domain
architecture of, **3:**149
description of, **3:**149
modification of, **3:**151–153
phosphorylation of, **3:**152
proteins interacting with, **3:**151–152
Su(H)-mediated repression of, **3:**149–150, **3:**153–154
target genes activated by, **3:**154–155
ubiquination of, **3:**153
signaling pathways. *see* Notch signaling pathways
transcriptional activity of, **3:**153–156

Notch receptors, **2:**813–814
Notch response elements, **3:**155
Notch signaling pathways
  description of, **1:**370
  discovery of, **2:**813
  in *Drosophila* eye development, **2:**827–831
  vertebrate development
    basic helix-loop-helix factors, **2:**819
    description of, **2:**817
    lateral inhibition, **2:**819
    limbs, **2:**820–821
    lymphoid development, **2:**821
    neurogenesis, **2:**817–819
    notch ligands, **2:**814–817
    notch receptors, **2:**813–814
    organ systems, **2:**821–822
    segmentation, **2:**819–820
    signal transduction, **2:**815–816
    vascular development, **2:**821
Nox, **2:**709
3-NPA, **3:**520–521
NR1a, **2:**385
NR2A-D units, **2:**329
Nrarp, **3:**151
NT-3, **2:**840
Nuclear envelope, **3:**420
Nuclear export sequences, **3:**125
Nuclear export signals, **3:**249, **3:**421
Nuclear factor 45. *see* NF45
Nuclear factor 90. *see* NF90
Nuclear factor 110. *see* NF110
Nuclear factor of activated T cells, **1:**718
Nuclear factor of activated T cells-c, **3:**467
Nuclear import signals, **3:**419–421
Nuclear inhibitor of protein phosphatase 1, **1:**629
Nuclear localization sequences, **3:**125
Nuclear localization signals
  adaptor-independent, **3:**420
  classical, **3:**419–420
  description of, **2:**697, **3:**249, **3:**419
  importin β binding, **3:**422
Nuclear pore complex
  architecture of, **3:**426
  definition of, **3:**426
  description of, **2:**695, **2:**781, **3:**419
  facilitated diffusion through, **3:**427
  passive diffusion through, **3:**427
  in *Saccharomyces cerevisiae,* **3:**426
  translocation, **3:**427–428
  transport mechanisms, **3:**427–428
Nuclear receptor corepressors
  description of, **3:**23, **3:**29
  developmental role of, **3:**31
  disease and, **3:**31
  histone deacetylase activity and, **3:**64
  mechanism of action, **3:**29
  mSiah2 interactions with, **3:**30
  N-terminus of, **3:**30
  purification of, **3:**30
  regulatory mechanisms, **3:**30–31
  signaling pathways effect on subcellular distribution of, **3:**31
  transcription factors associated with, **3:**30
  transcription repression by, **3:**29, **3:**31
Nuclear receptor(s)
  activation of, **3:**22–23, **3:**62
  classical, **3:**47
  coactivators of
    amino acid sequences, **3:**25–26
    CARM-1, **3:**27
    complexes, **3:**27
    description of, **3:**25
    histone acetyltransferase activity of, **3:**27
    recruitment mechanisms, **3:**25–26
    signal transduction pathway targets, **3:**27
  cystol receptor complex, **3:**8
  definition of, **3:**25
  description of, **2:**289, **3:**7, **3:**25, **3:**47, **3:**53, **3:**57, **3:**62
  dimerization, **3:**23
  discovery of, **3:**7, **3:**47
  DNA-binding domain of, **3:**47
  domains of, **3:**21
  Drosophila
    description of, **3:**69
    ecdysone regulatory hierarchies, **3:**69, **3:**71
    embryonic pattern formation and, **3:**69
    neuronal development role of, **3:**71
    subfamilies, **3:**69–70
  forms of, **3:**7–8
  function of, **3:**8
  hormone response elements, **3:**25
  immediate hormone responses, **3:**63
  inhibitory role of, **3:**62–63
  ligand-binding domain of
    antagonists, **3:**23
    description of, **3:**47
    ligand-binding pockets, **3:**21–22
    repression by, **3:**23
    structure of, **3:**21–22
  ligand-mediated activation of, **3:**22–23
  orphan. *see* Orphan nuclear receptors
  research areas for, **3:**7
  structure of, **3:**8
  transactivation domain of, **3:**62
  transcription activation by, **3:**25
  transcription factor modulation by, **3:**63–65
Nuclear transport
  regulated, **3:**125–127
  transcriptional control through, **3:**125–127
Nuclear transport factor 2, **3:**425
Nuclear transport receptors
  description of, **3:**421
  FG-rich nucleoporins and, **3:**427
  importin β, **3:**421–424
Nuclear transport signals
  export, **3:**249, **3:**421
  import, **3:**419–421
Nucleic acids, **1:**560
Nucleoporins, **3:**419, **3:**426
Nucleotide binding domain, **1:**117
Nucleus
  Abl function, **3:**252–254
  calcium levels, **2:**55
  endoplasmic reticulum signaling pathways to, **3:**263–265
  stress signals from, **3:**179–180
N-WASP, **2:**210, **2:**324

O

Obesity
  insulin resistance and, **3:**39
  tumor necrosis factor α levels in, **3:**43
OGR1, **2:**254
Okadaic acid, **1:**608, **1:**622
Olfaction, **1:**145
Olfactory receptors, **1:**145–146
Oligomerization
  cytokine receptors, **1:**362–364
  description of, **1:**361
  guanylyl-cyclase-containing receptors, **1:**362, **1:**364
  insulin receptor, **1:**311
  Mx protein, **2:**774
  tumor necrosis factor receptors, **1:**364
  tyrosine-kinase containing receptors, **1:**361–362
Oligosaccharides
  description of, **1:**87
  in vivo synthesis of, **1:**88
Omega-3 polyunsaturated fatty acids, **2:**283
Oncogenes, **3:**244
Online Mendelian Inheritance in Man, **1:**668
Opa52 protein, **1:**720
ORF74, **1:**174–175
ORP150, **3:**280
Orphan nuclear receptors
  constitutive androstane receptor
    activation of, **3:**54–55
    description of, **3:**50–51
    ligands for, **3:**54–55
    sequestration of, **3:**54
  COUP-TF, **3:**58–59
  definition of, **3:**47
  description of, **3:**25
  farnesoid X receptor
    bile acids that bind to, **3:**54
    description of, **2:**289, **3:**48
    ligands for, **3:**54
  ligands for, **3:**47, **3:**53–55
  liver X receptor
    cholesterol regulation by, **3:**53–54
    description of, **2:**289, **3:**48–49
    ligands for, **3:**53–54
    LXRα
      characteristics of, **3:**39, **3:**48–49
      fatty acid synthase targeting, **3:**48
    LXRβ, **3:**48
  peroxisome proliferator-activated receptors. *see* Peroxisome proliferator-activated receptors
  pregnane X receptors
    description of, **2:**289, **3:**21
    ligands for, **3:**54–55
  retinoid X receptor, **3:**39, **3:**48
  SXR, **3:**49–50
  vascular development and
    angiogenesis, **3:**57–58
    vasculogenesis, **3:**57
osk, **3:**327–328
Osteoblasts, **3:**499
Osteoclasts, **3:**498
Osteocytes, **3:**499
Osteoporosis, **3:**497–498
Osteoprotegerin, **3:**498
Osteotesticular protein tyrosine phosphatases, **1:**666
Ovary
  cell–cell interactions in, **3:**535–538
  cellular biology of, **3:**535
  description of, **3:**531
  follicle growth and differentiation, **3:**535–536
  theca cells, **3:**537
Oxidative stress
  in bacteria, **3:**191
  definition of, **1:**688, **3:**191
  mitochondrial dysfunction as cause of, **3:**520
Oxyanions, **1:**678–679
Oxygen dependent degradation domain, **3:**278
Oxygen homeostasis, **3:**277
OxyR protein, **3:**193–194
Oxysterol binding protein, **2:**166
Oxysterols, **2:**287

P

p27, **1:**508, **3:**405
p38
  characteristics of, **2:**367
  description of, **1:**320
  mitogen-activated protein kinases
    catalytic site activation, **3:**518
    description of, **1:**495, **2:**594
    docking domain, **3:**517
    family of, **3:**516
    isoforms, **3:**519
    mitochondrial dysfunction and, **3:**520–521
    nucleocytoplasmic transport of signal, **3:**519
    protein–protein interactions, **3:**516–518
    schematic diagram of, **3:**516
    stress response signaling by, **3:**515–521
    stress signal transduction, **3:**517
    subcellular localization of, **3:**519
  tristetraprolin and, **3:**321
p50, **3:**131
p50α, **2:**138
p53
  activation of
    description of, **3:**239–241
    genotoxic stress-induced, **3:**241–243
    hypoxia-induced, **3:**243
    ionizing radiation-induced, **3:**241–242
    microtubule disruption-related, **3:**243–244
    nongenotoxic stressor-induced, **3:**243–244
    oncogene activation-related, **3:**244
    replicative senescence, **3:**244
    ribonucleotide depletion, **3:**243
    ultraviolet light-induced, **3:**242
  ATM and, **3:**228
  cancer and, **3:**237
  carboxy-terminal DNA damage-induced modifications, **3:**242–243
  characteristics of, **3:**237
  C-terminal region, **3:**238, **3:**242
  description of, **3:**130–131, **3:**181
  gene expression inhibited by, **3:**237

Mdm2 effects, **3:**239
N-terminal region, **3:**238
p300 targeting, **3:**241
posttranslational modifications, **3:**238, **3:**241
protein domains of, **3:**237–238
regulation of, **3:**238–241
stabilization of, **3:**239
structure of, **3:**237–238
ubiquination of, **3:**239
p74, **3:**338–339
p85, **1:**409
p107, **2:**408
p110γ, **2:**139
p386, **2:**367
p140 factor, **3:**31
p42 KKIALRE, **2:**368–369
p56 KKIALRE, **2:**368–369
P_2 receptors, **3:**586
P site inhibitors, **2:**422
p70 S6K
description of, **1:**524, **2:**193
EF2 kinase regulation by, **1:**571
kinase, **1:**528
mTOR regulation of, **1:**527–528
murine studies, **2:**195–196
protein phosphatase 2A interactions with, **2:**409
p40 Syt1, **3:**395
PAC1, **1:**705
p21-activated kinases, **1:**499–501, **2:**409, **2:**610–611
Palmitoyl thioesterases, **2:**652
Palmitoyl transferases, **2:**652
Palmitoylation
definition of, **2:**651
description of, **1:**332–333
functions of, **2:**651
G-protein α subunit
activation-regulated, **2:**651–652
depalmitoylation and, **2:**653
description of, **2:**586, **2:**651
plasma membrane localization by, **2:**653
reversible, **2:**652–654
sites of, **2:**651
Ras, **2:**676
regulators of G protein signaling, **2:**651
reversible
functions of, **2:**653–654
mechanisms of, **2:**652–653
palmitoyl thioesterases in, **2:**652
palmitoyl transferases in, **2:**652
proteins that regulate, **2:**652–653
Pancreas
cell–cell interactions in, **3:**472–473
cell–matrix interactions in, **3:**472–473
β-cells, **1:**415–416
description of, **3:**471
development of, **3:**471–472
endocrine, **3:**472–473
endocrine cells, **3:**472
exocrine, **3:**473
islets of Langerhans, **3:**472
ontogeny of, **3:**471–472
studies of, **3:**471–472
Pantophobiacs, **1:**230
Papain, **1:**29
Paracrine, **3:**54
Paramecium
adenylyl cyclase, **2:**535–536
cAMP signaling in, **2:**535–536
cGMP formation in, **2:**536–537
cyclic nucleotide formation in, **2:**537
guanylyl cyclase, **2:**536–537
phosphodiesterases in, **2:**537
Parathyroid hormone, **3:**500–501
Parathyroid hormone-related protein, **3:**420, **3:**500–501
Paraxial protocadherin, **2:**891
Particulate guanylyl cyclases, **2:**427
Parvalbumin, **2:**67–70
PAS domains, **2:**525
Patched, **2:**793
Paxillin, **1:**464–465, **2:**409, **3:**481
p130Cas, **1:**730
p300/CBP, **1:**488
$p21^{Cip1}$, **2:**711
p120ctn, **2:**893–894
PD 184352, **1:**458
$P388D_1$ macropahges, **2:**262
PDE1, **2:**442
PDE2, **2:**442–443
PDE4, **2:**434
PDE10, **2:**444
PDE11, **2:**444
PDE4D3, **2:**386, **2:**438–439
PDK1
activation of, **2:**389
AGC kinase activation by, **1:**515, **2:**195–197
catalytic domain of, **1:**517–518, **2:**197–198
characteristics of, **2:**194
kinases activated by, **1:**515, **2:**195
mechanism of action, **1:**516–517, **2:**196–197
murine studies, **1:**515–516, **2:**195–196
phosphorylation by, **1:**518, **2:**198
protein kinase B activated by, **1:**514–515, **1:**517, **2:**194–195
protein kinase C interactions with, **2:**390
RSK phosphorylation by, **2:**198
SGK phosphorylation by, **2:**198
S6K phosphorylation by, **2:**198
PDZ binding motif, **1:**423
PDZ domain
Eph receptor signaling via, **1:**423
EphrinB reverse signaling via interactions with, **1:**424
protein kinase C, **2:**390
PSD-95, **2:**330
Peptide recognition modules, **2:**311–315
Peptide YY, **3:**478
Peptidoglycan recognition proteins, **2:**781
Peptidylglycine-a-amidating mono-oxygenase, **2:**409
Pericyte smooth muscle cells, **3:**457
Peripheral supramolecular activation cluster, **1:**80
PERK, **3:**264, **3:**316, **3:**360
Peroxisome proliferator-activated receptors
characteristics of, **3:**48
description of, **3:**21
ligands for, **3:**53–54
PPARα, **3:**48

Peroxisome proliferator-activated receptors *(continued)*
PPARδ
description of, **3:**22
ligands for, **3:**53
PPARγ
activation of, **3:**39–40
adipose tissue development and, **3:**39–40
angiogenesis inhibition by, **3:**58
animal model studies of, **3:**39
cell cycle regulation and, **3:**43
description of, **3:**22, **3:**48
gene structure, **3:**40
homozygous inactivation of, **3:**40
insulin resistance and, **3:**42–43
murine studies of, **3:**40
retinoid X receptor binding, **3:**39
subtypes of, **3:**53
Pertussis toxin, **2:**613–615, **2:**879
Pervanadate, **1:**679
PEST-1, **1:**577
Pfeiffer syndrome, **2:**862
Phagocyte oxidase homology domain. *see* PX domain
Phagocytosis, **2:**702
Phorbol ester
cell signaling and, **2:**120, **2:**190
description of, **2:**120
Phorbol myristate acetate, **3:**385
Phosducin, **2:**577, **2:**587, **2:**641
Phosphatase and tensin homology deleted on chromosome 10
activity of, **2:**143–144
chemotactic role of, **2:**647
C-terminus of, **2:**144
function of, **2:**143–144
glioblastomas and, **3:**278
history of, **2:**143
in human genome, **2:**144
hydrogen peroxide-mediated inactivation of, **2:**114–115
Ins(1,3,4,5,6)P_5 interactions with, **2:**233–234
murine studies, **2:**143
mutations of, **2:**143, **3:**278
NFκB inhibitors and, **3:**112
PDZ-binding domain of, **2:**144–145
phosphoinositide 3-kinase and, **2:**115, **2:**138, **2:**144, **2:**220
regulation of, **2:**144–145
second messenger production by, **2:**156
structure of, **2:**156
Phosphatases
CD45, **1:**433
chemotactic role of, **2:**647
description of, **1:**431
dual-specificity, **1:**643, **1:**653
inhibitors of, **1:**628
mitogen-activated protein kinase, **1:**643
myotubularin-like, **1:**745–746
serine/threonine. *see* Serine/threonine phosphatases
SHP-1, **1:**431–432
STAT, **1:**433
Phosphate binding cassette, **2:**521
Phosphatidic acid, **2:**728
description of, **2:**174
phosphatidylinositol 4-phosphate 5-kinase type I
stimulated by, **2:**125
phospholipase D release of, **1:**3, **2:**249
phospholipid metabolism and, **2:**237
regulation of, **2:**237
Phosphatidyl D-*myo*-inositol, **2:**153
Phosphatidylcholine, **2:**238
Phosphatidylethanolamine, **2:**289
Phosphatidylinositol
bradykinin-stimulated turnover of, **1:**324
phosphoinositide 3-kinase phosphorylation of, **2:**136
Phosphatidylinositol 3
description of, **1:**295
insulin receptor substrate-protein's role in signaling, **1:**412–413
insulin stimulation of glucose transport role of, **1:**296
p85 regulatory subunit of, **1:**485
Phosphatidylinositol biphosphate, **1:**3
Phosphatidylinositol 3,4,5-bisphospate, **2:**7
Phosphatidylinositol 4,5-bisphosphate. *see also* PIP2
description of, **1:**513, **2:**5, **2:**64, **2:**129, **2:**193
phospholipase C hydrolysis of, **2:**225
pleckstrin homology domain binding to, **2:**162–163, **2:**193
Phosphatidylinositol 3-kinase
activation of, **2:**249, **2:**672
chemotaxis involvement by, **2:**645–647
FOXO transcription factor regulation by, **3:**84
mRNA turnover and, **3:**287
p110 catalytic subunit of, **3:**305
pleckstrin homology domain recognition of, **2:**163
protein kinase B effects, **2:**163
Phosphatidylinositol 3-kinase related protein kinase family of proteins
architecture of, **1:**558
ataxia-telangiectasia-related protein, **1:**558–560
characteristics of, **1:**557
description of, **1:**557
DNA-dependent protein kinase catalytic subunits, **1:**557, **1:**559
members of, **1:**557–558
mTOR, **1:**558
nucleic acids and, **1:**560
SMG-1, **1:**559–560
TRRAP, **1:**560
Phosphatidylinositol 3-OH kinase-related kinase, **3:**94
Phosphatidylinositol 3-phosphate
description of, **2:**177
effectors of, **2:**177–178
FYVE domain. *see* FYVE domains
localization of, **2:**179
membrane trafficking role of, **2:**177–178
metabolism of, **2:**182
Phosphatidylinositol 5-phosphate, **2:**129–131
Phosphatidylinositol phosphate 4-kinase, **2:**156
Phosphatidylinositol phosphate 5-kinase
Arf6-induced activation of, **2:**211
overexpression of
actin comet formation induced by, **2:**210–211
actin stress fiber formation secondary to, **2:**211
description of, **2:**210
signal transduction mediated by, **2:**156
Phosphatidylinositol 4-phosphate 5-kinases
description of, **2:**123
type I
α, **2:**126
β, **2:**125

Arf family regulation of, **2:**125
casein kinase I and, **2:**125
characteristics of, **2:**123
cloning of, **2:**123
function of, **2:**126
isoforms of, **2:**126
localization of, **2:**125
phosphatidic acid effects on, **2:**125
phosphorylation of, **2:**125–126
properties of, **2:**123–125
regulation of, **2:**125–126
structure of, **2:**123–124
substrate specificity of, **2:**124–125
type II
α isoform, **2:**130
β isoform, **2:**130
γ isoform, **2:**130
description of, **2:**129
extracellular factors that affect, **2:**130
function of, **2:**131–132
history of, **2:**129
isoforms of, **2:**130
kinase domain of, **2:**130
membrane receptor interactions with, **2:**130–131
models of, **2:**131–132
phosphatidylinositol-5-phosphate use by, **2:**129–131
phosphorylation of, **2:**131
pulse labeling of, **2:**131
regulation of, **2:**130–131
structure of, **2:**129–130
subcellular localization of, **2:**130
substrate availability for, **2:**131
substrate specificity of, **2:**124–125
Phosphatidylinositol transfer proteins
α, **2:**225–227
β, **2:**225–227
amino acid sequence of, **2:**227
description of, **2:**225
function of, **2:**226
location of, **2:**226
mechanism of action, **2:**226
murine studies, **2:**227
RdgB family of, **2:**227
synthesis site for, **2:**226
Phosphatidylinositol 3,4,5-triphosphate
Arf GTPase-activating proteins regulated by, **2:**205–206
Arf guanine nucleotide exchange factors regulated by, **2:**205
description of, **1:**671
Phosphatidylserine, **3:**397
Phospho-dependent docking protein, **1:**382
Phosphodiesterase-5
allosteric sites in, **2:**449
catalytic domain of, **2:**448–449
cGMP sequestration by, **2:**467–469
description of, **2:**447
dimerization of, **2:**450
expression of, **2:**447–448
gene organization of, **2:**447
inhibitors of, **2:**449
regulatory domain of, **2:**448–450
structure of, **2:**448
Phosphodiesterase-6
γ subunit, **2:**453–455
activated, **2:**454–455
catalytic, **2:**454–455, **2:**525
cGMP sequestration in rod photoreceptor cells by, **2:**466–467
description of, **2:**453
GAF domains in regulation of, **2:**455, **2:**525–526
holoenzyme, **2:**466
light-induced activation of, **2:**466–467
nonactivated, **2:**454–455
in photoreceptor cells, **2:**466–467, **2:**525
structure of, **2:**453–454
subcellular localization of, **2:**453–454
subunits of, **2:**453–454
Phosphodiesterase(s)
activation of, **2:**562
A-kinase anchoring proteins and, **2:**433
cAMP-specific
catalytic domains of, **2:**438
cGMP-inhibited, **2:**443–444
description of, **2:**437–438, **2:**441, **2:**461
expression of, **2:**439
PDE2, **2:**442–443
phosphorylation-related regulation of, **2:**439
regulation of, **2:**439
regulatory domains of, **2:**438
structure of, **2:**437–438
subcellular targeting of, **2:**438–439
in T cells, **3:**550
catalytic core of, **2:**441
characteristics of, **2:**525
compartmentalization of, **2:**433
description of, **1:**233, **2:**431
expression of, **2:**433
functions of, **2:**429
future of, **2:**434
GAF domains, **2:**432, **2:**449–450, **2:**525–526
gene families, **2:**431–433, **2:**525
history of, **2:**431
inhibitors of, **2:**434
localization of, **2:**433
N-terminus domains of, **2:**432, **2:**441
in *Paramecium,* **2:**537
PDE1, **2:**442
PDE2, **2:**442–443, **2:**525
PDE4, **2:**434
PDE5. *see* Phosphodiesterase-5
PDE6. *see* Phosphodiesterase-6
PDE10, **2:**444
PDE11, **2:**444
PDE2A, **2:**526–528
PDE10A, **2:**526
PDE11A, **2:**526
PDE4D3, **2:**438–439
photoreceptor. *see* Photoreceptor phosphodiesterase
retinal
description of, **2:**578
α-gustducin activation of, **2:**657
subfamilies, **2:**431–433, **2:**525
therapeutic uses of, **2:**433–434
in *Trypanosoma brucei,* **2:**540–541
variants, **2:**433

Phosphofructokinase, **1:**618
Phosphoinositide 1, **2:**163
Phosphoinositide 3-kinases
  activation of, **3:**560–561
  B-cell adaptor for, **3:**560
  binding domains of
    FYVE, **2:**136–137, **2:**158
    PX, **2:**136, **2:**158
  CD28 mediation of, **2:**343
  cellular responses, **2:**137–138
  chemotaxis control by, **2:**218–220
  class I, **2:**135
  class II, **2:**136
  D-3 lipid-binding proteins, **2:**136–137
  description of, **1:**513, **2:**135, **2:**157–158
  Dictyostelium directional movement controlled by, **2:**218
  domain structure of, **2:**136
  Drosophila studies, **2:**139
  effector proteins regulated by, **2:**137–138
  enzymes associated with, **2:**135–136
  FYVE domain of, **2:**136–137, **2:**158
  γ-, **2:**154–155
  genetics of, **2:**139
  G-protein coupled receptor signaling and, **2:**593
  inhibitors of, **2:**193
  insulin responses and, **2:**139
  isoforms, **2:**135
  lipid second messengers, **2:**154–156
  lipid-binding domains of, **2:**136–137
  mTOR regulation by, **1:**528–529
  overexpression of, **2:**193
  phosphatase and tensin homology deleted on chromosome 10 effects, **2:**138
  phosphatidylinositol phosphorylation by, **2:**136
  phosphorylation of, **1:**513
  Pleckstrin homology domains, **2:**136
  products of, **2:**136
  PTEN antagonism of, **2:**115
  PX domains, **2:**136, **2:**158
  Rac activation by, **2:**203–204
  Ras family GTPases modulated by, **2:**206
  regulatory modes of, **2:**135
  structure of, **2:**136
  substrate binding, **2:**155–156
Phosphoinositide-binding domains
  ENTH, **2:**156–157
  FERM, **2:**157
  pleckstrin homology domains, **2:**157
  Tubby C-terminal DNA-binding domain, **2:**157
Phosphoinositide-dependent kinase-1
  description of, **2:**138
  phosphorylation of, for protein kinase C maturation, **2:**188
3-Phosphoinositide-dependent protein kinase 1. *see* PDK1
Phosphoinositide(s)
  actin-binding proteins affected by, **2:**209
  cdc42 guanine nucleotide exchange factor binding, **2:**204
  Cdc42 interactions with, **2:**203
  cytoskeletal protein regulation by, **2:**209
  Rac and, **2:**203
  rho GTPase interactions with, **2:**203
Phosphoinositide-specific phospholipase C
  description of, **2:**156
  phosphatidylinositol phosphate 4-kinase hydrolysis by, **2:**156
  phosphatidylinositol phosphate 5-kinase hydrolysis by, **2:**156
Phospholamban, **2:**58–59
Phospholipase A_2
  activation of, **2:**263
  arachidonate generated by, **2:**263
  arachidonic acid release by, **2:**261, **2:**265
  C2 domain of, **1:**12
  C_2 domain of, **2:**96
  calcium-dependent ($cPLA_2$), **2:**262
  cellular function of, **2:**262–263
  classification of, **2:**262
  cytosolic, **1:**12, **2:**156
  cytosolic calcium-independent ($iPLA_2$), **2:**262–263
  description of, **1:**3, **2:**261
  family members, **2:**261
  groups, **2:**261–262
  lipid second messengers produced by, **2:**156
  phospholipid cleavage by, **2:**261
  secreted ($sPLA_2$), **2:**262–263
  signal transduction mechanisms, **2:**262–263
Phospholipase C
  activation mechanisms, **2:**5–7
  anatomy of, **2:**5
  calcium release and, **2:**248
  chemokine binding-related activation of, **1:**151
  knockout mice studies of, **2:**8
  nuclear function role of, **2:**230–231
  phosphatidylinositol biphosphate hydrolysis by, **1:**3
  physiology of, **2:**7–8
  plasma membrane association, **2:**6
  PLC-β
    description of, **2:**5–6
    Gβγ subunit and, **2:**640
    G-protein coupled receptors effect on, **2:**5
    signal transduction, **2:**6
  PLC-δ, **2:**7
  PLC-ε
    characteristics of, **2:**7
    G-protein βγ subunit and, **2:**641
    N-terminus of, **2:**7
  PLC-γ
    activation of, **3:**558–560
    characteristics of, **2:**6
    diacylglycerol production by, **3:**560
    fibroblast growth fibroblast receptor binding, **2:**862
    growth factor-dependent activation of, **2:**6
    isoforms, **2:**6
  sphingosine 1-phosphate receptor signaling and, **2:**248–249
  subfamilies of, **2:**5
Phospholipase Cβ, **1:**130
Phospholipase Cδ1, **2:**96
Phospholipase Cγ, **1:**268
Phospholipase D
  activators of, **2:**239
  Arf family regulation of, **2:**239, **2:**728
  C-terminus, **2:**237–238
  description of, **2:**237
  diacylglycerol production by, **2:**240, **2:**243
  domain structure of, **2:**237–238
  enzymatic cleavage by, **2:**238
  family members, **2:**238

future studies of, **2:**241
G-protein-coupled receptors and, **2:**240
isozymes, **2:**237
localization of, **2:**241
mammalian, **2:**239
modifications of, **2:**239
phosphatidic acid released by, **1:**3, **2:**249
phosphorylation of, **2:**239
physiological function of, **2:**240
PIP_2 regulation of, **2:**240
pleckstrin homology domain of, **2:**239
protein kinase C regulation of, **2:**239, **2:**392
PX domain, **2:**174
regulatory inputs for, **2:**239
regulatory pathways of, **2:**240
Phospholipids
C_2-domain binding, **2:**98–99
description of, **1:**331
enzyme catalysis and, **2:**119
phosphatidic acid's role in metabolism of, **2:**237
radiation-induced turnover of, **3:**260
research of, **2:**119
ultraviolet light exposure effects, **3:**260
Phosphonodifluoromethyl phenylalanine, **1:**679
Phosphorylated state of myosin light chain, **1:**502
Phosphorylation
acetylation and, **3:**93–94
calpain, **2:**106–107
CREB, **3:**116
effects of, **1:**379
eIF4B, **3:**346
eIF-4E, **3:**344
eIF4G, **3:**346
G protein
cGMP-dependent protein kinase in, **2:**611
description of, **2:**609, **2:**665
epidermal growth factor receptor, **2:**611
insulin receptor in, **2:**611
p21-activated protein kinase, **2:**610–611
protein kinase C, **2:**609–610
serine, **2:**609–611
Src tyrosine kinases in, **2:**611
tyrosine, **2:**611
histidine kinases, **1:**564
histone
acetylation and, **3:**93–94
apoptosis and, **3:**94–95
cellular processes associated with, **3:**95–96
chromatin structure alterations and, **3:**91
DNA repair and, **3:**94
gene activation and, **3:**91–94
mitosis and, **3:**95
hypoxia-inducible factor 1, **3:**278
IκBs, **3:**109
insulin-mediated, **2:**443
multisite, **1:**382–383
Notch intracellular domain, **3:**152
phosphatidylinositol 4-phosphate 5-kinases
type I, **2:**125–126
type II, **2:**131
phospholipase D isozymes, **2:**239
protein kinase C regulation by, **1:**552–553, **2:**188–189
protein kinase-mediated, **1:**183
protein tyrosine phosphatases, **1:**656
p90RSK, **2:**197
receptor protein tyrosine phosphatases regulated by, **1:**686–687
Phospho-serine/threonine phosphorylated proteins
14-3-3 proteins, **1:**410, **1:**505–506
description of, **1:**505
forkhead-associated domains, **1:**506–507
leucine-rich repeats, **1:**508
summary of, **1:**508–509
WD40 domains, **1:**508
WW domains, **1:**507–508
Phosphotyrosine binding domain, **1:**380
Phosphotyrosyl phosphatase activator, **2:**410
Photobleaching, **2:**307–308
Photocadherins, **2:**890–891
Photoreceptors
Drosophila studies, **1:**349–351
inactivation/noafterpotential D protein in, **1:**349–351
light responsiveness of, **1:**351
Phototransduction
definition of, **1:**349
Drosophila studies, **1:**349
inactivation/noafterpotential D protein signaling complexes in, **1:**351
Phox domain. *see* PX domain
Phylogenetic profile of protein, **1:**16
Pib1, **2:**181
Pib2, **2:**181
Piccolo/aczonin, **2:**98
PIF pocket, **2:**197
PI3K. *see* Phosphoinositide 3-kinases
Pik3ca, **1:**412
Pik3cb, **1:**412
PIKfyve, **2:**181
$p16^{ink4a}$, **2:**711
Pins, **2:**572–573
PIP_2. *see also* Phosphatidylinositol 4,5-bisphosphate
actin polymerization and, **2:**209–210
actin-membrane linkers localized or activated by, **2:**211–212
α-actinin binding, **2:**211
calcium-induced ligand scrambling regulated by, **2:**213
cytoskeleton control by, **2:**210
description of, **2:**209
phosphoinositide 3-kinase activation and, **3:**560–561
phosphoinositide phosphatase manipulation effects, **2:**211
talin activation by, **2:**212
PIP5K. *see* Phosphatidylinositol phosphate 5-kinase
Piston model, of integrin signaling, **1:**125
PITPs. *see* Phosphatidylinositol transfer proteins
Pitx2, **3:**464
PIX, **2:**204
$p27^{kip1}$, **3:**551
Placenta growth factor, **1:**285
Placental lactogen, **1:**242
Plakoglobin, **2:**893
Plasma membrane
description of, **2:**57
G protein localization in
$G\alpha_z$ subunit, **2:**602
reversible palmitoylation's role in, **2:**653

Plasma membrane *(continued)*
Ras proteins at
activation of, **2:**677
drugs that affect binding, **2:**678
stress signals caused by, **3:**180
Plasma proteins, **2:**850
Plasticity
erythropoietin receptors, **1:**254
Fc, **1:**51–54
phosphatase's role in, **2:**400–401
Platelet basic protein, **1:**150
Platelet-activating factor, **2:**261
Platelet-derived growth factor
Abl kinase activated by, **3:**252
ASAP/ACAP Arf GTPase-activating proteins and, **2:**205
B chain, **1:**397–398
C chain, **1:**397–398
cellular responses induced by, **1:**401
A chain, **1:**397–398
D chain, **1:**397–398
description of, **1:**286, **1:**397
dimerization induced by, **1:**399
isoforms, **1:**397
lung development and, **3:**510
mitogen-activated protein kinase activation by, **1:**423
Rac activation by, **2:**701
Sertoli cell production of, **3:**533
sphingosine kinase recruitment by, **2:**248
vascular endothelial growth factor and, similarities between, **1:**287
Platelet-derived growth factor receptors
α-
description of, **1:**397, **1:**401
in vivo signaling, **2:**845–846
mutations of, **2:**847
β-
description of, **1:**393–394, **1:**397, **1:**399
in vivo signaling, **2:**845–846
mitogenesis activated by, **2:**846
angiogenesis and, **1:**455
autophosphorylation of, **1:**645
characteristics of, **1:**391, **1:**397–399
description of, **1:**286, **1:**362
homodimerization, **1:**393
integrins and, **1:**402
intracellular kinase domain of, **1:**397–398
intracellular signaling by, **2:**847
ligand-induced phosphorylation, **1:**402, **1:**645
proteins associated with, **1:**399–401
SH2 domains and, **1:**393
Shp2 and, **1:**401, **1:**716
signaling
cell-cycle progression and, **1:**401
in vivo, **2:**845–847
in mouse development, **2:**845–847
pathways for, **1:**400–401
regulation of, **1:**401–402
types of, **1:**286
tyrosine phosphorylation, **1:**399
Platelet(s)
calpain-induced activation of, **2:**108
sphingosine 1-phosphate storage by, **2:**248
PLC1, **2:**230
plc1, **2:**230
Plcb3, **2:**7
Pleckstrin homology domains
C-terminal, **2:**161
DAPPP1, **2:**162
Db family
characteristics of, **2:**752–753
description of, **2:**751
regulation of, **2:**754
definition of, **2:**161
description of, **2:**11, **2:**136
electrostatic sidedness, **2:**161
Golgi targeting of, **2:**166
identification of, **2:**161
membrane recruitment of, **2:**166
non-phosphoinositide ligand binding, **2:**165–166
nonspecific phosphoinositide binding by, **2:**165
N-terminal, **2:**161–162
phosphatidylinositol 4,5-bisphosphate binding, **2:**162–163, **2:**193
phosphatidylinositol 3-kinase recognition by, **2:**163
phosphoinositide binding by, **2:**157, **2:**161–165
phospholipase D, **2:**239
protein kinase B, **1:**514–515, **2:**194–195
PtdINs(3,4)P_2 binding, **2:**163
PtdINs(3,4,5)P_3 binding, **2:**163
structure of, **2:**161
Pleiotropic drug resistance, **3:**369
Pleiotropin, **2:**869
Plexins, **2:**878
Plk 1, **1:**695
P-loop, **1:**541, **1:**664
PMCA pump
calmodulin regulation of, **2:**59
discovery of, **2:**59
genetic diseases involving defects of, **2:**60
inhibitors of, **2:**57–58
isoforms of, **2:**59
reaction cycle of, **2:**57–**2:**59
regulation of, **2:**59–60
$p75^{NTR}$, **1:**281–283, **2:**839, **3:**488–489, **3:**596–597
Pocket factor, **1:**95
Polo-like kinase, **3:**87
Poly(ADP-ribose)polymerases, **2:**613
Polyomavirus middle tumor antigen, **2:**410
Polyomavirus small tumor antigen, **2:**410
Polypeptide growth factors, **1:**2
Polyubiquitylation, **1:**484
Position specific scoring matrix, **2:**312
Postsynaptic density, **2:**397, **2:**400
Postsynaptic-density-95/Discs-large/ZO1, **1:**349
posttranslational control of, **3:**100–101
Potassium channels
calcium-dependent flux in, **1:**227
carbon monoxide effects, **3:**578
description of, **1:**204–205
G-protein-gated inwardly rectifying, **2:**639–640, **2:**664, **2:**667
KcsA, **1:**215
opening of, **1:**206
small-conductance Ca^{2+}-activated
biophysical profiles, **1:**228
Ca^{2+} ions, **1:**228–229

calmodulin's role in gating, **1:**228–229
cDNAs, **1:**227–228
chemomechanical gating model for, **1:**230
clones encoding, **1:**227–228
description of, **1:**227
flux, **1:**227
gating mechanisms, **1:**228–230
pharmacological profiles, **1:**228
structure of, **1:**215
PP1. *see* Protein phosphatase 1
pp185, **1:**409
PP2A. *see* Protein phosphatase 2A
PP2B, **1:**603
PP2C
in *Arabidopsis thaliana,* **1:**638
in *Bacillus subtilis,* **1:**639
cystic fibrosis transmembrane conductance regulation chloride channel controlled by, **1:**638
description of, **1:**591, **1:**604, **1:**637, **1:**703
eukaryotic, **1:**637
plant hormone abscisic acid signaling by, **1:**638
sexual dimorphism role of, in nematodes, **1:**638–639
stress-activated mitogen-activated protein kinase cascades regulated by, **1:**637–638
PP1G/R_{GL} effects, **1:**614
PP1/Hck complex, **1:**584
$p47^{phox}$, **2:**705
$p67^{phox}$, **2:**705, **2:**747–748
PP-$InsP_4$, **2:**234
PPM
characteristics of, **1:**594
description of, **1:**603–604
PPP family vs., **1:**604
sequences of, **1:**604
structure of, **1:**604
PPP family
amino acid sequences, **1:**597–598
catalytic activities of, **1:**594–596, **1:**601–602
catalytic domain of, **1:**594, **1:**602
characteristics of, **1:**593–594
dephosphorylation catalyzed by, **1:**602
description of, **1:**703
domain organization of, **1:**596–598
evolution of, **1:**594
features of, **1:**594
medical importance of, **1:**598
Ppp2, **1:**596–597
Ppp5, **1:**598
Ppp6, **1:**598
Ppp7, **1:**598
Ppp1c, **1:**597, **1:**602
regulatory subunit interactions, **1:**603
structure of, **1:**601–602, **1:**604
subfamilies, **1:**596–598
subunit structure, **1:**597–598
PR48, **2:**411
$p21^{Ras}$, **2:**691
Pref-1, **3:**42
Pregnane X receptors
description of, **2:**289, **3:**21
ligands for, **3:**54–55
Pre-ligand-binding assembly, **1:**278
Prenylated proteins, **1:**332
Prenylation
alternative, **2:**739–740
definition of, **2:**737
G-protein γ subunit, **2:**586–587
Presenilin-1, **2:**895, **3:**162
Presynaptic inhibition, **3:**386
P-Rex, **2:**204
p190RhoGAP, **1:**423
Priming, **3:**382–383
PRL-3, **1:**647
PRMT1, **3:**145, **3:**147
PRMT2, **3:**145
PRMT3, **3:**145
PRMT4, **3:**145
PRMT5, **3:**145, **3:**147
PRMT6, **3:**145
Proangiogenic factors, **3:**456
Probabilistic models, **1:**18
Pro-caspase 9, **2:**353
Procaspase 8, **1:**275
Progesterone, **3:**23
Progesterone receptor
description of, **3:**35
PR-A, **3:**35
PR-B, **3:**35
Programmed cell death. *see* Apoptosis
Prolactin, **3:**595
Prolactin hormone
binding energetics, **1:**244–245
binding sites for, **1:**243–244
cross-reactivity of, **1:**243
description of, **1:**241
extracellular domains, **1:**243–244
growth hormone receptor interactions, **1:**244
rPRL-R, **1:**244
specificity of, **1:**243
Prolactin hormone–receptor complex, **1:**242
Prolactin receptor
description of, **1:**24
structure of, **1:**256
Proliferating cell nuclear antigen, **3:**198, **3:**340
Proneural enhancement, **3:**154
Proneurotrophins, **3:**487
Prostacyclin, **2:**267
Prostaglandin D_2, **2:**268
Prostaglandin E_2, **2:**268–269
Prostaglandin $F_2\alpha$, **2:**269
Prostaglandin G/H synthase, **2:**265
Prostaglandin J_2, **2:**268
Prostaglandin mediators
cyclooxygenase-1
amino acid sequence of, **2:**265
expression of, **2:**265
thromboxane A_2 production by, **2:**266–267
cyclooxygenase-2
amino acid sequence of, **2:**265
deletion of, **2:**265
expression of, **2:**265
patent ductus arteriosus and, **2:**265
prostacyclin production by, **2:**267
description of, **2:**265

Prostaglandin mediators *(continued)*
prostaglandin D_2, **2:**268
prostaglandin E_2, **2:**268–269
prostaglandin $F_2\alpha$, **2:**269
thromboxane A_2, **2:**266–267
Prostaglandin(s), **3:**577–578
Prostanoids
G-protein coupled receptors for, **2:**266
production of, **2:**265
receptors, **2:**266
Prostate gland
adult, **3:**594–597
aging and
cAMP signaling pathway, **3:**598–599
description of, **3:**591
growth factor signaling, **3:**599
steroid hormones, **3:**597–598
apoptosis, **3:**595–597
benign prostatic hyperplasia of, **3:**597
cancer of, **3:**598–600
cellular renewal in, **3:**594
description of, **3:**591
development of
androgens in, **3:**592
description of, **3:**592
estrogens in, **3:**594
fibroblast growth factors involved in, **3:**592
inhibitors of, **3:**593
insulin-like growth factors in, **3:**592
mitogenic factors involved in, **3:**592–593
vascular endothelial growth factor in, **3:**593
fibroblast growth factor effects, **3:**592, **3:**594
insulin-like growth factors effect, **3:**592, **3:**595
mitogenic signals, **3:**594
neuroendocrine cells of, **3:**596
proliferation control in, **3:**594–595
transforming growth factor β effects, **3:**595
Protease signaling, **2:**351
Protease-activated receptors
activation of, **1:**167–169
cleavage of, **1:**168
description of, **1:**167
endothelial, **1:**171
exofacial domains of, **1:**168
family of, **1:**169
in vivo role of, **1:**169, **1:**171
PAR1, **1:**167
PAR2, **1:**169
PAR3, **1:**169, **1:**171
PAR4, **1:**169, **1:**171
properties of, **1:**170
sensory neuron activation, **1:**171
SFLLRN peptide, **1:**167–168
thrombin, **1:**167–168
Proteasomes
signaling function of, **2:**352
20S, **2:**349
26S, **2:**349
Protein. *see also specific protein*
arginine methylation. *see* Arginine methylation
carbohydrate interactions with, **1:**91
ceramide interactions with, **2:**259
chemosensing, **1:**18
degradation of, **3:**129
ERM, **2:**211
flagellar, **1:**16, **1:**18
function of
computational methods for inferring, **1:**16
genomic context and, **1:**15–18
homology methods, **1:**16, **1:**18
nonhomology methods, **1:**16, **1:**18
functional linkages, **1:**16, **1:**18
FYVE domain-containing, **2:**179, **2:**181
genomic context of, **1:**15–16
glycogen synthase kinase 3 phosphorylation of, **1:**547
immunoglobulin superfamily, **1:**58
ion channel, **1:**203
lipid modifications of, **1:**332
lipid rafts, **1:**324, **1:**333–334
phospho-dependent docking, **1:**382
phylogenetic principles, **1:**16
platelet-derived growth factor receptors, **1:**399–401
PPI-actin binding regulation of, **2:**212–213
prenylated, **1:**332
PX domain-containing, **2:**172
scaffold, **2:**330–331, **2:**360
ubiquitination, **1:**383, **2:**349
ubiquitin–proteasome system processing of, **3:**129, **3:**131–132
ubiquitin-related, **2:**347
voltage-gated calcium channel sensing in, **1:**209–210
Protein A, **1:**52–53
Protein acetyltransferase, **1:**333
Protein binding
electrostatic interactions in, **1:**11
lipophilic modification, **1:**12
principles of, **1:**11–12
Protein complex
association of, **1:**30
dissociation of, **1:**30–31
Protein Data Bank, **1:**24–25
Protein dephosphorylation, **1:**591
Protein disulfide-bond isomerases, **3:**311
Protein export
fibroblast growth factor, **3:**393–394
interleukin-1, **3:**396–397
nonclassical pathways, **3:**393–398
studies of, **3:**393
Protein G, **1:**52–53
Protein inhibitors of activated signal transducers and activators of transcription
arginine methylation effects, **3:**146
effects of, **1:**434
family of, **1:**433
mechanism of action, **1:**433–434
PIAS1, **1:**433
PIAS3, **1:**433
signal transducers and activators of transcription interactions with, **1:**433
Protein inhibitors of signal transducers and activators of transcription, description of, **1:**347, **1:**433
Protein kinase
activation loop, **1:**443, **1:**541
adenosine triphosphate binding site, **1:**541

AMP-activated
description of, **1:**531, **1:**535
medical uses of, **1:**536
regulation of, **1:**535–536
structure of, **1:**535
α-subunits, **1:**535
atypical
ChaK
catalytic domain of, **1:**568–569
characteristics of, **1:**568
description of, **1:**568
hydrophobic ATP-binding pocket of, **1:**569
kinase domains of, **1:**568–569
protein kinase A vs., **1:**569
regulation of, **1:**571
description of, **1:**567
domain organization of, **1:**568
EF2 kinase, **1:**567–568
calcium/calmodulin and, **1:**570
cloning of, **1:**568
description of, **1:**567
p70 S6K effects, **1:**571
regulation of, **1:**570–571
functions of, **1:**571–572
identification of, **1:**567–568
regulation of, **1:**570–571
structure of, **1:**568–570
substrate specificity of, **1:**570
autoinhibitory mechanisms, **1:**443
cadherins, **2:**891
calcium-dependent, **1:**377
cAMP-dependent. *see* Protein kinase A, cAMP-dependent
cancer-related activation of
chromosomal translocations, **1:**444–445
gene amplification and overexpression, **1:**445
mutation, **1:**445–446
upstream regulators, **1:**446
catalysis by, **1:**539
Cbl proteins effect on, **1:**483–484
cGMP-dependent
activation of, **2:**480
biochemistry of, **2:**479–481
cAMP cross-activation of, **2:**545–546
β-catenin phosphorylation by, **2:**483
cell function roles of, **2:**481–482
description of, **2:**479
inhibitors of
ATP binding site-targeted, **2:**488–491
cyclic nucleotide binding site-targeted, **2:**488
description of, **2:**487–488
peptide binding site-targeted, **2:**491
properties, **2:**489–490
isoforms of, **2:**479–481
knockout mice studies of, **2:**481
mitogen-activated protein kinase regulation by, **2:**482
myosin light chain phosphatase binding subunit, **2:**481–482
peptide substrates of
acceptor loci, **2:**496
description of, **2:**495
optimum recognition sequences, **2:**496–498
phosphorylation sites, **2:**497–498
recognition of, **2:**495–496
specificity of, **2:**498
physiologic roles of, **2:**481–483
protein tyrosine phosphatase regulation by, **2:**482
Rp-cyclic nucleotide phosphorothioates, **2:**488
smooth muscle cell functions of, **2:**480
specificity of, **2:**498
substrates
peptide, **2:**495–498
physiological, **2:**502, **2:**507–508
type I, **2:**479–480
type Iα, **2:**480–481
type Iβ, **2:**481
type II, **2:**479, **2:**481
Csk, **1:**477
description of, **1:**483, **1:**539
DNA damage checkpoints and, **3:**406
downregulation of, by polyubiquitylation, **1:**484
engineering of, **1:**583–586
eukaryotic
Caenorhabditis elegans, **1:**375–376
catalytic domain of, **1:**373–374
comparative kinomics, **1:**376–377
discovery of, **1:**373
Drosophila melanogaster, **1:**375
Homo sapiens, **1:**375–376
nematodes, **1:**376
Saccharomyces cerevisiae, **1:**374–375
Schizosacharomyces pombe, **1:**374–375
structure of, **1:**373
flanking segments, **1:**541–542
function of, **1:**387
helix C, **1:**541
improper activation of, **1:**387
inactivated, **1:**388–390
inhibitors
adenosine triphosphate competitive types of, **1:**455
Akt inhibitors and, **1:**457–458
cancer prevention uses of, **1:**456–457
chemistry of, **1:**452–453
description of, **1:**451–452
development of, **1:**452–453
diagnostic uses of, **1:**457
discovery of, **1:**451
non-cancer diseases treated with, **1:**456
pro-apoptotic agents and, **1:**455–456
substrate-competitive, **1:**455
inhibitors of, **1:**583
intramolecular mechanisms that affect, **1:**443–444
mitogen-activated protein kinase-related, **2:**368–369
N-terminal lobe of, **1:**442–444, **1:**540
oncogenic
activation of
chromosomal translocations, **1:**444–445
gene amplification and overexpression, **1:**445
mutation, **1:**445–446
treatment for, **1:**446–447
upstream regulators, **1:**446
treatment for, **1:**446–447
p21-activated, **1:**499–501, **2:**409, **2:**610–611
physiological regulation of, **1:**441–444
P-loop, **1:**541

Protein kinase *(continued)*
regulation of, **1:**441–444
allosteric, **1:**540
intrasteric, **1:**540–541
sites, **1:**541–542
retrovirus activation of, **1:**444
serum-induced, **2:**193
SH2 domains, **1:**380
Src, **1:**475–476
structure of, **1:**387–388, **1:**539–540
substrate binding site, **1:**541
substrates for, **1:**583
ZAP-70, **1:**477–478
Protein kinase A
A-kinase anchoring proteins subcellular targeting of
AKAP15/18α, **2:**379–380
AKAP75/79/150, **2:**379
cellular functions of, **2:**380
description of, **2:**377, **2:**390, **2:**596
determinants, **2:**378–379
domains of, **2:**378–379
hydrophobic interactions, **2:**378–379
structure, **2:**378–379
targeting domains, **2:**379–380
C subunit isoforms, **2:**378
cAMP-dependent
activation, **1:**614, **1:**627, **1:**631, **2:**326, **2:**330, **2:**377, **2:**383, **2:**419, **2:**471, **3:**598
catalytic domain of, **2:**497
catalytic subunits of, **2:**471–474
cGMP cross-activation of, **2:**546
CRH-induced adrenocorticotropic hormone release mediated by, **3:**379
D/D domain, **2:**475
description of, **2:**495, **2:**596
discovery of, **2:**471
domain structure of, **2:**487–488
exocytosis secretion and, **3:**384
inhibitors of
ATP binding site-targeted, **2:**488–491
cyclic nucleotide binding site-targeted, **2:**488
description of, **2:**487–488
peptide binding site-targeted, **2:**491
properties, **2:**489–490
motifs, **2:**501–502
peptide substrates of
acceptor loci, **2:**496
description of, **2:**495
optimum recognition sequences, **2:**496–498
phosphorylation sites, **2:**497–498
recognition of, **2:**495–496
specificity of, **2:**498
phosphorylation sites, **2:**501–502, **3:**91
regulatory subunits, **2:**471, **2:**474–475
specificity of, **2:**498
T-cell proliferation and, **3:**551
catalytic domain of, **1:**569, **2:**497
ChaK vs., **1:**569
C-terminal residue of, **2:**198
Cubitus interruptus regulation by, **3:**169
cytosolic form of, **2:**461
description of, **1:**518
holozymes, **2:**378
inactive, **2:**460
isoforms, **1:**586
localization of, **1:**617
motifs, **2:**501–502
N-terminal lobe, **1:**568
phosphorylation sites, **2:**501–502
R subunits, **2:**378
substrates
description of, **2:**501
peptide
acceptor loci, **2:**496
description of, **2:**495
optimum recognition sequences, **2:**496–498
phosphorylation sites, **2:**497–498
recognition of, **2:**495–496
specificity of, **2:**498
phosphorylation sites, **2:**501–502
physiological, **2:**502–507
in *Trypanosoma brucei,* **2:**541
Protein kinase A binding proteins, **1:**553
Protein kinase B
description of, **1:**513
isoforms of, **1:**513–514, **2:**193
mechanism of activation of, **1:**513–514, **2:**193–194
murine studies, **1:**515–516
PDK1 activation of, **1:**514–515, **1:**517, **2:**194–195
phosphatidylinositol 3-kinase and, **2:**163, **2:**194
phosphoinositide 3-kinase effects, **1:**513–514
pleckstrin homology domain of, **1:**514–515, **2:**194–195
Protein kinase C
activation loop, **1:**552
activation of, **2:**120, **2:**390–392, **2:**430, **2:**606
atypical isoforms, **2:**120
C1 domain, **2:**187
C2 domain, **2:**187
calcineurin-mediated activation of, **1:**634
catalytic domain of, **1:**387–388
classical isoforms, **2:**120
degradation of, **2:**393
dephosphorylation of, **1:**552–553, **2:**188–189, **2:**393
description of, **1:**3
diacylglycerol activation of, **1:**369, **2:**187, **2:**390–391, **3:**260–261, **3:**550
discovery of, **1:**551, **2:**119, **2:**187, **2:**389
domains of, **1:**552, **2:**187
exocytosis role of, **3:**385–386
family members, **1:**551–552, **2:**187–188, **2:**389
function of, **1:**554, **2:**190
G protein phosphorylation by, **2:**609–610
gastrointestinal hormone-stimulated signal transduction and, **3:**480–481
history of, **2:**187
inactivation of, **2:**392–393
inhibitors of, **1:**458
inositol phospholipid hydrolysis and, **2:**120
ion channel interactions with, **2:**391
isoforms of, **1:**517, **1:**554, **2:**120, **2:**197
isozymes, **1:**551–552, **2:**187–188
knockout mice studies of, **2:**392
lipid-mediated translocation of, **2:**121
maturation of, **1:**552–553, **2:**188–189

mRNA turnover and, **3:**286
novel isoforms, **2:**120
nuclear receptor corepressors and, **3:**31
pathways for, **2:**392
PDK1 interactions with, **2:**390
PDZ domain, **2:**390
phosphoinositide-dependent kinase-1 effects on, **2:**188
phospholipase D regulation by, **2:**239, **2:**392
priming, **2:**389–390
protein tyrosine kinase signaling controlled by, **1:**395
receptor for activated, **2:**165, **2:**391–392
regulation of
anchoring proteins, **1:**553, **2:**189
description of, **2:**188
membrane translocation, **1:**553, **2:**189
model for, **1:**553–554, **2:**189–190
phosphorylation/dephosphorylation, **1:**552–553, **2:**188–189
second messengers involved in, **2:**189–190
Rho effected by, **2:**748–749
structure of, **1:**387–388, **2:**120
substrates, **2:**392, **2:**609
summary of, **1:**554
targeting of, **2:**390
translocation, **2:**121
transmembrane protein interactions with, **2:**391
tyrosine phosphorylation of, **2:**390
Protein kinase Cα
C_2 domain of, **2:**96
dephosphorylated, **2:**389
integrin-PKCα-ezrin association, **2:**392
phospholipid binding mechanism, **2:**98–99
protein phosphatase 2A interaction with, **2:**409
synedecan interactions with, **2:**391
Protein kinase Cβ, **1:**12, **2:**96
Protein kinase Cδ
C_2 domain of, **2:**96
protein phosphatase 2A interaction with, **2:**409
Protein kinase Cε, **2:**96
Protein kinase inhibitor, **2:**471, **2:**474, **2:**491
Protein kinase-mediated phosphorylation, **1:**183
Protein phosphatase 1
α, **2:**398
β, **2:**398
γ_1, **2:**398
binding motif, **2:**397–398
catalytic subunits, **1:**613, **2:**397–398
characteristics of, **1:**613–614
description of, **1:**603, **1:**613
endoplasmic reticulum targeting, **1:**617–618
evolution of, **1:**614
GADD34, **1:**618
glycogen targeting subunits, **1:**614–616
holoenzymes of, **1:**618
I-2 deactivation of, **1:**629
inhibitors of, **1:**603, **1:**614, **1:**627–628
isoforms of, **2:**398
membrane targeting subunits of, **1:**617
modulators of, **1:**614
myosin targeting subunits of, **1:**616–617
nuclear inhibitor of, **1:**629
nuclear targeting subunits of, **1:**617
$PP1_c$, **1:**613, **1:**616–618, **2:**398, **2:**400
ribosome targeting, **1:**617–618
targeting proteins, **2:**398–399
targeting subunits of, **1:**614–616
Protein phosphatase 5, **2:**410
Protein phosphatase 2A
Aα mutations, **1:**623
Aβ mutations, **1:**623
A subunit, **1:**621
alteration of, **1:**622
axin dephosphorylation by, **1:**623
B subunit, **1:**621
C subunit, **1:**621
cancer development and, **1:**622–625
catalytic subunits of, **2:**405
β-catenin and, **1:**623–624
cellular proteins and, **1:**622
composition of, **2:**405
core enzyme, **1:**621
description of, **1:**591, **1:**603, **1:**621, **2:**405
forms of, **1:**621
holoenzyme, **1:**621
inhibition of, **1:**622, **1:**629
interacting proteins with, **2:**412
mitogen-activated kinase pathway and, **1:**624
phosphorylase *a* dephosphorylated by, **1:**627
structure of, **1:**621–622
subunits
B, **1:**621
C, **1:**621
catalytic, **2:**405
description of, **1:**621–622, **2:**405–406
functions of, **2:**406
nomenclature of, **2:**406
R2, **2:**407, **2:**410
R3, **2:**410–411
R5, **2:**411–412
Wnt signaling and, **1:**623–624
Protein phosphatase methylesterase, **2:**410
Protein phosphatase(s)
ceramide-activated, **2:**258
description of, **1:**369, **1:**591
inhibition of, **1:**394
PP1. *see* Protein phosphatase 1
PP2A. *see* Protein phosphatase 2A
PPP. *see* PPP
types of, **1:**703
tyrosine. *see* Protein tyrosine phosphatases
Protein phosphorylation, **2:**879, **2:**894–895
Protein tyrosine kinase
Syk, **1:**477–478
Tec, **1:**478
Protein tyrosine kinase receptors
activation of, **1:**393
antagonists for, **1:**395
autophosphorylation of, **1:**393
control of, **1:**394–395
cross-talk between signaling pathways, **1:**395
C-terminal tail, **1:**393
degradation, **1:**394
description of, **1:**391
disease-related activation of, **1:**395

Protein tyrosine kinase receptors *(continued)*
epidermal growth factor receptor. *see* Epidermal growth factor receptor
genes that encode, **1:**391
heterodimerization, **1:**393
homodimerization, **1:**393
insulin receptor. *see* Insulin receptor
internalization, **1:**394
ligand-induced dimerization of, **1:**392–393
mechanism of activation, **1:**392–394
overactivity of, **1:**395
platelet-derived growth factor receptors. *see* Platelet-derived growth factor receptors
signaling, **1:**394–395
subfamilies of, **1:**391–392
Protein tyrosine phosphatases
amino acid sequences of, **1:**654, **1:**662, **1:**677
Asp residue, **1:**641
background of, **1:**641
bioinformatics of, **1:**661–668
catalytic domains of, **1:**653, **1:**663, **1:**687
cGMP-dependent protein kinase regulation of, **2:**482
characteristics of, **1:**591, **1:**653, **1:**677
chromosomal localization of genes, **1:**665, **1:**668
classical, **1:**642–643, **1:**659
conserved regions of, **1:**662–663
covalent inactivators of, **1:**677–678
cysteine nucleophile, **1:**655
description of, **1:**399, **1:**591, **1:**653, **1:**729
discovery of, **1:**641
diseases and, **1:**646–647, **1:**667–668
function of, **1:**643–644
genomes, **1:**662
genomic complement of, **1:**665–667
high-molecular-weight, **1:**733
human genome, **1:**665–666
hydrogen peroxide-mediated inactivation of, **2:**114–115
inhibitors of
bidentate, **1:**679–681
covalent, **1:**677–678
description of, **1:**664, **1:**677
miscellaneous types, **1:**680–681
oxyanions, **1:**678–679
pTyr surrogates, **1:**679
interferon-α signaling suppressed by, **3:**525
intracellular, **1:**662
intron/exon structures, **1:**665
knockout mice studies of, **1:**645–646
loop of, **1:**641, **1:**664
low-molecular-weight
activators of, **1:**736–737
amino acid sequence alignments of, **1:**734
biological role of, **1:**737
bovine, **1:**733, **1:**735–736
catalytic mechanisms, **1:**735–736
characteristics of, **1:**733
cysteine residues, **1:**736
description of, **1:**604, **1:**654
diethyl pyrocarbonate inactivation of, **1:**736
discovery of, **1:**733
inhibitors of, **1:**736–737
isoenzymes, **1:**737
phosphate binding loop of, **1:**734
pyridoxal phosphate binding to, **1:**736
regulation of, **1:**737
structure of, **1:**735
substrate specificity of, **1:**737
vascular endothelial growth factor and, **1:**737
in yeast, **1:**737
mechanisms of, **1:**655
membrane-proximal domains of, **1:**662
mRNA, **1:**662
nonconserved residues, **1:**664–665
nontransmembrane, **1:**642, **1:**662
novel, **1:**666–667
osteotesticular, **1:**666
oxidation of, **1:**644–645
pharmaceutical uses of, **1:**646
phosphoprotein classification of, **1:**644
phosphorylation of, **1:**656
phosphotyrosyl residue specificity of, **1:**642
phylogenetic analysis of, **1:**662
primary sequence alignments, **1:**663
protein sequences, **1:**662
protein tyrosine kinases and, **1:**644
pseudogenes
dead-phosphatases vs., **1:**741
description of, **1:**665–666
PTP1B
characteristics of, **1:**668, **1:**680–681, **1:**729–730
crystal structure of, **1:**645
description of, **1:**641, **1:**729
gene polymorphisms, **1:**730
inhibitors of, **1:**665, **1:**681
insulin receptor dephosphorylated by, **1:**729
insulin signaling and, **1:**729
insulin-mediated modulation of, **1:**730
substrates, **1:**730–731
PTP-ER, **1:**704
reactive oxygen species effects, **1:**401–402
receptor-like
crystal structure of, **1:**664
D2 domains, **1:**664, **1:**687–688, **1:**744–745
description of, **1:**642, **1:**644
dimerization of, **1:**685–686
extracellular domain of, **1:**685
oxidative stress effects, **1:**688
phosphatase activity of, **1:**685
phosphorylation of, **1:**686–687
regulation of, **1:**685–688
RPTPα, **1:**686
SH2 domain, **1:**686
structure of, **1:**685
tandem domain, **1:**662, **1:**664
regulation of, **1:**643–644, **1:**656, **1:**704, **1:**709–710
schematic representation of, **1:**660
sequence alignments, **1:**662–663
signal transduction and, **1:**646
signature motif of, **1:**641–642, **1:**653, **1:**655, **1:**663, **1:**677
structure of, **1:**641–642, **1:**653–655
substrate
specificity of, **1:**645–646, **1:**654, **1:**719
trapping, **1:**671–674
subtypes of, **1:**662

thiol-specific alkylating agents effect on, **1:**677–678
trapping mutants, **1:**672, **1:**674
tyrosine phosphorylation-dependent signaling, **1:**644–645
vertebrate sequences, **1:**662
WPD loop, **1:**664
Protein–ligand interactions, **2:**302–303
Protein–protein interactions
affinity chromatography for, **2:**302–303
analysis of, **2:**293
binding affinity, **1:**27
calpain activated by, **2:**107
categories of, **1:**42
coprecipitation experiments
antibodies, **2:**297
considerations for, **2:**295–296
epitope tags for, **2:**297–298
glutathione S-transferase-tagged proteins, **2:**296–297
description of, **1:**12, **1:**27
domains of, **1:**380–381, **2:**171
free energy landscape of, **1:**28–29
hydrophobic effect in, **1:**40
immunoglobulin G-superfold-mediated, **1:**59
kinetics of, **1:**28–29
mass spectrometric approaches, **2:**298–299
microcystin-sepharose chromatography for studying, **2:**302
nuclear transport regulation by, **3:**125
p38 mitogen-activated protein kinases, **3:**516–518
peptide recognition modules for studying, **2:**311–315
phosphorylation effects, **1:**379–380
phosphotyrosine-dependent, **1:**379–380
recognition, **1:**23–25
SH3 domain, **2:**313
sphingosine kinase activated by, **2:**20
thermodynamics of, **1:**27–28
transcription factor modulation by, **3:**64
transition state, **1:**29–30
Proteoglycans, **1:**90
Proteomics
definition of, **2:**301
γ-phosphate linked ATP-sepharose, **2:**301–302
proteome isolation, **2:**301–302
p90RSK, **1:**517, **2:**197
PS-20, **3:**593
4PS, **1:**414
PSD-95, **2:**330, **3:**443
P-selectin, **1:**88
P-SMAC, **2:**342
P-smads, **2:**835
Ptc gene, **2:**796
PtdIns(3)(P). *see* Phosphatidylinositol 3-phosphate
PtdIns(3,4,5)P_3, **1:**513–514, **2:**163
PTEN. *see* Phosphatase and tensin homology deleted on chromosome 10
Ptp-2, **1:**712
PTP1B
characteristics of, **1:**668, **1:**680–681, **1:**729–730
crystal structure of, **1:**645
description of, **1:**641, **1:**729
gene polymorphisms, **1:**730
inhibitors of, **1:**665, **1:**681
insulin receptor dephosphorylated by, **1:**729
insulin signaling and, **1:**729
insulin-mediated modulation of, **1:**730
substrates, **1:**730–731
PTPN11, **1:**668, **3:**467
pTyr
description of, **1:**664
surrogates
protein tyrosine phosphatase inhibition by, **1:**679
structure of, **1:**680
Puc phosphatase, **2:**785
Pumilio, **3:**328
Purinergic nucleotides, **3:**581
Purinoceptors, **3:**581
Pvf1, **1:**397
PX domains
Arg residue, **2:**173
classification of, **2:**171
history of, **2:**171
ligand-binding specificity of, **2:**171
overview of, **2:**171
phosphoinositide 3-kinases, **2:**136, **2:**158
phosphoinositide specificity of, **2:**171
phospholipase D, **2:**174
$p40^{phox}$Px:PtdIns(3)P structure, **2:**173
proteins with
function of, **2:**174
properties of, **2:**172
in *Saccharomyces cerevisiae,* **2:**174
sequence alignment of, **2:**171, **2:**173
sorting nexins, **2:**171–172
structure of, **2:**171–174
p38y, **2:**367–368
Pygopus, **3:**161, **3:**164
Pyridoxal phosphate, **1:**736

Q

Q mutant, **1:**199

R

R2, **2:**407, **2:**410
R3, **2:**410–411
R5, **2:**411–412
Rab1, **2:**691
Rab5
description of, **2:**691, **3:**413
rho GTPases and, **3:**416–417
Rab23, **2:**794
Rab proteins
description of, **2:**689
disease and, **2:**692
domains, **2:**690
dysfunction of, **2:**692
effectors of, **2:**691–692
GDI recycling of, **2:**692
GDP-bound state conversion, **2:**691–692
GTPases and, **2:**689–690
guanine nucleotide exchange factors and, **2:**691
localization of, **2:**691
posttranslational modification of, **2:**691
sequence alignment of, **2:**690
β-strands of, **2:**691

Rab proteins *(continued)*
structural organization of, **2:**690–691
subfamilies of, **2:**690
Rab3A, **2:**691
Rab27a, **2:**692
Rabenosyn-5, **2:**179–181
Rabip-4, **2:**180–181
Rac
cell cycle progression and transformation, **2:**712–713
cell migration and, **2:**701–702, **2:**851
description of, **1:**499
downstream effectors of, **2:**703
effectors of, **2:**747
function models of, **2:**707–708
guanine nucleotide exchange factor, **2:**203
NADPH oxidase regulation by, **2:**706–709
phosphoinositide interactions with, **2:**203–204
platelet-derived growth factor activation of, **2:**701
Rac2, **2:**707
semaphorin response mediated by, **2:**878
sequence alignment of, **2:**746
signaling pathways, **2:**702
Rac1, **3:**415, **3:**561
rac-GAP, **2:**326
RACKs. *see* Receptors for activated c-kinases
Rad9, **3:**200
Rad17, **3:**199
Rad24, **3:**198
Rad52, **3:**227
Rad53, **3:**200
RAD17, **3:**208
RAD24, **3:**208
RAD53, **3:**208
rad9, **3:**206
Rad53 kinase, **3:**199
Radiation
cytoplasmic signaling induced by
activation methods, **3:**259
overview of, **3:**257–258
toxic agents that activate, **3:**258
DNA damage caused by
Drosophila melanogaster studies
apoptosis, **3:**214, **3:**216
cell cycle arrest secondary to, **3:**213–215
description of, **3:**213
DNA damage, **3:**213
DNA repair, **3:**214
effectors, **3:**213–214
sensors, **3:**213
transmitters, **3:**213
sensing of, **3:**226–227
p53 activation by, **3:**241–242
phospholipid turnover secondary to, **3:**260
Radical fringe, **2:**820–821
RAF-1, **2:**409, **2:**677
Raf-MAP kinase, **2:**671–672
Rafts
description of, **1:**315–316, **1:**319
lipid
epidermal growth factor receptor localization to, **1:**323–325
proteins, **1:**324
Ral-guanine nucleotide dissociation, **2:**737
Ran
cellular asymmetry of, **3:**424–425
characteristics of, **2:**695
conformational changes of, **3:**424
C-terminal element of, **2:**757
definition of, **2:**695
mitotic progression and, **2:**697
nuclear import of, **3:**425
nuclear transport role of, **2:**696–697
nucleotide exchange factor, **2:**695–696
postmitotic nuclear assembly role of, **2:**697–698
spindle assembly function of, **2:**697
structure of, **2:**695–696, **3:**424
summary of, **2:**698
RanBP1, **2:**695–696
RanBP2, **2:**695
RanGAP1, **2:**695
RanGTP, **2:**695, **3:**423
RANK ligand, **3:**498
Rap1
cAMP-binding guanine nucleotide exchange factors for. *see* Epacs
cellular functions, **2:**524
description of, **1:**338
Rap1 GAP
$G\alpha_o$ and, **2:**605
$G\alpha_z$ and, **2:**602–603
Rapamycin
target of. *see* TOR
treatment uses of, **1:**458, **1:**523, **1:**525
Rapid signal transduction, **1:**115, **1:**130
RAR. *see* Retinoic acid receptors
Ras association domain, **2:**758–759
Ras signaling
ERK1 as target of, **2:**365
ERK2 as target of, **2:**365
Ras superfamily proteins, **1:**127
Ras-binding domain, **2:**758
RasGAP, **1:**400, **1:**402, **2:**759–760
Ras/Ras proteins
activation of, **1:**401
from brain. *see* Rab proteins
cancer and, **2:**671–673
cell surface movement, **2:**675–677
cyclin D1's role in transformation of, **2:**672
cytosolic, **2:**675
description of, **2:**681, **2:**737–738
effectors of, **2:**745
farnesyl transferase inhibitors and, **2:**740
G domain, **2:**757
GTPase reaction for, **2:**759
guanine nucleotide exchange factor interactions with, **2:**677, **2:**682, **2:**758
H-
amino acid sequence of, **2:**682
description of, **2:**675–676
farnesyl transferase inhibitors effect on, **2:**739
prenylation of, **2:**739
inhibitors of, **1:**458
K-, **2:**739
kinase suppressor of, **2:**295, **2:**369

localization of, **2:**676, **2:**738
M-, **2:**686–687
mutations, **2:**671
N-, **2:**675–676
palmitoylation, **2:**676
pathways downstream of, **2:**671–672
plasma membrane
activation at, **2:**677
drugs that affect binding, **2:**678
localization to, **2:**738
properties of, **2:**558–559
R-
amino acid sequence of, **2:**682–683
biochemistry of, **2:**682–683
carboxyl-terminal CAAX tetrapeptide motif, **2:**682, **2:**738
description of, **2:**681
effectors of, **2:**683–684
expression of, **2:**682
GDP/GTP regulation, **2:**684
GTPases, **2:**684
guanine nucleotide exchange factor interactions with, **2:**682
R-Ras-2, **2:**685–686
R-Ras-3, **2:**686–687
signal transduction, **2:**682–685
structure of, **2:**681
TC21, **2:**685–686
Rho GTPases. *see* Rho GTPases
signaling pathways, **2:**684
topology of, **2:**764
trafficking, **2:**676
RCC1, **2:**695–696
RdgB phosphatidylinositol transfer proteins, **2:**227
Reaction coordinate, **1:**28–29
Reactive oxygen species
chemical properties of, **2:**113–114
description of, **1:**401–402, **1:**644
NADPH oxidase production of, **2:**705
sources of, **2:**113–114
Receptor for activated C kinase 1, **1:**339
Receptor protein tyrosine phosphatases
axonal signaling by, **2:**869–870
binding partners of, **2:**869
catenin targeting, **2:**870
description of, **2:**867
downstream signals, **2:**869–870
in *Drosophila,* **2:**867
expression of, **2:**867
in neuromuscular system, **2:**868
substrates, **2:**869
visual system and, **2:**867–868
Receptor tyrosine kinases
activation of, **3:**260
autophosphorylation sites, **1:**300–301
control of, **3:**443–444
description of, **1:**299, **1:**398, **1:**405
insulin, **1:**299–302
insulin-like growth factor 1, **1:**301
ligand-binding domains, **1:**389
mutation activation of, **1:**445
radiation-induced activation of, **3:**260
residues, **1:**299–300
retrovirus activation of, **1:**444
Shp2's role in signaling of, **1:**714, **1:**716–717
signaling
cell surface, **3:**441
endocytic compartments, **3:**441–443
specificity of, **2:**846
Src family of, **1:**389–390
types of, **1:**299
Tyr972, **1:**300
Receptor–ligand interactions
factors that affect, **1:**21–22
local environment effects, **1:**21–22
stabilization of, **1:**22–23
Receptor-like cytoplasmic kinases, **1:**579–580
Receptor-like kinases, **1:**579
Receptor-operated calcium channels, **2:**52
Recoverins, **2:**80–81
Redox sensing, **3:**194
Regulated nuclear transport
description of, **3:**125
transcription factors, **3:**125–127
Regulators of G protein signaling
A-subfamily, **2:**634
B-subfamily, **2:**634–635
C-subfamily, **2:**635
description of, **1:**335, **2:**566–567, **2:**602
domain structure of, **2:**634
D-subfamily, **2:**636
E-subfamily, **2:**636
F-subfamily, **2:**636
function of, **2:**634
Gα interactions with
description of, **2:**634–637
palmitoylation effects, **2:**654
GEF-subfamily, **2:**636
GRK-subfamily, **2:**637
G-subfamily, **2:**637
H-subfamily, **2:**637
overexpression of, **2:**633
palmitoylation sites, **2:**651
RA-subfamily, **2:**636
R4-subfamily, **2:**634–635
R7-subfamily, **2:**635–636
R12-subfamily, **2:**636
RZ-subfamily, **2:**634
SNX-subfamily, **2:**637
Rel proteins, **2:**781
Rel transcription factors, **3:**62
RelA, **2:**408
Relenza, **1:**109
Renin, **3:**580
RENT complex, **1:**698
Replicative senescence, **3:**244
Resensitization, **1:**181, **1:**184
Response regulator, **1:**563–565
Retinal degeneration mutant phenotype type B. *see* RdgB
Retinoblastoma protein, **2:**711, **3:**251, **3:**551
Retinoic acid, **2:**724
Retinoic acid receptors, **3:**29
Retinoid X receptor, **3:**39, **3:**48
Retrograde regulation
aging and, **3:**371
definition of, **3:**365

Retroviral oncogenesis, **1:**444
Retroviral transduction, **1:**444
Retroviruses, **1:**444
Reverse signaling, **1:**424
rfc-4-2, **3:**208
RGS-box, **2:**631–634
18*R*-HEPE, **2:**283–284
Rheumatoid factors, **1:**52
Rhinoviruses
  classification of, **1:**95
  description of, **1:**95
  intercellular adhesion molecule-1 binding to, **1:**95–97
  low-density lipoprotein receptor binding to, **1:**95
  receptor sites of, **1:**95–96
  very-low-density lipoprotein receptor binding to, **1:**95–97
  viral coat proteins, **1:**95, **1:**97
Rho activation, **1:**465
Rho associated kinase, **2:**703
Rho GTPases
  actin cytoskeleton and
    cell migration, **2:**701–702, **2:**851
    description of, **2:**701, **3:**294
    remodeling, **3:**414–416
    signaling, **2:**702–703
  actin:myosin filament assembly, **2:**702
  Cdc42. *see* Cdc42
  cell cycle progression and transformation, **2:**712–713
  cyclin D1 expression and, **2:**713
  deactivation of, **3:**416
  description of, **1:**499, **2:**203, **2:**701
  DH domain, **2:**758
  effectors of, **2:**747–749
  Eph receptors signaling through, **1:**422–423
  focal adhesion assembly and, **2:**701
  functions of, **2:**701
  guanosine nucleotide exchange factors
    allosteric regulation of, **2:**753
    DB family. *see* DB family
    description of, **2:**751
    external regulation of, **2:**754
    modulation of, **2:**754
    nucleotide exchange mechanisms, **2:**753
    pleckstrin homology domains, **2:**753
  neuronal morphology and guidance regulated by, **2:**702
  oncogenesis and, **2:**751
  p120ctn function affected by, **2:**893
  phagocytosis and, **2:**702
  phosphoinositide interactions with, **2:**203
  protein kinase effector proteins of, **1:**500
  Rab5 and, **3:**416–417
  Ras vs., **2:**745
  signaling pathways, **2:**702
  structural features of, **1:**501
  substrates, **2:**753–754
  targets of
    CRIB proteins, **2:**745–747, **2:**759
    description of, **2:**745
    protein kinase C, **2:**747–749
Rho kinase
  autoinhibitory region of, **1:**502
  characteristics of, **1:**5023
  description of, **1:**499
RhoB, **2:**737, **2:**740
RhoB-GG, **2:**740
Rhodopsin
  activation of, **1:**142
  amino-terminal tail of, **1:**140
  characteristics of, **1:**139–140
  crystal structure of, **1:**139
  cytoplasmic domain of, **1:**141
  description of, **1:**178
  extracellular loops, **1:**158–159
  G-protein catalysis, **2:**453
  interhelical loops of, **1:**140
  ligand-binding pocket of, **1:**139
  membrane-embedded domain of, **1:**140
  molecular structure of, **1:**140–141
  R* transition, **1:**141–142, **2:**453
  solvent-accessible surface area analysis, **1:**156
  transmembrane helices, **1:**142
  transmembrane segments of, **1:**155
Rhodopsin kinase, **1:**182
RhoGAP, **2:**759–760
Rho-GEF, **2:**562
Rhomboid, **2:**352
Ribosomal S6 kinase, **1:**524
Ribotoxic stress response, **3:**260
Rickets, **3:**500
RIM, **2:**99
RIP 140, **3:**31
RNA polymerase II
  description of, **3:**11
  general transcription factors
    covalent modification regulation of, **3:**16
    description of, **3:**11
    loss of, **3:**14
    negative control of, **3:**16
    preinitiation complex. *see* RNA polymerase II, preinitiation complex
  phosphorylation of, **3:**14
  preinitiation complex
    downstream promoter element, **3:**13
    gene-specific regulation of, **3:**14–16
    global mechanisms of, **3:**13–14
    mediator function, **3:**15–16
    nonstandard core promoters, **3:**13
    promoter clearance, **3:**14
    promoter melting, **3:**13–14
    schematic diagram of, **3:**12
    structural organization of, **3:**11–13
    transcriptional activators that regulate, **3:**14–16
  subunits, **3:**12
  TATA box binding protein-associated factors, **3:**11, **3:**13
RNA-binding proteins
  description of, **3:**335
  NF90 family of. *see* NF90
  signaling by, **3:**335
ROCK. *see* Rho associated kinase
RPB1, **3:**11
RPB2, **3:**11
Rp-8-B-cAMPS, **2:**551
Rp-cyclic nucleotide phosphorothioates, **2:**488
RsbP, **1:**639

R-Smads
characteristics of, **1:**487–489, **2:**557, **3:**171–172
degradation of, **3:**173
RTg1p, **3:**367–368
RTg2p, **3:**367–368
RTg3p, **3:**367–368
Ruthenium red, **2:**73–74
Ryanodine receptors
calcium release channels, **2:**46–47
cardiac diseases and, **2:**47–48
central core disease and, **2:**47
description of, **2:**32, **2:**41, **2:**52
FKBP and, **2:**47
function of, **2:**45
genes, **2:**47–48
isoforms, **2:**45
malignant hyperthermia and, **2:**47
molecular biology of, **2:**47–48
regulation of, **2:**47
RYR1, **2:**45–46
RYR2, **2:**45
RYR3, **2:**45
structure of, **2:**45–46
RYR1, **2:**47
RYR2, **2:**47
RYR3, **2:**47

S

S4
coupling gating to, **1:**213
gating current generated by, **1:**212
helical screw motion of, **1:**212–213
positive charges, **1:**211
residues in, **1:**211–212
sequence and charge pairing, **1:**210–211
transmembrane motion in, **1:**211
S45, **3:**162
20S proteasome, **2:**349
26S proteasome, **2:**349
S100 proteins
A1, **2:**91
A2, **2:**91
A3, **2:**88
A4, **2:**91
A8, **2:**91
A9, **2:**91
Alzheimer's disease and, **2:**91
B, **2:**88
cellular compartment localization of, **2:**87, **2:**92
chromosomal localization of, **2:**89–90
C-terminal EF-hand, **2:**87
description of, **2:**87
diseases and, **2:**91–92
exons of, **2:**89
extracellular levels of, **2:**87
family members, **2:**87, **2:**91
functions of, **2:**90
genes, **2:**87–88
genomic organization of, **2:**89–90
nomenclature of, **2:**90
N-terminal EF-hand, **2:**87
paracrine effects of, **2:**92
phylogenetic tree of, **2:**89–90
secretion of, **2:**90
size of, **2:**87
structure of, **2:**88
summary of, **2:**92
target binding of, **2:**88–89
translocation of, **2:**90
zinc binding, **2:**89
S100A13, **3:**394–396
Saccharomyces cerevisiae. see also Yeast
cdc42 expression in, **2:**715
cell cycle arrest in, **3:**204–205
CIT2 expression in, **3:**367
description of, **1:**374–375, **1:**523, **1:**637
DNA damage studies in, **3:**204
dynamin in, **2:**767
GTPase studies in, **2:**733–735
heterotrimer G protein signaling in, **2:**571–572
life cycle of, **2:**358
mitogen-activated protein kinases in, **1:**703–704, **2:**358–359
NF90 function in, **3:**340
nuclear pore complex in, **3:**426
PX domain-containing proteins in, **2:**174
replicative aging in, **3:**371
SH3 domain of, **2:**312
transcription profiles of, after exposure to damaging agents, **3:**182
unfolded protein response in, **3:**312
S-adenosylmethionine, **3:**145
SAPK, **1:**494–495
SARA, **2:**182–183
Sarcoplasmic reticulum, **2:**102
Scaffold proteins
anchor-. *see* Anchoring proteins
description of, **2:**330–331, **2:**360, **2:**369
ft-arrestin, **2:**370–371
G-protein coupled receptor signaling and, **2:**595–596, **3:**444
JIP, **2:**370, **2:**595–596
JSP-1, **2:**371
KSR, **2:**369, **2:**595
MPI, **2:**370
nomenclature of, **2:**370
SKRP1, **2:**371
Scaffolding adapters, **1:**708
SCAP. *see* SREBP cleavage-activating protein
Scar/WAVE, **2:**324–326, **2:**384, **2:**703
SCF complex, **3:**130–131
Schizosacharomyces pombe
cdc14, **1:**699–700
description of, **1:**374–375
Scissors model, of integrin signaling, **1:**125
Second messengers
C1 domains and, **2:**159
characteristics of, **2:**153
description of, **2:**153
enzyme recognition of, **2:**153–156
inositol polyphosphate 5-phosphatase production of, **2:**156
non-phosphoinositide recognition of, **2:**159
phosphatase and tensin homology deleted on chromosome 10 production of, **2:**156
phosphatidylinositol phosphate 4-kinase production of, **2:**156

Second messengers *(continued)*
phosphatidylinositol phosphate 5-kinase production of, **2:**156
phosphoinositide 3-kinases, **2:**154–156
phosphoinositide-binding domains
ENTH, **2:**156–157
FERM, **2:**157
pleckstrin homology domains, **2:**157
Tubby C-terminal DNA-binding domain, **2:**157
phospholipase A_2 production of, **2:**156
sequestration of, **2:**465
taste transduction, **2:**660
Second mitochondrial activator of caspases. *see* SMAC
Secretagogues, exocytosis
calcium influx regulation by, **3:**380
properties of, **3:**377
signaling, **3:**387
target cell receptor functions of, **3:**377–378
types of, **3:**376
γ-Secretase, **2:**352
Secreted phospholipase A_2, **2:**262–263
Secretory granules, **3:**377
SEL-10, **3:**153
Selective estrogen receptor modulator, **3:**37
Semaphorins
central nervous system development role of, **2:**877
classes of, **2:**877
definition of, **2:**877
family members, **2:**872–873
immune system signaling, **2:**879
intracellular signaling pathways, **2:**878–879
neuropilins, **2:**872, **2:**877–878
plexins, **2:**878
Rac mediation of, **2:**878
receptors for, **2:**877–878
Sema3A, **2:**878
Sema4D, **2:**877
Seminiferous crods, **3:**533
Sensor histidine kinase, **1:**563
Sensor proteins, **2:**67
Separase, **2:**352
24*(S),*25-Epoxycholesterol, **3:**54
Sequestration, **1:**182
Ser82, **3:**43
Ser142, **3:**116
Ser216, **3:**407
Ser241, **2:**196
Ser304, **2:**131
Ser315, **1:**700
Ser376, **3:**242
Ser378, **3:**242
Ser473, **2:**194
Ser535, **1:**547
Ser831, **2:**400
Ser^{2809}, **2:**47
SERCA pump
discovery of, **2:**58
genetic diseases involving defects of, **2:**60
inhibitors of, **2:**57–58
isoforms, **2:**58
N domain, **2:**58
P domain, **2:**58
phospholamban interactions with, **2:**58–59
reaction cycle of, **2:**57–58
regulation of, **2:**58–59
structure of, **2:**58–59
Serine kinase GSKβ, **3:**152
Serine palmitoyltransferase, **2:**257
Serine/threonine kinase
description of, **1:**387
inhibitors of, **1:**457–458
PAK family of, **2:**703
signaling pathways, **1:**457
Serine/threonine kinase receptors
characteristics of, **1:**362, **1:**364
description of, **1:**2
interacting proteins, **1:**490
Serine/threonine phosphatases
background of, **1:**593
catalytic sites of, **1:**602
catalytic subunits, **1:**593
classification of, **1:**593
description of, **2:**405
family of, **2:**405
FCM, **1:**594
naturally occurring inhibitors of
calyculin A, **1:**608–609
cantharidin, **1:**609
cell-based experiments of, **1:**607, **1:**609
chemical synthesis of, **1:**609
description of, **1:**607
fostriecin, **1:**607–609
limitations, **1:**607
microcystin-LR, **1:**608–609
okadaic acid, **1:**608
structure of, **1:**607
tautomycin, **1:**608
toxin binding, **1:**609
PP1
description of, **1:**603
inhibition of, **1:**603
PP2B. *see also* Calcineurin
description of, **1:**591, **1:**603
inhibition of, **1:**629
suppression of, **1:**629
PP2C
in *Arabidopsis thaliana,* **1:**638
in *Bacillus subtilis,* **1:**639
cystic fibrosis transmembrane conductance regulation chloride channel controlled by, **1:**638
description of, **1:**591, **1:**604, **1:**637, **1:**703
eukaryotic, **1:**637
plant hormone abscisic acid signaling by, **1:**638
sexual dimorphism role of, in nematodes, **1:**638–639
stress-activated mitogen-activated protein kinase cascades regulated by, **1:**637–638
PPM
characteristics of, **1:**594
description of, **1:**603–604
PPP family vs., **1:**604
sequences of, **1:**604
structure of, **1:**604
PPP family
amino acid sequences, **1:**597–598
catalytic activities of, **1:**594–596, **1:**601–602

catalytic domain of, **1:**594, **1:**602
characteristics of, **1:**593–594
dephosphorylation catalyzed by, **1:**602
description of, **1:**703
domain organization of, **1:**596–598
evolution of, **1:**594
features of, **1:**594
medical importance of, **1:**598
Ppp2, **1:**596–597
Ppp5, **1:**598
Ppp6, **1:**598
Ppp7, **1:**598
Ppp1c, **1:**597, **1:**602
regulatory subunit interactions, **1:**603
structure of, **1:**601–602, **1:**604
subfamilies, **1:**596–598
subunit structure, **1:**597–598
protein phosphatase 2A
Aα mutations, **1:**623
Aβ mutations, **1:**623
A subunit, **1:**621
alteration of, **1:**622
axin dephosphorylation by, **1:**623
B subunit, **1:**621
C subunit, **1:**621
cancer development and, **1:**622–625
β-catenin and, **1:**623–624
cellular proteins and, **1:**622
core enzyme, **1:**621
description of, **1:**591, **1:**603, **1:**621
forms of, **1:**621
holoenzyme, **1:**621
inhibition of, **1:**622, **1:**629
mitogen-activated kinase pathway and, **1:**624
structure of, **1:**621–622
subunits, **1:**621–622
Wnt signaling and, **1:**623–624
type 2, **1:**629
Sertoli cells, **3:**531–533
Serum response elements, **2:**711, **3:**100
Serum response factor
definition of, **2:**711
mitogen-induced activation of, **2:**713
Serum-induced protein kinase, **2:**193
Set-binding factor 1, **1:**746
Seven-pass transmembrane cadherins, **2:**891
Sex combs reduced, **2:**408
SFLLRN peptide, **1:**167–168
SGK, **1:**518
SGK1, **2:**197
SG2NA, **2:**410
SH2 domain
binding proteins, **3:**251
description of, **1:**380
docking of signaling proteins, **1:**393–394
inositol phosphatases, **1:**513
Itk, **1:**478
receptor protein tyrosine phosphatases, **1:**686
Shps, **1:**708–709
SH3 domain, **2:**313, **3:**250–251
SH2 domain-containing inositol 5-phosphatases. *see* SHIP
Shank, **2:**331
Shc, **1:**382, **2:**408, **2:**592
Shh signaling, **2:**884, **2:**886
SHIP
-1. *See* SHIP1
-2. *See* SHIP2
chemotactic role of, **2:**647
description of, **2:**147
s-, **2:**148–149
SHIP1
α isoform, **2:**147–148
description of, **2:**147
expression of, **2:**148–149
function of, **2:**149
hematopoietic cell expression of, **2:**148–149
isoforms, **2:**147–148
knockout mice studies of, **2:**149
NPNY motif, **2:**147
NPXY motifs, **2:**147
SHIP2 vs., **2:**150
structure of, **2:**147–148
SHIP2
cellular expression of, **2:**150
C-tail region of, **2:**150
description of, **2:**147
expression of, **2:**150
function of, **2:**150
SHIP1 vs., **2:**150
structure of, **2:**148–150
tissue expression of, **2:**150
tyrosine phosphorylation of, **2:**150
shotgun gene, **2:**890
Shp1
CD46 binding, **1:**720
cloning of, **1:**707
C-tail, **1:**707, **1:**710
death receptors and, **1:**713
deficiency of, **1:**720
description of, **1:**329, **1:**395, **1:**431–432
diseases and, **1:**647
erythropoietin signaling regulated by, **1:**432
expression of, **1:**708
isoforms, **1:**708
JAK–STAT signaling pathway deactivated by, **1:**347
lymphocyte signaling regulated by, **1:**714
natural killer cell signaling regulated by, **1:**714
neutrophil signaling affected by deficiency of, **1:**713
phagocytosis and, **1:**713
point mutations in, **1:**711
signaling pathways
bone marrow macrophage studies of, **1:**712–713
description of, **1:**712
in epithelial cells, **1:**714
in erythroid cells, **1:**714
lymphocytes, **1:**714
in myeloid cells, **1:**712–713
natural killer cells, **1:**714
schematic diagram of, **1:**715
Shp2
catalytic activity of, **1:**656
C-tail, **1:**707
CTLA-4 and, **1:**718
cytokine signaling and, **1:**718

Shp2 *(continued)*
deficiency of, invertebrate models of, **1:**711–712
dephosphorylation by, **1:**716
description of, **1:**358
diseases and, **1:**647, **1:**719–720
expression of, **1:**708
human, **1:**708
inappropriate activation of, **1:**720
integrin signaling and, **1:**718
interferon signaling regulated by, **3:**525
mouse, **1:**708
mutations of, **1:**719–720
Noonan syndrome and, **1:**719–720
N-SH2 domain, **1:**656, **1:**709, **1:**719
platelet-derived growth factor receptor and, **1:**401, **1:**716
Ras signaling, **1:**716
regulation of, **1:**656
reversible oxidation effects on, **1:**711
signaling by, **1:**714–719
specificity of, **1:**719
substrates, **1:**716
T-cell antigen receptor signaling and, **1:**718–719
vertebrate, **1:**712
Shp(s)
biological functions of, **1:**711–712
C-tails
regulation by, **1:**710–711
variations in, **1:**707–708
expression of, **1:**708
history of, **1:**707
motheaten phenotype, **1:**711
nomenclature of, **1:**707
phospholipid's effect on, **1:**710
pTyr ligand added to, **1:**709
regulation of, **1:**709–710
serine–threonine phosphorylation of, **1:**710
SH2 domain function, **1:**708–709
structure of, **1:**707
Siah-1, **3:**162
Sialic acid, **1:**107
Sialic acid binding immunoglobulin G-superfold family, **1:**59
Sic1, **1:**383–384
Side-chain rearrangements, in Fab fragment, **1:**34–35
Siglecs, **1:**89
Signal transducers and activators of transcription. *see also* JAK–STAT signaling
amino-terminal domain of, **3:**79
arginine methylation of, **3:**146
biological responses mediated by, **3:**79
cytokine activation of, **1:**429
definition of, **1:**343
description of, **1:**251, **1:**343, **3:**77
DNA-binding domain of, **3:**79
domains of, **3:**79
family of, **3:**79
function of, **1:**346, **3:**79
hematopoietin signaling by, **3:**77
linker domain of, **3:**79
phosphatases, **1:**433
protein inhibitors of, **1:**347
protein inhibitors of activated STAT and, **1:**433
SH2 domain of, **3:**79
signal decay, **3:**80
Src homology 2 domains, **1:**346
STAT-1, **1:**433
STAT1, **1:**401
STAT5, **2:**408, **3:**631
STAT6, **1:**414
structure of, **1:**346
transcription factor interactions with, **3:**80
Signal transduction. *see also* Cell signaling
Abl in, **3:**252–254
arginine methylation's role in, **3:**147
calcium channels and, **2:**24
calcium-mediated, **1:**369
cytokine receptor activation of, **1:**429
definition of, **2:**557
G-protein coupled receptor endocytosis effects on specificity of, **1:**184–185
initiation of, **1:**361
lipid-derived second messengers in, **1:**369
nuclear receptor coactivators as targets of, **3:**27
phospholipase A_2, **2:**262–263
protein tyrosine phosphatases and, **1:**646
rapid, **1:**115
therapeutic targeting of, **1:**451
transforming growth factor β, **1:**487–490
vascular endothelial growth factor, **2:**856
Signaling
ATM, **3:**231–232
CD45 effects, **1:**690
FYVE domains in, **2:**182–183
hypoxia-inducible factor 1, **3:**277–279
inositol, **2:**229–230
integrin, **1:**123–125
modular interaction domains' role in, **1:**471–473
myelin-associated glycoprotein, **2:**873
phorbol ester and, **2:**120, **2:**190
protease, **2:**351
T-cell receptor, **2:**340
ubiquitin regulation of, **2:**349
Signaling pathways
angiopoietins, **2:**849
Breast, **3:**566–568
carbohydrates and, **1:**87
Cdc14, **1:**698
cdc42, **2:**702
cytokine receptors and, **1:**429
DNA damage, **3:**203
downstream
description of, **1:**471
insulin, **3:**329
modular interactions, **1:**471–473
Ras/Ras proteins, **2:**671–672
receptor protein tyrosine phosphatases, **2:**869–870
sphingosine 1-phosphate receptors, **2:**248–249
endoplasmic reticulum, **3:**263–265
epidermal growth factor receptors, **1:**407
Escherichia coli, **1:**16, **1:**18
fibroblast growth factor receptor, **2:**862–863
insulin receptors, **1:**299
intracellular compartments, **3:**351–352
kidney, **3:**576
LET-23, **2:**805–807

mapping of, **1:**583
mTOR, **1:**528, **1:**558, **3:**300–302
neurotrophin(s), **3:**489–490
platelet-derived growth factor receptors, **1:**400–401
rac, **2:**702
ras/Ras proteins, **2:**684
rho GTPases, **2:**702
serine/threonine kinases, **1:**457
SHP1
bone marrow macrophage studies of, **1:**712–713
description of, **1:**712
in epithelial cells, **1:**714
in erythroid cells, **1:**714
lymphocytes, **1:**714
in myeloid cells, **1:**712–713
natural killer cells, **1:**714
schematic diagram of, **1:**715
Smad-independent, **1:**489–490
T-cell antigen receptor
coreceptors, **3:**548–549
schematic diagram of, **3:**547
toll receptors, **2:**335–336
Signalosomes, **1:**319, **3:**546, **3:**548
Silencing mediator for retinoid and thyroid hormone receptors
description of, **3:**23
dissociation of, **3:**31
purification of, **3:**30
regulatory mechanisms, **3:**30–31
Su(H) interactions with, **3:**150
transcription factors associated with, **3:**30
sim, **3:**154–155
Sis-inducible enhancer, **3:**100
S6K1, **1:**516, **2:**196, **3:**302
SK channels. *see* Small-conductance Ca^{2+}-activated potassium channels
SKIP, **3:**151
SKRP1, **2:**371
Sli-1, **1:**483–484
Slit-ROBO-GTPase activating protein 1, **2:**873
Slits, **2:**873
SMAC
C-, **2:**340, **2:**342, **3:**548
definition of, **2:**354
P-, **2:**342, **3:**548
Smads
bone morphogenetic proteins and, **2:**833
cardiac valve formation, **3:**467
characteristics of, **1:**487–489
Co-, **3:**171
cross-regulation of, **3:**173
degradation of, **3:**173
description of, **1:**487, **3:**171, **3:**442
DNA-binding partners, **1:**488
downregulation of, **3:**173
family of, **3:**171
function of, **3:**173
I-, **3:**171
MH2 domain, **3:**172
oligomerization of, **3:**171–172
p-, **2:**835
phosphorylation regulation of, **3:**173
R-
characteristics of, **1:**487–489, **3:**171–172
degradation of, **3:**173
receptor regulation of, **3:**171–172
signaling pathways, **1:**489–490
Smad4, **1:**489, **2:**182
Smad6, **1:**489
Smad7, **1:**489
transcriptional regulation by, **3:**172–173
transforming growth factor β signaling and, **1:**488–489, **2:**884
ubiquitin–proteasome system and, **1:**489
Small intestine peptides, **3:**478–479
Small nuclear ribonucleoprotein particles, **3:**331
Small soluble saccharides, **1:**90
Small t-antigen, **2:**407
Small ubiquitin-like modifier peptides, **3:**163
Small-conductance Ca^{2+}-activated potassium channels
biophysical profiles, **1:**228
Ca^{2+} ions, **1:**228–229
calmodulin's role in gating, **1:**228–229
cDNAs, **1:**227–228
chemomechanical gating model for, **1:**230
clones encoding, **1:**227–228
description of, **1:**227
flux, **1:**227
gating mechanisms, **1:**228–230
pharmacological profiles, **1:**228
SmD1, **3:**146
SmD3, **3:**146
SMG-1, **1:**559–560
SML1, **3:**208
SMN, **3:**339
Smooth muscle cells, vascular
cyclic GMP-dependent protein kinase expression by, **2:**511
description of, **3:**456
kidney, **3:**579–580
renal, **3:**579–580
vasoconstriction of, **3:**579–580
SMRT. *see* Silencing mediator for retinoid and thyroid hormone receptors
Smurf1, **2:**835
Smurf2, **2:**835
SNAP-25, **3:**382
SNARE proteins, **2:**23, **2:**27, **2:**691, **3:**382–383
SOCS. *see* Suppressors of cytokine signaling
SOCS box, **1:**348
Sodium channels, **1:**216
Sodium dodecyl sulfate polyacrylamide gel electrophoresis, **1:**673
Sodium/calcium exchanger. *see* Na^+/Ca^{2+} exchanger
Sog protein, **2:**834
Soluble adenylyl cyclase, **2:**423
Soluble guanylyl cyclases, **2:**427–428
Soluble N-ethylmaleimide-sensitive factor attachment protein receptor, **1:**296
Solvent-accessible surface area analysis, **1:**156–157
Sonic hedgehog, **2:**800–802, **3:**464
Sorting nexins, **2:**171–172
SOS response of *Escherichia coli*
description of, **3:**185
dinI gene, **3:**185
ending of, **3:**185–186
genes induced by, **3:**187

SOS response of *Escherichia coli (continued)*
LexA repressor, **3:**185–187
posttranscriptional control in, **3:**185
regulation of, **3:**186–187
self-cleavage responses, **3:**186
SoxR protein, **3:**191–193
S1P, **3:**355
Sp1, **2:**408
Spatzle, **2:**779
SPC. *see* Sphingosylphosphorylcholine
Sphingolipid Ca^{2+}-release mediating protein of the endoplasmic reticulum, **2:**21
Sphingolipid(s)
description of, **1:**3, **2:**19
metabolism of, **2:**19
Sphingomyelin cycle-derived ceramide, **2:**258
Sphingosine kinase
activation of, **2:**19–20
cellular distribution of, **2:**20
description of, **2:**19, **2:**247, **3:**393
extracellular stimuli that affect, **2:**20
protein–protein interactions effect, **2:**20
Sphingosine 1-phosphate
angiogenesis by, **2:**590–591
calcium release
description of, **2:**19, **2:**249
intracellular target for, **2:**20–21
description of, **2:**19
extracellular targets of, **2:**19
formation of, **2:**247
G-protein coupled receptors and, **2:**21, **2:**247
signaling by, **2:**248
$S1P_2$, **2:**247
$S1P_3$, **2:**248
sphingosine kinase's role in, **2:**247
Sphingosine 1-phosphate receptors
characteristics of, **2:**247–248
description of, **2:**247
downstream signaling from, **2:**248–249
phospholipase C effects, **2:**248–249
Sphingosine 1-phosphate signaling by, **2:**248
transactivation of, **2:**248
type 1, **2:**248
Sphingosylphosphorylcholine, **2:**20
description of, **2:**253
physiological and pathological functions of, **2:**253
receptors for, **2:**254
Spinal cord
description of, **2:**883
dorsal development, **2:**883–886
dorsoventral axis patterning, **2:**883
rostrocaudal specification of, **2:**886–887
Shh signaling, **2:**884, **2:**886
ventral development, **2:**886
Spindle assembly, **2:**697
Spinophilin, **2:**398–399
Spontaneous transient inward currents, **2:**54
Spontaneous transient outward currents, **2:**54
Spt23, **3:**132
SRB/Mediator, **3:**14–15
Src
description of, **1:**331
membrane binding regions, **1:**12
protein phosphatase 2A interactions with, **2:**409
protein tyrosine kinases, **1:**475–476
Src homology 2 domain, **1:**345, **1:**379–380
Src homology 3 domain, **2:**276
Src kinases
CD45 substrate, **1:**689–690
Eph receptor effects, **1:**422
Gα subunit phosphorylation by, **2:**611
structure of, **1:**389–390
SREBP cleavage-activating protein
description of, **3:**354–355
endoplasmic reticulum retention of, **3:**356
endoplasmic reticulum vesicle sorting of, **3:**355–356
function of, **3:**354
sterol regulatory element binding protein trafficking by, **3:**355
SRp38, **3:**333
SSecKS, **2:**392
s-SHIP, **2:**148–149
Sst2, **2:**572
Ssy1p, **3:**368
STATs. *see* Signal transducers and activators of transcription
Ste4, **2:**640
Ste7, **2:**571
Ste11, **2:**571
Ste18, **2:**640
Stem cell factor, **3:**618–619
Stem cell factor protein complexes, **1:**508
Stem cell factor receptor, **3:**618–619
Stem cell(s), neural
isolation and culture of, **3:**626–628
regulation of differentiation into neurons, **3:**628–631
SOCS2 regulation of, **3:**630–632
Steroid hormone receptors
activation of, **3:**35–36
description of, **3:**35, **3:**62
DNA binding domain of, **3:**35
drug clearance by, **3:**50
hormone response elements, **3:**35
ligand-binding domain of, **3:**35
nongenomic action of, **3:**35
selective modulators, **3:**37
subgrouping of, **3:**35
transcription factor cross-talk with, **3:**35
transcriptional activity of, **3:**36
Steroid hormones
biological effects of, **3:**35
description of, **3:**35
prostate gland aging and, **3:**597–598
Sterol regulatory element binding proteins
activation of, **3:**354
characteristics of, **3:**353–354
cleavage of, **3:**354
definition of, **3:**353
description of, **2:**288–289
endoplasmic reticulum retention of, **3:**356
endoplasmic reticulum vesicle sorting of, **3:**355–356
NH_2-terminal domain of, **3:**354
SREBP1, **3:**48, **3:**353
SREBP2, **3:**353
sterol-mediated regulation mechanisms, **3:**354–355
STICK proteins, **2:**392
Stimulus-secretion coupling, **3:**376–377, **3:**387
Stomach hormones, **3:**477–478

Store-operated calcium channels, **2:**31–32, **2:**52
STRAP, **1:**490
Stress
  aging liver response to, **3:**519–520
  genotoxic agents as cause of, **3:**179
  heat-shock transcription factor activation by, **3:**269–270
Stress fibers, **2:**850–851
Stress response
  aging liver, **3:**519–520
  endoplasmic reticulum, **3:**263–266, **3:**279, **3:**359–362
  FOXO transcription factors, **3:**88
  functional genomics study of, **3:**182
  integrated, **3:**263, **3:**265
  p38 mitogen-activated protein kinase pathway in signaling of, **3:**515–521
  ribotoxic, **3:**260
  transcriptional basis of, **3:**182
Stress signals
  cytoplasm, **3:**180
  eukaryotic cells, **3:**180
  extracellular, **3:**180
  mitogen-activated protein kinase pathways for, **3:**182
  mRNA turnover regulation by, **3:**285–287
  nucleus origin of, **3:**179–180
  origin of, **3:**179–180
  p53 transduction of, **3:**181
  plasma membrane, **3:**180
  protein modification for propagation of, **3:**181
  transduction methods, **3:**181–182
Stress-activated protein kinases, **3:**286
Striatin, **2:**410
STYX
  catalytic efficiency, **1:**744
  definition of, **1:**741
  dual-specificity phosphates and, **1:**745
  functions of, **1:**744–746
  structure of, **1:**741, **1:**744
SU5402, **2:**887
SU 6668, **1:**456
SUC1-associated neurotrophic factor, **1:**268
Su(H)
  Notch intracellular domain repressed by, **3:**149–150, **3:**153–154
  Notch signaling effects on, **3:**150–151
  proteins interacting with, **3:**151–152
  silencing mediator for retinoid and thyroid hormone receptors interactions with, **3:**150
  transcriptional repression by, **3:**149–150, **3:**153–154
Sulfolobus solfataricus, **3:**188
SUMO-1, **2:**774
Suppressors of cytokine signaling
  C-terminal box, **1:**435
  description of, **1:**347, **3:**521
  discovery of, **1:**434
  in vivo role of, **1:**436
  interferon-γ action regulated by, **1:**436
  knockout mice studies of, **1:**436
  leukemia inhibitory factor regulated by, **3:**630
  mechanisms of action, **1:**435–436
  mRNAs, **3:**521
  negative feedback regulation, **3:**523
  overview of, **1:**434
  proteins
    description of, **1:**434–435
    growth hormone signaling downregulation by, **3:**524–525
    hepatocellular carcinoma and, **3:**526
    insulin signaling downregulation by, **3:**523–524
    interferon signaling regulated by, **3:**525
    liver regeneration regulated by, **3:**525
    physiological function of, **3:**522–523
  SOCS2, **3:**630–632
  SOCS3, **3:**524
  transcription induction, **3:**521–522
Suprachiasmatic nuclei, **3:**139–140
Supramolecular activation cluster, **1:**80
SUR-2, **2:**805
sur-5, **2:**805–806
Survivin, **3:**432
SV40, **1:**622–623, **2:**407, **2:**410, **3:**412
SW14, **3:**208
SW16, **3:**208
SWI/SNF complexes, **3:**27
Syk
  B cell antigen receptor ligand binding effects, **3:**556–557
  description of, **1:**328
  kinase activity of, **1:**328
  protein tyrosine kinases, **1:**477–478
Synaptic plasticity, **2:**400–401
Synaptobrevin, **2:**663
Synaptojanin 1 gene, **2:**211
Synaptotagmin 1, **2:**95, **2:**97–98, **3:**383, **3:**394
Syndecans, **1:**90, **2:**391
SynGAP, **2:**330
Syntaxin, **2:**663
Synthetic genetic array, **1:**16

T

TAF_{II}, **3:**16
Talin, **1:**463, **2:**212
Tamiflu, **1:**109–110
TAP, **3:**424
TAP:p15, **3:**427
Taste
  qualities of, **2:**657
  transduction
    G proteins in
      description of, **2:**657
      Gβγ subunits, **2:**658
      α-gustducin, **2:**657–658
      α-transducin, **2:**658
    G-protein coupled receptors in, **2:**658–660
    second messenger pathways for, **2:**660
Taste receptor cells, **2:**657
TATA box binding protein-associated factors, **3:**11, **3:**13
Tau, **2:**409
Tautomycin, **1:**608
TC10, **2:**702
TCA cycle, **3:**368
T-cell factor, **1:**73
T-cell receptor
  αβ, **1:**63, **1:**83
  accessory molecule association with, **2:**340
  activation of, **2:**84
  antigen-specific, **3:**546

T-cell receptor *(continued)*
binding domains, **1:**83
BM3.3, **1:**65–66
complementarity-determining region loops, **1:**64–65
conformational variation and changes, **1:**65–66
costimulation of, **1:**73–75, **3:**549–550
crosslinking of, **1:**476
description of, **1:**59, **1:**355, **2:**339, **3:**545
downregulation of, **2:**343–344
encoding of, **1:**475
engagement of, **3:**546–548
FK506 effects, **1:**523
generation of, **1:**63
immunological synapse effects, **2:**343–344
immunoreceptor tyrosine based activation motif, **1:**475
ligand binding, **3:**546
major histocompatibility antigen and
alloreactivity, **1:**66–67
altered peptide ligands effect, **1:**65, **1:**67, **2:**341
antagonism, **1:**65
binding, **2:**339
complexes
bound water molecules in, **1:**67
description of, **1:**64–65
interactions
description of, **1:**63–64
signaling complex, **1:**64
superantagonism, **1:**65
mechanism of action, **2:**339–340
peptide–MHC complex interactions with, **3:**545
protein tyrosine kinases that affect, **1:**475–476
Shp2's role in signaling by, **1:**718–719
signaling pathways
coreceptors, **3:**548–549
description of, **2:**340
schematic diagram of, **3:**547
structure of, **1:**475
Syk and, **1:**477–478
ZAP-70 and, **1:**477
T-cell(s)
activation of
calcineurin's role in, **1:**634
CD28's role in, **1:**355–356, **2:**343
CTLA-4's role in, **1:**355–356
description of, **1:**74, **1:**79
effector T cells derived from, **2:**339–340
immunological synapse's role in, **1:**79–81
variations in, **2:**342
antigen stimulation of, **3:**550–551
antigen-presenting cell junction with, **1:**79, **1:**358
calcium mobilization and, **3:**550
cAMP-related activation of, **3:**550–551
CD4, **2:**342
$CD8^+$, **2:**342
description of, **3:**545
immunological synapse and, **1:**79, **2:**341–342
sources of, **3:**545
transcription factors that affect, **3:**550
TCP80, **3:**337
TCPTP, **1:**666
TC21/R-Ras-2, **2:**685–686
Tec protein tyrosine kinases, **1:**478
Ternary complex factor, **2:**711
Testis
anatomy of, **3:**531–532
cell–cell signaling in, **3:**531–535
cellular biology of, **3:**531–532
description of, **3:**531
development of, **3:**532–533
fibroblast growth factor expression by, **3:**533
growth factors that affect, **3:**533–535
Leydig cells, **3:**532–533
paracrine factors, **3:**533
Sertoli cells, **3:**531–533
Tetrathhiomolybdate, **3:**398
Tetratricopeptide repeat-containing proteins, **2:**747
TGase. *see* Tissue transglutaminase
TGN38, **2:**409
Theca cells
description of, **3:**537
Th1, **2:**340, **2:**342
Th2, **2:**340, **2:**342
T-helper cells, **3:**79
Thioredoxin peroxidase I, **3:**370
Thr286, **2:**400
Thr308, **1:**514, **2:**194
Threonine receptors
characteristics of, **1:**362, **1:**364
description of, **1:**2, **1:**362
interacting proteins, **1:**490
Thrombin, **1:**167–168, **1:**171
Thrombopoietin, **3:**620–621
Thrombopoietin receptor, **3:**620–621
Thromboxane A_2, **2:**266–267
Thymocytes, **3:**545
Thyroid hormone receptors, **3:**29
Thyrotropin receptors
activation of, **1:**161
description of, **1:**161
experiments of, **1:**163
serpentine domain of, **1:**164
Tiam-1, **2:**204, **3:**414
Tiam1, **2:**753
Tie, **1:**375
Tie 2, **2:**850
Tissue inhibitors of matrix metalloproteinases, **3:**459
Tissue transglutaminase
adenosine triphosphate binding of, **2:**722
arginine for, **2:**723
biochemical characteristics of, **2:**722
biological function of, **2:**724
catalytic activities of, **2:**721
definition of, **2:**721
future studies of, **2:**724–725
GTP-binding/GTPase, **2:**722–724
guanine-nucleotide-binding site for, **2:**723
mammalian tissue distribution of, **2:**722
overview of, **2:**721–722
protection-factor role of, **2:**724
retinoic acid regulation of, **2:**724
three-dimensional structure of, **2:**722–724
3T3-L1 preadipocytes, **3:**40–41
TLR-4 receptor pathway, **1:**90
Tlrs, **2:**659

TLS, **3:**339
Toll receptors
activation of, **2:**334–335
description of, **2:**333
discovery of, **2:**333
signaling pathways of, **2:**335–336
structure of, **2:**333–334
Toll-Dorsal signaling, **2:**779–781
Toll-like receptors
activation of, **2:**334–335
carboxy-terminal of, **2:**336
coreceptor association with, **2:**334
discovery of, **2:**333
domains of, **2:**334
TLR2, **2:**334, **2:**336
TLR3, **2:**334, **2:**336
TLR4, **2:**334, **2:**336
TLR6, **2:**334
TOR
cell growth and, **1:**525
description of, **1:**523
discovery of, **1:**523
FAT motif, **1:**526
functions of, **1:**523–525
mammalian. *see* mTOR
mRNA translation and, **1:**524
mutations, **1:**523
outputs, **1:**524
segments of, **1:**525–526
signaling from, **1:**525–528, **3:**300–302
T_3R. *see* Thyroid hormone receptors
TRAF6, **3:**132
TRAIL. *see* Tumor necrosis factor-α-related-apoptosis-inducing ligand
Tramtrack69, **3:**329
Transcription
initiation steps, **3:**64–65
Notch, **3:**153–156
nuclear receptor corepressor repression of, **3:**29, **3:**31
Smads regulation of, **3:**172–173
Transcription factors
activating protein 1
description of, **3:**99, **3:**490–491
DNA-binding domain of, **3:**99
Fos, **3:**99–100
function of, **3:**101–103
genotoxic agent effects, **3:**102
Jun, **3:**99–100
mitogen-activated protein kinase regulation of, **3:**101
murine studies of, **3:**101–103
NFAT protein interactions with, **3:**121
posttranslational control of, **3:**100–101
subunits of, **3:**99–100
target genes, **3:**102
transcriptional control of, **3:**100–101
types of, **3:**102
angiogenesis and, **3:**58
Cubitus interruptus
description of, **3:**167
expression patterns of, **3:**167
Hedgehog regulation of, **3:**167–169
N-terminus of, **3:**167
protein kinase A regulation of, **3:**169
protein structure of, **3:**167
transcriptional regulation of, **3:**169–170
heat shock 2, **2:**408
modulation of, **3:**63–65
NFAT
activator protein-1 interactions with, **3:**121
biological functions regulated by, **3:**121–122
calcineurin interactions with, **3:**120
description of, **3:**119
DNA-binding domain of, **3:**120
gene transcription, **3:**120
isoforms, **3:**121
ligand binding of, **3:**120
mass spectrometry analysis of, **3:**121
NFAT1, **3:**119–122
NFAT2, **3:**119–122
NFAT3, **1:**634, **3:**119–122
NFAT4, **3:**120–122
NFAT5, **1:**634, **3:**119, **3:**121–122
NFATc, **3:**126–127
regulation of, **3:**120–121
rephosphorylation of, **3:**120
structure of, **3:**120
transcriptional functions of, **3:**121
nuclear receptor corepressors and, **3:**30
nuclear receptor modulation of, **3:**63–65
regulated nuclear transport for control of, **3:**125–127
Rel, **3:**62
RNA polymerase II
covalent modification regulation of, **3:**16
description of, **3:**11
loss of, **3:**14
negative control of, **3:**16
preinitiation complex. *see* RNA polymerase II, preinitiation complex
silencing mediator for retinoid and thyroid hormone receptors and, **3:**30
steroid hormone receptor cross-talk with, **3:**35
Transducin, **1:**129, **2:**566, **2:**575, **2:**583
Transducisomes, **1:**351
Transforming growth factor α
description of, **3:**141
lung development and, **3:**510
ovary development and, **3:**536–537
testis development and, **3:**533
tumorigenesis and, **3:**566–567
Transforming growth factor β
bone development and, **3:**501–502
breast tissue and, **3:**566–658
description of, **1:**289, **1:**487
family of, **3:**567–568
Gdf11, **2:**887
interneuron differentiation and, **2:**884
intracellular signaling after activation of, **3:**442
kinases activated by, **1:**489
ligands, **1:**289–290
lung development and, **3:**511
ovary development and, **3:**536
prostate gland development and, **3:**593
receptor–ligand complex, **1:**291–292
receptors, **1:**289–290

Transforming growth factor β *(continued)*
signal transduction, **1:**487–490
signaling of, **1:**290–291
Smad cross-talk with, **1:**488–489, **3:**171. *see also* Smads
spinal cord development, **2:**883
structure of, **1:**289–290
superfamily members, **1:**487
type II, **1:**290, **1:**364
Transglutaminase, **3:**393
trans-Golgi network, **2:**729, **3:**386
Transient receptor potential, **2:**52
Transient receptor potential channels, **1:**568
Transition state
definition of, **1:**29
protein–protein interactions, **1:**29–30
Transmembrane proteins
description of, **1:**21
ion channels. *see* Ion channels
protein kinase C interactions with, **2:**391
Transmembrane signaling. *see also* Cell signaling
bacterial chemotaxis receptors, **1:**198–199
paradigms of, **1:**21–23
Transportin, **3:**420
TRAP, **1:**490
Tristetraprolin, **3:**320–321
Trk
characteristics of, **1:**281–282
domains, **1:**281–282
Trk receptors
description of, **1:**24
developmental role of, **2:**840–841
neural functions mediated by, **3:**489–490
TrkA receptors, **3:**445, **3:**487
TRRAP, **1:**560
Trypanosoma brucei
adenylyl cyclases in, **2:**422, **2:**540
antibody response to, **2:**541
calcium mobilization in, **2:**541
cAMP levels, **2:**539–540
cyclic nucleotide signaling in
cell proliferation, **2:**539–540
description of, **2:**539
pathways, **2:**540–541
phosphodiesterases, **2:**540–541
guanylyl cyclases in, **2:**540
infection process, **2:**541
protein kinase A in, **2:**541
Trypanosoma cruzi, cAMP levels, **2:**540
TSC1, **3:**301
TSC2, **3:**301
Tsg, **2:**834
Tubby family proteins, **2:**157
Tubuloglomerular feedback, **3:**581–582
Tumor necrosis factor
α-
description of, **1:**275
interferon-α levels affected by, **3:**525
obesity levels of, **3:**43
testis production of, **3:**534
β-
characteristics of, **1:**275
tumor necrosis factor-receptor 1 and, **1:**277–278
cell-surface receptors for, **1:**275
monomers, **1:**276
signaling of, **1:**275
structure of, **1:**275–276
types of, **1:**275
Tumor necrosis factor receptor-associated death domain, **1:**277, **1:**311, **1:**317
Tumor necrosis factor receptor-associated factors
CD40 signaling and, **1:**320–321
characteristics of, **1:**311–312
crystal structure of, **1:**311–312
domain organization of, **1:**312
functions of, **1:**311
intracellular trafficking of, **1:**312
multicellular organisms with, **1:**311
signaling, **1:**312
TRAF-1, **1:**312
TRAF-2, **1:**307, **1:**311, **1:**320–321
TRAF-5, **1:**320
TRAF-6, **1:**320
tumor necrosis factor receptor-associated death domain protein recruitment by, **1:**312
Tumor necrosis factor receptor(s)
activation of, **1:**364
aggregation of, **1:**315
associated factors, **1:**278
caspase recruitment domains, **1:**276
CD40. *see* CD**1:**40
characteristics of, **1:**276, **1:**364–365
death domains, **1:**276–277
death receptors. *see* Death receptors
description of, **1:**2, **1:**24, **1:**239, **1:**315
dimerization of, **1:**364–365
extracellular domains of, **1:**276, **1:**305
functions of, **1:**305
interactions among, **1:**316–317
ligand–receptor complexes
consequences of, **1:**277–278
description of, **1:**276–277
oligomeric nature of, **1:**364
$p75^{NTR}$, **1:**283
preassociation of, **1:**278–279
raft recruitment, **1:**315–316
signaling complexes for, **1:**315–317
structure of, **1:**256
TNF-R1, **1:**276–277, **1:**305
TNF-R2, **1:**276
TRAF6, **1:**316
ubiquitination, **1:**316
Tumor necrosis factor-α-related-apoptosis-inducing ligand, **3:**595
Tyk2 deficiency, **1:**428
Tyr239, **1:**646
Tyr292, **1:**478
Tyr551, **3:**558
Tyr766, **1:**268
Tyr925, **1:**466
Tyr972, **1:**300
Tyrosine kinase-containing receptors
description of, **1:**361
dimerization of, **1:**361–362
hematopoietic cytokine signaling through, **3:**618–620

Tyrosine phosphatases
description of, **1:**347
inhibition of, **1:**394
SHP-1, **1:**395
SHP-2, **1:**395
Tyrosine phosphorylation
cadherin cytoplasmic tail, **2:**894
G proteins, **2:**611
inhibitors of, **1:**451–452
insulin receptor substrate-proteins, **1:**410
SHIP2, **2:**150
Tyrosine-based inhibitory motifs, **1:**89
Tyrosine-based internalization motif, **1:**356
Tyrphostins, **1:**452–454
TZDs, **3:**43

U

U73122, **2:**249
U937 cells, **2:**206
Ubiquitin C-terminal hydrolase, **2:**348
Ubiquitin ligases
description of, **2:**348, **3:**129
HECT-type, **3:**153
regulation of, **3:**131
SCF class, **3:**130–131
SCF E3, **3:**405
Ubiquitin–activating enzyme, **2:**347–348
Ubiquitination
G-protein coupled factor degradation, **3:**443
hypoxia inducible factor-1α stimulation of, **3:**131
kinase activity modulated by, **3:**132
Notch intracellular domain, **3:**152
p53, **3:**130–131, **3:**239
protein, **1:**383
reaction cycle of, **3:**130
Smad regulation of, **1:**489
substrate modification regulation of, **3:**129–131
tumor necrosis factor receptors, **1:**316
Ubiquitin–conjugating enzyme, **2:**348
Ubiquitin-like proteins, **2:**347
Ubiquitin–proteasome system
C-terminus of, **2:**347
degradation signals, **2:**349
description of, **1:**489, **2:**347
deubiquination pathways, **2:**347–348
E1, **2:**347
E2, **2:**347
ligation pathways, **2:**347–348
overview of, **2:**347
protein degradation by, **3:**129
protein processing by, **3:**131–132
regulation by, **2:**349
20S proteasome, **2:**349
Ubiquitin-related proteins, **2:**347
Ubiquitin-specific processing proteases, **2:**348
Ultrasensitive biological switch, **1:**383
Ultraviolet light
p53 activation by, **3:**242
phospholipid turnover secondary to, **3:**260
receptor tyrosine kinase activation secondary to, **3:**260
UmuD, **3:**186
$UmuD_2$, **3:**187
UNC-5, **2:**871–872
Unfolded protein response
definition of, **3:**311, **3:**360
description of, **3:**180, **3:**264, **3:**279–280
physiological role of, **3:**315–316
in *Saccharomyces cerevisiae,* **3:**312
signaling of, **3:**314
transcriptional activation, in metazoan species, **3:**312–315
in yeast, **3:**312, **3:**360
Uniporter, **2:**73–74
US28, **1:**174–175

V

V3 loop, of human immunodeficiency virus-1, **1:**103
VAB-1, **1:**424
Vac1, **2:**180–181
VAMP-2, **3:**382–383
Vanadate, **1:**678–679
van't Hoff's law, **1:**29
Vascular endothelial growth factor
angiogenesis and, **2:**849, **2:**856, **3:**456, **3:**537, **3:**599
description of, **1:**285
endothelial cell proliferation and, **2:**850
ERK cascade activation, **2:**850
family of, **1:**285
heparin-binding domain of, **1:**285–286
hypoxia inducible factor-1 targeting of, **3:**278–279
low-molecular-weight protein tyrosine phosphatases and, **1:**737
lung development and, **3:**511
neuropilins and, **2:**878
plasma protein extravasation and, **2:**850
platelet-derived growth factor and, similarities between, **1:**287
prostate gland development and, **3:**593
receptor-binding domain of, **1:**286
signal transduction by, **2:**856
vascular permeability and, **2:**850–851
vasculogenesis role of, **3:**57
Vascular endothelial growth factor receptors
description of, **1:**285–286, **1:**391
ligands of, **2:**857
VEGFR-1
angiogenesis and, **2:**849, **2:**856–857
description of, **1:**285
vasculogenesis and, **2:**856–857
VEGFR-2
angiogenesis and, **2:**849, **2:**856–857
description of, **1:**285
vasculogenesis and, **2:**856–857
VEGFR-3, **2:**857
VEGFR-C, **2:**857
VEGFR-D, **2:**857
Vascular endothelial growth factor–*flt1*-D2 complex, **1:**286–288
Vascular permeability
Ang-1 effects, **2:**851
vascular endothelial growth factor effects, **2:**850–851
Vascular permeability factor, **3:**458
Vascular smooth muscle cells
cyclic GMP-dependent protein kinase expression by, **2:**511
renal, **3:**579–580
vasoconstriction of, **3:**579–580

Vasculogenesis, **2:**855–856
Vasopressin, **3:**575
Vav3, **3:**560
Vav proteins, **2:**204
VCP, **1:**672
V(D)J recombination, **1:**33
VEGFR-C, **2:**857
VEGFR-D, **2:**857
Ventral uterine precursor cell, **2:**810
Vertebrates
  development of
    CPEB-mediated translation in, **3:**323–325
    Notch signaling in. *see* Vertebrates, Notch signaling
    wnt signaling in, **2:**790
  inactivation/noafterpotential D protein signaling complexes, **1:**352
  left-right determination in, by hedgehog signaling
    description of, **2:**799
    genes involved in, **2:**799
    mice, **2:**800–802
  Notch signaling in development of
    basic helix-loop-helix factors, **2:**819
    description of, **2:**817
    lateral inhibition, **2:**819
    limbs, **2:**820–821
    lymphoid development, **2:**821
    neurogenesis, **2:**817–819
    notch function during neurogenesis, **2:**817–819
    notch ligands, **2:**814–817
    notch receptors, **2:**813–814
    notch signal transduction, **2:**815–816
    organ systems, **2:**821–822
    segmentation, **2:**819–820
    signal transduction, **2:**815–816
    vascular development, **2:**821
  protein tyrosine phosphatase sequences in, **1:**662
  retinotectal system, **2:**867–868
  Shp2 in, **1:**712
  wnt signaling in development of, **2:**790
Very-low-density lipoprotein receptor, **1:**95–97
Vesicle associated membrane protein, **2:**663
Vesicle budding, **2:**729
Vesicular stomatitis virus G protein, **3:**355
VH1, **1:**643, **1:**646
VHR, **1:**654
VILIP 1, **2:**80–81
VILIP 2, **2:**80–81
VILIP 3, **2:**80–81
Vimentin, **2:**409
Vinculin, **1:**463, **2:**318
Viral coat proteins, **1:**95, **1:**97
Virus-encoded 7TM receptors
  development of, **1:**173
  in vivo function of, **1:**176
  ligands recognized by, **1:**175–176
  multiple, **1:**174
  ORF74, **1:**174–176
  redundant chemokine system used for, **1:**173–174
  US28, **1:**174–175
Vitamin D, **3:**500
Vitamin D receptor, **2:**288
V_L-V_H rearrangements, in Fab fragment, **1:**36
Voltage-dependent anion channel, **2:**74, **3:**435
Voltage-gated calcium channels
  calcium currents
    L-type, **2:**23–26, **2:**583
    N-type, **2:**25, **2:**640
    pharmacological properties of, **2:**23–24
    physiological properties of, **2:**23–24
    P-type, **2:**24, **2:**26, **2:**640
    Q-type, **2:**24, **2:**26, **2:**640
    T-type, **2:**24, **2:**26
    types of, **2:**23
    voltage clamp studies of, **2:**24
  cardiac, **2:**25
  Ca_V 1, **2:**25
  Ca_V 2, **2:**25, **2:**27
  Ca_V 3, **2:**25
  depolarization, **1:**209
  description of, **1:**207, **2:**23, **2:**51–52
  diversity of, **2:**25–26
  function of, **2:**26–27
  gating
    G-protein βγ subunit effects on, **2:**664
    particles of, **1:**209
    S4 coupled to, **1:**213
  molecular properties of, **2:**24–26
  in neurons, **2:**23
  physiological roles of, **2:**23
  pore domain, **1:**209
  regulation of, **2:**27
  second messenger signal transduction pathways effect on, **2:**27
  sensing by
    description of, **1:**209
    in proteins, **1:**209–210
    S4, **1:**210–213
  structure of, **1:**210
  subunits
    α1, **2:**24–25
    β-, **2:**24–25
    γ-, **2:**25
    functions of, **2:**25
    S5 segments, **2:**26
    S6 segments, **2:**26
    structure of, **2:**24–25
  summary of, **2:**27
  transmembrane organization of, **2:**25–26
Voltage-gated sodium channels, **1:**217
Vps27, **2:**181
v-Src, **1:**584, **1:**673

W

WASp
  Arp2/3 complex, **2:**323–324
  binding interactions with, **2:**325
  description of, **2:**323
  domain structure of, **2:**324
  N-, **2:**323–324
  Scar/WAVE, **2:**324–326, **2:**384, **2:**703
  structure of, **2:**746
WD40 domains, **1:**508
Wee1, **3:**404

WIN compounds, **1:**97
Wiskott-Aldrich syndrome protein. *see* WASp
Wnt signaling
β-catenin and
description of, **2:**789, **3:**161
target genes of, **2:**790–791
description of, **1:**177–179, **1:**370, **1:**549, **2:**789
developmental role of, **2:**789–791
embryogenetic role of, **1:**623
events in, **3:**162
Frizzleds, **1:**177–179, **2:**789
invertebrate development role of, **2:**789–790
LEF-1/TCF proteins, **3:**163–164
Legless, **3:**164
PP2A and, **1:**623–624
Pygopus, **3:**164
vertebrate development role of, **2:**790
Wnt/Wg pathways, **3:**161
Wortmannin, **2:**249
Wrist epitope, **1:**290
WRP, **2:**326
WW domains, **1:**507–508

X

X-box binding protein 1, **3:**279
XBP1, **3:**315, **3:**362
Xenopus, CPEB in development of, **3:**324
XIP, **2:**64
X-linked inhibitor of apoptosis, **2:**353–354
X-linked myotubular myopathy, **2:**145
xNa^+/Ca^{2+} exchange, **2:**74
XRCC4, **3:**220

Y

Y542, **1:**710
Y580, **1:**710
Yeast. *see also Saccharomyces cerevisiae*
actin cytoskeleton in, **3:**411–413
cdc14
anaphase-promoting complex, **1:**697
description of, **1:**697
exit from mitosis and, **1:**697–698
FEAR pathway, **1:**698–699
MEN pathway, **1:**698–699
oligomerization of, **1:**697
regulation of, **1:**698–699
signaling pathways, **1:**698
structure of, **1:**697–698
substrates, **1:**698
Tyr residues, **1:**697
$G\alpha_o$ studies, **2:**606
heterotrimeric G protein signaling in, **2:**571–572
low-molecular-weight protein tyrosine phosphatases in, **1:**737
mitochondrial signaling in, **3:**367–371
mitogen-activated protein kinases in
activation of, **2:**357–358
cascades
cell architecture in, **2:**361–362
downregulation, **2:**361–362
dynamic localization of, **2:**360–361
inactivation of, **2:**361
kinase interactions, **2:**359–360
cascades in
description of, **2:**357–358
regulatory mechanisms, **2:**359–362
scaffold proteins, **2:**360
crossregulation of, **2:**362
description of, **2:**357
unfolded protein response in, **3:**312, **3:**360
UPR, **3:**264–265
Y-family polymerases, **3:**188
YFP, **3:**136
YKG9, **2:**526–527
YopH, **1:**647
Yotiao, **2:**399
Ypt1, **2:**691

Z

Z4.aaa, **2:**810
ZAKI-4, **1:**629
ZAP-70, **1:**380, **1:**477, **1:**485, **3:**546
ZFR, **3:**337
Zn-RING domain, **1:**316
Z1.ppp, **2:**810
Zymogen, **2:**353